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An 'OFF–ON' fluorescent probe for specially recognize on Cys and its application in bioimaging

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ABSTRACT

Up to date, the design of highly selective probes for Cys, Hcy, and GSH is still a challenge and very few examples have been reported so far. In this work, the compound, N-[4-Methylcoumarin-7-yl] maleimide was synthesized and it can specially recognize on Cys as an 'OFF–ON' fluorescent probe. For specific recognition on Cys of probe depends on its maleimide group, the structural and acidic differences of biothiol itself. Furthermore, the ability of probe to detect Cys in living cells (HepG2 cells) via an enhancement of the fluorescence was proved.

1. Introduction

Thiols play essential roles in biological systems. As important biothiols, cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) which have the similar structures play crucial roles in human physiology [1–3]. Their abnormal levels are linked to a number of diseases. The deficiency of Cys is involved in many human diseases, such as delayed growth, lethargy, hair depigmentation, skin lesions, liver damage, muscle and fat loss, and so on [4–10]. As determined by elevated plasma levels, Hcy is a risk factor for Alzheimer's disease [11,12]. As the most abundant intracellular non-proteinogenic thiol, GSH plays important roles in maintaining the reducing environment in cells and acts as a redox regulator [12–18]. Therefore, a rapid, selective and sensitive detection of thiols in biological samples has been the subject of much attention. Various methods for the determination of thiols were reported. Colorimetric and fluorescent methods to detect thiols have received increasing attention since they are simple, sensitive, efficient and applicable for intracellular detection among the various strategies [19]. Many mechanisms were based on Michael addition [20–28], cyclization with aldehydes [29–32], ligand displacement of metal complexes by thiols [33–36] and cleavage reaction induced by thiols [37–40]. The sensors for thiol utilizing thiol addition to the maleimide moiety have been developed when Sippel reported N-(4′-(7-diethylamino-4-methylcoumarin-3-yl)phenyl)maleimide as one of the first examples of thiol probes in 1981 [41,42].

Most of the reported examples are designed to detect biothiols such as Cys, Hcy, and GSH. However, the common functional group sulfydryl makes it difficult to distinguish them from each other. In fact, deep reason can be explained as following. First, the GSH is a tripeptide in which the thiol is located in the middle of the molecule. The inherent reactivity of the SH group is thus expected to be reduced as the result of steric shielding. This should be particularly true for nucleophilic reactions involving hindered electrophiles. Second, in Cys the SH proton is relatively acidic (lower pKa value for the sulfhydryl group compared to other thiols) in aqueous media [43]. As a consequence, the thiolate/thiol ratio is higher for Cys at neutral pH than it is for most other biologically important thiols, including Hcy and GSH. This difference is expected to translate into greater relatively reactivity in nucleophilic reactions. Up to date, the design of highly selective probes for Cys, Hcy, and GSH is still a challenge and very few examples have been reported so far. Particularly, only a few sensors can detect Cys over Hcy and GSH in biological systems [44–48]. In our group, we always expect to obtain the specific probe for distinguishing among Cys, Hcy and

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GSH. We aimed excellent coumarin fluorophore and maleimide bonding groups to design a series of fluorescent probes [49–52]. The result is outstanding, we found that N-[4-Methylcoumarin-7-yl]maleimide can specially recognize on Cys, and Hcy, GSH did not disturb the determination of Cys. Furthermore, this probe was successfully applied to the detection of cysteine in water and can detect cysteine in living cells (HepG2 cells).

2. Materials and methods

2.1. Materials

All analytes and solvents were of analytical grade without further purification. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma–Aldrich (St. Louis, MO). All spectroscopic measurements were performed in HEPES (10 mmol/L, pH 7.4) buffer. Sodium hydroxide solution (0.1 mol/L) was added to aqueous HEPES (10 mmol/L) to adjust the pH to 7.4. Probe was synthesized using a modification of a literature method [53]. H_2O was deionized. The solutions of anions were prepared from their sodium salts.

2.2. Instruments

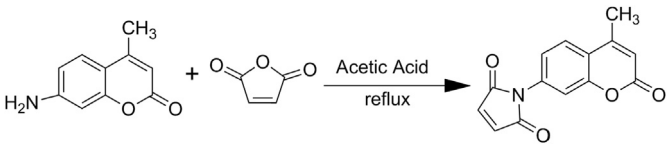
A pH meter (Mettler Toledo, Switzerland) was used to determine the pH. Ultraviolet–visible (UV–Vis) spectra were recorded on a Cary 50 Bio UV–Visible spectrophotometer. Fluorescence spectra were measured on Cary Eclipse fluorescence spectrophotometer. A PO-120 quartz cuvette (10 mm) was purchased from Shanhai Huamei Experiment Instrrument Plants, China. ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker AVANCE-300 MHz and 75 MHz NMR spectrometer, respectively (Bruker, Billerica, MA). ESI was measured with an LTQ-MS (Thermo) instrument. The ability of probe reacting to Cys in the living cells was also evaluated by laser confocal fluorescence imaging using a Leica TCS SP5 laser scanning microscope.

