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A fluorescent turn-on probe for highly selective detection of cysteine and its bioimaging applications in living cells and tissues

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ABSTRACT

Due to the abnormal concentration of cysteine (Cys) related to various diseases, it is of great significance in disease diagnosis to monitoring Cys under pathophysiological conditions. Fluorescence probe capable of fast detecting Cys would be beneficial to the study of mechanisms of certain diseases. In this paper, we designed and synthesized a novel fluorescent probe **LC-Cys**, which employed the acrylate group and α, β-unsaturated ketone as the two response groups. It could swiftly respond to Cys within 10 min, and exhibit satisfactory sensitivity and excellent selectivity to Cys. Furthermore, probe **LC-Cys** can be conveniently used to detect cysteine in living HepG2 cells and tissues with low cytotoxicity, indicating it would have great promise for biological applications.

1. Introduction

Cysteine(Cys), serving as one of the essential biological thiols, plays various roles in a vast scope of human physiology [1–3]. Cys has a thiol group on the side chain due to homocysteine (Hcy) [4,5], the precursor compound of Cys in the mammal. Furthermore, cysteine functions as a prevalent nucleophile in many biological activities [6]. It is involved in protein and glutathione (GSH) synthesis, biological reduction activity and the like [7,8]. Moreover, the abnormality of Cys would result in symptoms as wide-ranging, for instances, metabolic hair depigmentation, liver damage, Alzheimer's disease and debility [9–12]. It can be seen that detecting the concentration of cysteine under physiological conditions is a significant research topic, and there is the imperative demanding to develop a particular analytical technique for high selectivity, good sensitivity and more conveniently testing cysteine under pathophysiological conditions.

Detecting Cys in the biological environment has always been a challenging field owing to the low level of cysteine and similar activity to Hcy and GSH. In the past decades, electrophoresis, high-performance liquid chromatography (HPLC), UV–vis spectroscopy, the electrochemical method, chemical method and fluorescence analysis have been applied in the monitoring of cysteine [13,14]. Among these methods mentioned above, fluorescence analysis draws great attention because of its rapid response, excellent sensitivity and easy accessibility [15–17]. Recently, some fluorescent probes possessing specific functional groups have been developed for the detection of Cys [18,19]. The near-infrared fluorescent probe with dual models based on the Michael reaction and nucleophilic substitution was designed to monitor Cys with low detection limit [20]. However, long response time is a non-negligible drawback for most of the reported probes. And some fluorescence probes are failed to discriminate Cys from Hcy and GSH, which limit their applications in specific detection of Cys in biological samples. Hence, it is eager to develop fluorescent probes with the rapid response and high selectivity to inspect cysteine in biological samples and organism.

Our group previously reported a dual-site fluorescent probe, which could generate turn-on and ratio modes response to the low and high concentration range of Cys respectively. Although the dual-site fluorescent probe exhibited satisfactory response toward Cys, the dual-mode under two different excitation wavelengths might complicate the detection of the unknown concentration range of Cys. We suppose that the dual-mode was ascribed to the intramolecular hydrogen bond between the hydroxyl and carbonyl group. The removal of the intramolecular hydrogen bond would engender the only turn-on mode while maintaining the rapid response to Cys. In this paper, we designed a naphthalene-based probe **LC-Cys**, which employed a conventional fluorescent dye, 2-acetyl-6-methoxynaphthalene, with high quantum yield as the fluorophore without intramolecular hydrogen bond. Moreover, the acrylate group and α, β-unsaturated ketone as the response sites could mainly react with the thiol of cysteine within 10 min. The probe **LC-Cys** displayed improved sensitivity toward Cys with a detection limit of 2.2×10^{-8} M and exhibited satisfactory selectivity to Cys over

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other amino acids, particularly GSH and Hcy. Importantly, this probe was successfully applied to monitor Cys in living cells and tissues with low cytotoxicity.

2. Experimental section

2.1. Materials and instruments

All reagents were obtained from commercial sources as analytical reagent grade and used without further purification. The One Drop spectrophotometer (OD-1000+, Nanjing, China) was used to measure the UV–vis absorption spectra. Fluorescence spectrophotometer (PerkinElmer, LS55) was applied for detecting fluorescence emission spectra. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance at 300-MHz; values of δ in ppm were measured based on TMS. High-resolution mass spectra was gained on Agilent 6200 series TOF/6500 series. The laser confocal fluorescence microscopy (FluoView™, FV1000, Olympus, Japan) was used to acquire laser confocal cell and tissue imaging. Fluorescence quantum yields were determined by standard methods, using quinine sulfate (Φ_F = 0.54 in 0.1 M H_2SO_4 aqueous solution) as a standard.

2.2. Synthesis of compound LC-OH

1-(6-Methoxynaphthalen-2-yl)ethan-1-one (200 mg, 1.0 mmol) and 4-hydroxybenzaldehyde (120 mg, 1.0 mmol) were dissolved in absolute ethyl alcohol (20 mL). Two drops of piperidine were added to the mixture solution. Then the mixture was stirred for 4 h under reflux. The solution was concentrated under rotary evaporator to give the crude product, which was purified by silica gel column chromatography to afford the desired compound **LC-OH** (228 mg, 75% yield). ^{1}H NMR (300 MHz, DMSO-*d6*) *δ* 10.08 (s, 1H), 8.82 (s, 1H), 8.04-8.12 (m, 2H), 7.88-7.94 (m, 2H), 7.76-7.79 (m, 3H), 7.42 (s, 1H), 7.29 (d, *J* = 8.9 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 2H), 3.93 (s, 3H). ^{13}C NMR (75 MHz, DMSO-*d6*) *δ* 188.7, 160.5, 159.8, 144.3, 137.2, 133.6, 131.6, 131.3, 130.4, 128.0, 127.6, 126.4, 125.2, 119.8, 119.0, 116.3, 106.6, 55.8. HRMS (ESI): calcd. for $C_{20}H_{16}O_3Na$ 327.0992 $[M + Na]^+$, found 327.0994.

