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A colorimetric, ratiometric and water-soluble fluorescent probe for simultaneously sensing glutathione and cysteine/homocysteine

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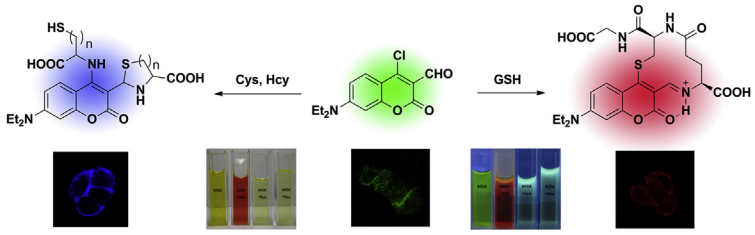
HIGHLIGHTS

- A colorimetric, ratiometric and water-soluble fluorescent probe was developed.
- The probe could simultaneous distinguish GSH and Cys/Hcy by visual determination.
- This probe was successfully used to achieve living cell ratio imaging.

GRAPHICAL ABSTRACT



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ABSTRACT

A chlorinated coumarin-aldehyde was developed as a colorimetric and ratiometric fluorescent probe for distinguishing glutathione (GSH), cystenine (Cys) and homocysteine (Hcy). The GSH-induced substitution-cyclization and Cys/Hcy-induced substitution-rearrangement cascades lead to the corresponding thiol-coumarin-iminium cation and amino-coumarin-aldehyde with distinct photophysical properties. The probe can be used to simultaneously detect GSH and Cys/Hcy by visual determination based on distinct different colors – red and pale-yellow in PBS buffer solution by two reaction sites. From the linear relationship of fluorescence intensity and biothiols concentrations, it was determined that the limits of detection for GSH, Hcy and Cys are 0.08, 0.09 and 0.18 μM, respectively. Furthermore, the probe was successfully used in living cell imaging with low cell toxicity.

1. Introduction

Low molecular weight thiols, like glutathione (GSH), cysteine (Cys) and homocysteine (Hcy), are crucial cellular components that play numerous roles in metabolism and homeostasis as well as in varying functions of physiological and pathological processes [1–3]. Abnormal levels of biothiols are related to many diseases, such as neurodegenerative diseases, liver damage and renal diseases [4–7]. Fluorescent probes are powerful molecular tools for monitoring trace amounts of analytes in live cells or tissues because of their simplicity and high sensitivity [8–20]. However, the discrimination between three biothiols is still challenging because of their similarity in structure and reactivity.

Numerous fluorescent probes have been developed for the

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detection of biothiols by utilizing different mechanisms, such as cleavage reaction, Michael addition, redox reaction and nucleophilic substitution [21–24]. The addition and cyclization reaction of Cys/Hcy with aldehydes or acrylates and native chemical ligation (NCL) reaction have been developed to discriminate Cys and Hcy over GSH [25–35]. Relatively speaking, the discriminating of GSH over Cys/Hcy remains a tough mission. Our group reported two fluorescent probes to detect GSH over Cys and Hcy using the acrylate derivatives [36,37]. The Michael addition products of the probes with GSH have strong fluorescence; on the contrary, the addition-cyclization products of the probes with Cys/Hcy are non-fluorescence. Yang group demonstrated a GSH fluorescent probe based on the thiol-halogen nucleophilic substitution reaction between GSH and a monochlorinated Bodipy [38]. The attractive strategy is distinct from that induced nucleophilic substitution-rearrangement cascade reaction by Cys/Hcy. In recent years, some fluorescent probes have been designed based on this strategy [39–41]. Furthermore, a chlorinated coumarin-hemicyanine dye with three potential reaction sites was reported for sensing GSH and Cys through different emission channels [41]. However, a simple and effective fluorescent probe for simultaneously sensing GSH, Cys and Hcy is extremely necessary.

Herein, we combine the above strategies to study a colorimetric and ratiometric fluorescent probe, 4-chloro-7-(diethylamino)-3-carbaldehyde coumarin, with two reaction sites. The probe could simultaneously distinguish GSH and Cys/Hcy through different emission channels in PBS buffer solution. No matter *in vivo* or *in vitro*, we could observe the significant different colors by visual determination. Furthermore, the probe was successfully used in living cell ratio imaging.

2. Experimental

2.1. Apparatus and chemicals

Thin-layer chromatography (TLC) was conducted on silica gel 60 F_{254} plates (Merck KGaA) and column chromatography was conducted over silica gel (mesh 200–300). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were carried out on a Bruker Avance 400 spectrometer, using DMSO as solvent and tetramethylsilane (TMS) as internal standard. Melting points were determined on an XD-4 digital micro melting point apparatus. IR spectra were performed with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). HRMS spectra were obtained on a Q-TOF6510 spectrograph (Agilent). UV–vis spectra were measured by using a Hitachi U-4100 spectrophotometer. Fluorescent measurements were recorded on a Perkin–Elmer LS-55 luminescence spectrophotometer. Quartz cuvettes with a 1 cm path length and 3 mL volume were used for all measurements. The pH measurements were done on a Model PHS-3C pH meter. Unless otherwise stated, all reagents were purchased from J&K, Sinopharm Chemical Reagent Co. and Kermel and used without further purification. Twice-distilled water was used throughout all experiments.

