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A colorimetric and fluorescent probe for detecting intracellular biothiols

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ABSTRACT

A new rapid and highly sensitive coumarin-based probe (probe **1**) has been designed and synthesized for detecting intracellular thiols. Probe **1** was prepared by a 4-step procedure as a latent fluorescence probe to achieve high sensitivity and fluorescence turn-on response toward cysteine and homocysteine over GSH and other various natural amino acids under physiological conditions. Owing to specific cyclization between thiols and aldehyde group, probe **1** displayed a highly selectivity toward cysteine and homocysteine. Above all, probe **1** was successfully used for fluorescence imaging of biothiols in Hela cells, and quantitative determination had been achieved within a certain range. Then specific fluorescence imaging of mice organ tissues was obtained for proving the permeability of probe **1**. Simultaneously, the viability was measured to be more than 80%, which shows probe **1** can be a rapid and biocompatible probe for biothiols in cells. Furthermore, the measurement of thiols detection in 5 kinds of animal serum showed that probe **1** can be used in determination of biothiols in blood.

1. Introduction

Compounds with thiols (–SH) functionality are very important as low molecular weight aliphatic thiols containing amino acids (cysteine and Homocysteine) and peptides (glutathione) play pivotal roles in biological systems. For example, cysteine (Cys) is the only amino acid with a thiols functional group that serves as a unique unit in protein construction, enzyme active sites and cofactors (Albers et al., 2013; Lipton et al., 2002; Marino and Gladyshev, 2010). On the other hand, glutathione (GSH) is critical in maintaining redox homeostasis in the intracellular environment, which is important for maintenance of cellular defense against reactive oxygen species and for a number of biological processes (Kanzok et al., 2000; Wu et al., 2004). Recently, much research interest has been paid to Homocysteine (Hcy) because of its special role as a biomarker in many diseases (Austin et al., 2004; Xiao et al., 2011). Generally, the levels of intracellular biothiols have been associated to toxic agents and diseases. A low level of biothiols would be a dangerous signal for various syndromes, such as slow growth in children, hair depigmentation, lethargy, psoriasis, liver damage, substance abuse, muscle and fat loss, edema and weakness. While an elevated level of biothiols in human plasma is a risk factor for cardiovascular, Alzheimer's disease, neural tube defect, inflammatory bowel disease, osteoporosis, cancer, and AIDS (Refsum et al., 1998; Seshadri et al., 2002; Shahrokhian, 2001; Townsend et al., 2003). Therefore, the determination and quantification of cellular biothiols is of great importance and has attracted much attention.

Of all the traditional detection methods for biothiols, one of the most common analytical techniques is liquid chromatography (LC) (Ivanov et al., 2000; Michaelsen et al., 2009; Refsum et al., 2004; Tcherkas and Denisenko, 2001). Although this method offers high accuracy, it typically requires staff with a certain skill level, cumbersome preconditioning procedures and expensive instruments for the analysis, which makes it unsuitable for on-site trials and household testing. Among the available techniques to detect and quantify thiols, fluorescent methods have attracted increasing interest due to their high sensitivity, inexpensiveness, selectivity, easy operation and nondestructive (Chen et al., 2010; Kaur et al., 2012; Moragues et al., 2011; Wang et al., 2014). Accordingly, during the past decade, considerable efforts have been devoted to developing fluorescent probes for thiols (Jung et al., 2013; Kim et al., 2008; Yin et al., 2013). Among these probes, there are two main mechanisms used to design fluorescent probes for thiols. One is based on cleavage reaction mechanism (Bouffard et al., 2008; Ji et al., 2009; Pires and Chmielewski, 2008; Tang et al., 2007; Yuan et al., 2012), the other one is based on nucleophilic reaction mechanism (Jung et al., 2013; Yin et al., 2013). By comparing the two kinds of mechanisms, nucleophilic reaction mechanism are more actively developed because of the diversity of

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the electrophiles. Especially in recent years, the selective reaction of aldehydes with thiols to form thiazolidines was applied to the detection of Cys and Hcy, as sensors with an aldehyde functionality can form a rapid ring with 1, 3- or 1, 2-aminothiols, while other biothiols, like GSH, cannot (Rusin et al., 2004; Wang et al., 2005; Yang et al., 2011). However, it has been known that the selective detection of Cys, Hcy and GSH is still a challenge due to their reactivity of thiols and similarity in structure.

Based on the abovementioned consideration and our earlier work (Chen et al., 2015; Shi et al., 2014; Yang et al., 2014), we report a new type fluorescent probe (probe **1**) containing coumarin fluorophore and aldehydes moiety. Coumarin was selected as the fluorophore by reason of its desirable photophysical properties, such as a large Stokes' shift, visible emission wavelengths, and high fluorescence quantum yields (Lim and Bruckner, 2004; Lim et al., 2005; Trenor et al., 2004). Simultaneously, aldehydes was chosen because it serves as not only an electrophile but also a quencher of the coumarin fluorophore. The aldehydes moiety plays an electron acceptor for the photoinduced electron transfer (PET) process, leading to a low fluorescence of probe **1**. After treatment with thiols, ring between fluorophore and thiols are formed, and the blocking of PET process can cause recovery of fluorescence (Scheme 1).