2.3. Preparation and characterization of probes

Probe was synthesized using a modification of a literature method [53] (Scheme 1). 7-Amino-4-methylcoumarin (175 mg, 1 mmol) and maleic anhydride (98 mg, 1 mmol) were dissolved in glacial acetic acid (10 mL), the solution was heated under reflux for 6 h, and the solvent was removed under reduced pressure, the product obtained filtered and washed with sodium carbonate solution. A yellow solid was obtained (158 mg, 62%) (Scheme 1). ^{1}H NMR (DMSO-d_6): δ 7.89 (d, J = 8.2 Hz, 1H), 7.82 (d, J = 13.7 Hz, 1H), 7.39 (s, 2H), 7.24 ((s, 1H), 6.44 (d, J = 1.2 Hz,1H), 2.46 (s, 3H).^{13}C NMR (DMSO-d_6): δ = 172.7, 168.9, 156.5, 156.1, 148.2, 138.1, 128.9, 125.1, 122.1, 117.8, 117.1, 20.9. Elemental analysis (calcd. %) for $C_{14}H_9NO_4$: C, 65.88; H, 3.55, Found: C, 65.82; H, 3.63. (ESI-MS) m/z: $[M + H]^+$ Calcd for $C_{14}H_9NO_4$ 256.06, Found 256.17.

2.4. General UV–Vis and fluorescence spectra measurements

Stock solutions of probe (2 mmol/L) were prepared in CH_3OH. Stock solutions (2.0 mmol/L) of various analytes amino acids, cysteine (Cys), homocysteine (Hcy), glutathione (GSH), phenylalanine (Phe), threonine (Thr), arginine (Arg), histidine (His), asparagines (Asp), leucine (Leu), alanine (Ala), proline (Pro), valine (Val), glycine (Gly), lysine (Lys), glutamine (Gln), methionine (Met), serine (Ser), isoleucine (Ile), tyrosine (Tyr), tryptophane (Trp), glutamic acid (Glu), aspartic acid (Asp) Mercaptoacetic acid (MPA), mercaptoethanol (ME), hydrogen sulfide, cyanide ion and thiocyanate ion, bisulfite were prepared in deionized water. All UV/Vis and fluorescence titration experiments were performed using HEPES (10 mmol/L, pH 7.4) solution with at room temperature. And any changes in the fluorescence intensity were monitored using a fluorescence spectrometer (E_x = 300 nm, E_m = 398 nm, slit: 5 nm/5 nm).



Scheme 1. The synthesis of compounds.

2.5. Imaging of HepG2 cells

The HepG2 cells were grown in 1 × SPP medium (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA ferric sodium salt) at 37 °C. The HepG2 were treated with 2 μmol/L probe in culture media for 30 min at 37 °C and washed three times with phosphate-buffered saline (PBS). For the control experiment, the cells were treated with 4 μmol/L N-ethylmaleimide (NEM) in culture media for 30 min at 37 °C. After washing with PBS to remove the remaining NEM, the cells were further incubated with 2 μmol/L of probe in culture media for 30 min at 37 °C, and then with 40 μmol/L cysteine for another 30 min at 37 °C.

3. Results and discussion

3.1. The selective response of probe to Cys

The high selectivity toward the target molecular is a major issue in the field of molecular sensing. To validate the selectivity of probe, the fluorescence spectral changes for probe provided, various analytes including Phe, Thr, Arg, His, Asp, Leu, Ala, Pro, Val, Gly, Lys, Gln, Met, Ser, Ile, Tyr, Trp, Glu, Asp, Hcy, GSH, MPA, hydrogen sulfide, cyanide ion and thiocyanate ion, bisulfate, Cys, ME under HEPES (pH 7.4) solution condition are shown in Fig. 1. The fluorescence intensity of probe was highly enhanced only by the addition of Cys and ME (small increase). The other amino acids did not cause any significant changes in the fluorescence emission intensity. A competitive binding assay was performed by adding Cys to 0.1 μmol/L of probe in the presence of 500 equiv. of other analytes also served to confirm that probe provides a selective response to Cys even in the presence of analytes (Fig. S2).

A large increase in the fluorescence intensity of Cys can be perceived by the naked eye. When probe was excited at 365 nm in the presence 500 equiv. of other analytes in HEPES buffer at pH 7.4, the bright blue fluorescence responses were observed only with Cys in the solution of 0.1 μmol/L of probe (Fig. S2).

3.2. The fluorescence and UV–vis spectra of detecting Cys

To measure the fluorescence sensitivity of probe for Hcy and Cys, fluorescence titration was carried out in aqueous solution (HEPES buffer, pH 7.4). When Cys was added to the HEPES (10 mmol/L, pH 7.4) solution containing probe (0.1 μmol/L), the fluorescence intensity at 398 nm gradually increases with the increase of concentration of Cys. A visual fluorescence change (from colorless to blue) was observed (Fig. 2). The corresponding UV–vis spectral changes were also carried out. Fig. S3 shows the change in the UV–Visible spectrum upon addition of Cys to a solution of the probe (30 μmol/L) in HEPES (10 mmol/L, pH 7.4) solutions. With increasing concentration of Cys, the UV–vis absorption of probe slightly decreases and blue shifts gradually with one isosbestic points at 287 nm, indicating a new compound generated.