2.2. Synthesis of 4-chloro-7-(diethylamino)-3-carbaldehyde coumarin (probe ***ACCA***)

7-(Diethylamino)-4-hydroxy coumarin (**4**) was synthesized according to literature methods [41]. Under nitrogen, dry DMF (2.5 mL) was added dropwise to $POCl_3$ (1.0 mL) at room temperature. After stirred for 30 min, compound **4** (3 mmol) dissolved in 10 mL DMF was added dropwise to the above solution. The solution was stirred at 60 °C for 10 h until TLC indicated the end of the reaction. After cooling to room temperature, the mixture was poured into ice water (50 mL), and was adjusted to neutral with diluted NaOH solution. The ensuing precipitate was filtered and washed with water. The crude product was purified by the recrystallization from absolute ethanol to give 4-chloro-7-(diethylamino)-3-carbaldehyde coumarin in 73% yield. Yellow solid; mp: 138–140 °C. IR (KBr) ν: 3090 (ArH), 2972 (−CH_3), 2928 (−CH_2−), 2879 (H−C═O), 1719 (C═O), 1177(C−O−C). ^{1}H NMR (DMSO, 400 MHz): δ = 10.30 (1H, s, −CHO), 7.85 (1H, d, J = 9.3 Hz, ArH), 6.70 (1H, dd, J = 2.4 and 9.3 Hz, ArH), 6.44 (1H, d, J = 2.4 Hz, ArH), 3.49 (4H, q, J = 7.2 Hz, −CH_2−), 1.26 (6H, t, J = 7.2 Hz, −CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): 12.44 (2C), 45.40 (2C), 96.75, 107.82, 110.60, 111.15, 129.31, 153.58, 154.00, 156.45, 159.95, 187.02. HRMS: m/z $[M+H]^+$ calcd for $[C_{14}H_{14}ClNO_3 + H]^+$: 280.0740, found 280.0731.

2.3. Preparation for UV–vis and fluorescence spectral measurements

Probe **ACCA** was dissolved in DMSO to afford the stock solution (2×10^{-3} M). Amino acids (GSH, Cys, Hcy, arg, asp, glu, gly, his, lys, pro, ser, thr, trp), cation (Ca^{2+}, Fe^{3+}, K^+, Mg^{2+}, Na^+, Zn^{2+}), H_2O_2, glucose were all dissolved in deionized water at a concentration of 2×10^{-2} M for absorption and fluorescence spectral analysis. Test solutions were prepared by placing 100 μL or 50 μL of the stock solution and an appropriate aliquot of each testing species solution into a 10-mL volumetric flask, and the solution was diluted to 10 mL in an aqueous solution with pH 7.4 (PBS buffer). The resulting solution was shaken well and incubated for 60 min at room temperature before recording the spectra.

2.4. HPLC-MS traces

HPLC-MS spectra were recorded with a Thermo LCQ Fleet coupled with a thermo Ultimate 3000 HPLC system. HPLC analysis was accomplished with an Atlanti C18 reversed-phase column (2.1 × 150 mm), with CH_3CN (0.1% of HCOOH) and water as the eluent. Injection volume: 5 μM; mobile phase: A-0.1% methanoic acid/acetonitrile, B-water; gradient elution: 0–7.9 min, 20%A; 8–15 min 90%A; flow rate: 0.2 mL min^{-1}.

2.5. Cell culture and cell imaging

HeLa cells were cultured in RPMI-1640 with 10% CBS at 2×10^4 cells per well. The probe was dissolved in DMSO at a storage concentration of 10 mM. Cells were adherent-cultured in 24-well culture plates for 12 h. After washing away the culture medium with phosphate-buffered saline solution (PBS), HeLa cells of control group were loaded with 10.0 μM probe solution at 37 °C for 40 min. Other test groups were pretreated with GSH (0.5 mM) or Cys (0.1, 0.5, 1.0 and 5.0 mM) at 37 °C for 40 min, followed by incubation with 10.0 μM probe solution for 40 min. Then washed 2 times with PBS and underwent imaging measurement by ultraviolet light with a confocal microscope (LSM700). Fluorescence values were quantified by the fluorescence analysis software Image J. The exciting light was 405 nm for the emission range of the blue channel (405–475 nm). The exciting light was 488 nm for the emission range of the green channel (488–520 nm) and the red channel was (560–700 nm).

2.6. Cytotoxicity assay

The *in vitro* cytotoxicity of the probe to HeLa cells was measured by a standard sulforhodamine B (SRB) assay. Briefly, HeLa cells were loaded in 96-well culture plates at 4×10^4 cells per well. After culture for 24 h, cells were incubated with fresh RPMI 1640 containing 1.0, 5.0 and 10.0 μM probe for 24 h, respectively. Then cells were fixed with 4% TCA for 1 h at 4 °C, and then washed 5 times