2. Experimental details

2.1. Materials and instruments

All reagents and solvents were obtained commercially and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL ECS 400M spectrometer and referenced to the solvent signals. Mass spectra (ESI) were obtained on a LQC system (Finnegan MAT, USA). UV–visible spectra were gathered on a Varian Cary 100 spectrophotometer. The melting points were measured with an X-6 melting point apparatus without calibration (Beijing Fuka Keyi Science and Technology Co., LTD). Fluorescence spectra were performed on a Hitachi F-7000 luminescence spectrometer.

2.2. Preparation of amino acids solutions for fluorescent study

Stock solutions (2 mM) of amino acids including Cys, Hcy, GSH, Glu, Phe, Ala, Pro, Thr, His, Ile, Arg, Lys, Val, Leu, Met, Gln, Trp, Ser, Gly, Tyr, Asn and Asp in ultrapure water were prepared, and stock solution of probe **1** was prepared in 10 mL of DMSO. In a typical experiment, examine solutions were prepared by placing 10 μL of the probe stock solution into the solution of 2 mL phosphate buffer. Fluorescence spectra were measured after addition of analytes for 10 min at 20 °C. An excitation and emission slit widths of 5.0 nm were used for the fluorescent measurements.

Phosphate Buffered Saline (PBS) buffer was prepared with the following method. Dissolve 1.775 g of Dibasic Sodium Phosphate in ultrapure water, and dilute to 250 mL, which is the solution A. Then dissolve 0.680 g of Potassium Phosphate Monobasic in ultrapure water, and dilute to 100 mL, which is the solution B. Afterwards, solution B was added to solution A until the pH comes to 7.40, then the final PBS buffer was preserver at 4 °C.

2.3. Cell and tissue culture

Hela cells were obtained from the school of life science of Lanzhou University (Gansu, China). The cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 μg/mL), and streptomycin (100 μg/mL). Cells were maintained under a humidified atmosphere of 5% CO_2 and at 37 °C incubator.

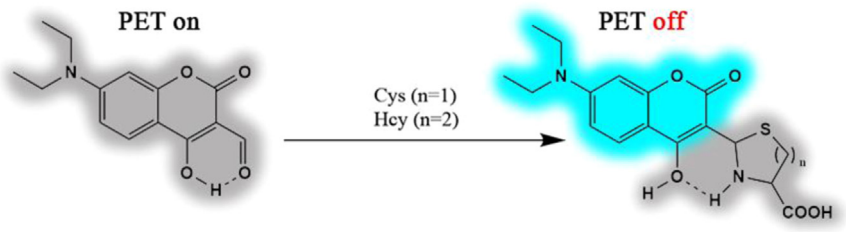
The cytotoxic effect of compound probe was determined by an MTT assay following the manufacturer instruction (Sigma-Aldrich, MO). Hela cells were initially propagated in a 25 cm^2 tissue culture flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 μg/mL), and streptomycin (100 μg/mL) in a CO_2 incubator. For cytotoxicity assay, cells were seeded into 96-well plates (approximately 104 cells per well), and various concentrations of compound probe (20, 40, 60, 80, 100 and 120 μM) made in DMEM were added to the cells and incubated for 24 h. Solvent control samples (cells treated with DMSO alone) was also included in parallel sets. Following incubation, the growth media was removed, and fresh DMEM containing MTT solution 5 mg/mL was added. The plate was incubated for 2–3 h at 37 °C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured in a microtiter plate reader (Infinite M200, TECAN, Switzerland) at 480 nm. The assay was performed in five sets for each concentration of compound probe complex. Data analysis and calculation of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation). For statistical analysis, a one way analysis of variance (ANOVA) was performed using Sigma plot.

2.4. Fluorescence microscope experiment

Fluorescence images of dye labeled cells and tissues were obtained by Olympus FV1000 laser confocal microscope IX81 with 40× objective (cells) and 10× objective (tissues), numerical aperture (NA)=0.65. The images signals at 400–500 nm range were collected by internal PMTs in a 12 bit unsigned 1024*1024 pixels at 40 Hz scan speed.

2.5. Synthesis

Initially, compound **3** was synthesized according to the previous report (Scheme S1) (Bochkov et al., 2013; Liu et al., 2014). Then a solution of compound **3** (1.165 g, 5 mmol), phosphorus oxychloride (0.7 mL, 6.75 mmol) in 5 mL *N*, *N*-Dimethylformamide (DMF) and stirred at room temperature for 24 h. Then the mixture was poured into aqueous sodium acetate (5 g in 100 mL) and



Scheme 1. Design concept of probe **1** for thiols.