2.3. Synthesis of probe LC-Cys

Triethylamine (TEA, 102 mg, 1.0 mmol) and compound LC-OH (152 mg, 0.5 mmol) were dissolved into dry CH_2Cl_2 (10 mL) and cooled to 0 °C. Then acryloyl chloride (55 mg, 0.6 mmol) was dropped into the solution and stirred at room temperature for 3 h. After removed the solvent by rotary evaporator, the desired product **LC-Cys** was obtained by silica gel column chromatography in 88% yield. ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 8.53 (s, 1H), 8.14 (d, *J* = 8.6 Hz, 1H), 7.89-7.96 (m, 3H), 7.68-7.78 (m, 3H), 7.27-7.31 (m, 4H), 6.69 (d, *J* = 17.3 Hz, 1H), 6.39 (dd, *J* = 17.3, 10.4 Hz, 1H), 6.10 (d, *J* = 10.4 Hz, 1H), 4.02 (s, 3H). ^{13}C NMR (75 MHz, DMSO-*d6*) *δ* 188.8, 164.4, 159.9, 152.2, 142.8, 137.4, 134.3, 133.2, 131.7, 130.9, 130.8, 130.5, 127.9, 125.2, 122.8, 122.7, 122.0, 119.9, 114.6, 106.6, 55.9. HRMS (ESI): calcd. for $C_{23}H_{18}O_4Na$ 381.1097 $[M + Na]^+$, found 381.1098.

2.4. Spectroscopic properties and optical response to Cys

The reactions between **LC-Cys** (10 μM) with different concentrations of Cys in PBS buffer solution (10 mM, pH 7.4, 10% DMSO) were carried out at 37 °C for 10 min. Then the absorption and fluorescence spectra were recorded.

2.5. Sensing mechanism study of LC-Cys with Cys

The solution of **LC-Cys** (100 μM) and Cys (10 mM) was oscillated in shaking table at 37 °C for 10 min. The solution was extracted with dichloromethane for three times. The collected organic layers were removed under reduced pressure. The residue was analyzed by mass spectrum. MS-ESI: 448.1$[M + H]^+$.

2.6. Cell experiments and confocal fluorescence imaging

The human cell line HepG2 (human liver hepatocellular cells) was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) Medium containing 10% fetal bovine serum (FBS, Hyclone, containing 80 U/mL penicillin and 0.08 mg/mL streptomycin) at 37 °C in a 5% CO_2/95% air (v/v) incubator. One day before imaging, cells were seeded in the laser scanning confocal microscope (LSCM) culture dishes at a density of 5×10^5 cells/wall. Subsequently incubated at 37 °C in a humidified atmosphere containing 5% CO_2. For the control group, probe **LC-Cys** (10 μM) was added to the dishes and incubated at 37 °C for 30 min. In another group, Cys (1 mM) was put into the cells for 30 min. Then the probe **LC-Cys** (10 μM) was added to the medium and incubated at 37 °C for another 30 min. In the blocking group, the cells were incubated with *N*-ethylmaleimide (NEM, 2 mM) for 30 min, followed by probe **LC-Cys** (10 μM) for another 30 min. Fluorescence imaging experiments were performed using FV1000 confocal laser scanning microscope.

2.7. In vitro cytotoxicity assay

HepG2 cells were cultured in 96-well plates at the density of 5×10^5 cells per well and cultured at 37 °C in a 5% CO_2/95% air (v/v) incubator for 24 h. The medium was replaced with different concentrations of probe **LC-Cys** (0, 10, 20, 30, 40, 50 μM) were added in the following, and the cells were cultivated for 12 h. After 4 h, the MTT solution was abandoned, and 150 μL of DMSO was added to each well to dissolve the formed formazan. Finally, the absorbance of each well of 96-well plates was measured at 490 nm through a multi-well plate reader. The cell viability was calculated using the following formula: Cell viability = (the optical density of test wells − the optical density of medium control wells)/(the optical density of untreated wells − the optical density of medium control wells) × 100%. Each concentration was measured in triplicate and used in three independent experiments.

2.8. Tissue imaging

Mice with six weeks to eight weeks old were purchased for tissue imaging. The liver was taken from the healthy mice, embedded in the embedding medium (PVA), and frozen in −80 °C refrigerator for 1 h. Then the fresh liver slice (100 μm) was obtained by freezing-microtome. Liver slice was stained with probe **LC-Cys** (50 μM) for 1 h. Then the solution was replaced by Cys (500 μM) and further standing for another 1.5 h. Before tissue imaging, the tissue slice was washed with PBS (10 mM, pH 7.4) for three times. Finally, the Z-scan mode of the laser scanning confocal microscope was utilized to record the fluorescence intensities at different depths of the slice.

3. Results and discussion

3.1. Design and synthesis of probe LC-Cys

Naphthalene ring is a greatly widely used fluorophore owing to its high fluorescence quantum yield. The mechanism of emission fluorescence for naphthalene group is known as Photoinduced Electron Transfer (PET). Different substituent groups on the naphthalene ring would lead to the different effect on the progress of PET. Herein, we utilized acrylate and α, β-unsaturated ketone as the rapid response groups to adjust the PET process of naphthalene for monitoring Cys. And 2-acetyl-6-methoxynaphthalen was employed as the fluorophore without intramolecular hydrogen bond. Compared with 2-acetyl-1-naphthol, the absence of intramolecular hydrogen bond might improve