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NEM assisted real-time fluorescence detection of Cys in cytoplasm and mice imaging by a Coumarin probe containing carboxyl group

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ABSTRACT

Alterations of the homeostasis balance of cysteine (Cys) are associated with a variety of diseases and cellular functions, and therefore, Cys dynamic real-time living cell intracellular imaging and quantification are important for understanding the pathophysiological processes. Thus, Cys probe that can permeate high efficiently is the first one to be affected. In fact, it is difficult for organic molecular probes to infiltrate cells because of the unique structure of the cell membrane. In this work, we found that probe containing-carboxyl just stagnated in cytomembrane due to carboxyl of probe and amino group of membrane protein forming peptide chains, nevertheless, the addition of NEM, improved membrane permeability by NEM reacting with sulfhydryl of membrane protein, which made probe permeate high efficiently and sequentially real-time detect the Cys in cytoplasm. It is the first time noted that NEM can regulate Cys probe containing-carboxyl for high efficient detection in cytoplasm. Additionally, probe was successfully applied to image Cys in mouse.

1. Introduction

Cell as the basic units of the structure and function of organisms is inseparable from the existence of cytomembrane [1–5]. Cytomembrane have irreplaceable and significant physiological functions in biological systems, which not only enable cells to maintain a stable metabolic intracellular environment, but also regulate and select substance to entry and exit the cell, specific performance in the following six aspects: (a) to provide a relatively stable internal environment and improve biological function; (b) barrier function; (c) selective material transport; (d) biological function: including that hormone effect, enzymatic reaction, cell recognition, electron transfer, etc.; (e) recognize and transmit information function; (f) material transport function. Cell membranes consist of phospholipid bilayers and embedded proteins that pass through the cell membranes and are adsorbed on the surface of the cell membranes. Depending on the difficulty of protein separation and the location of protein distribution in the membrane, membrane proteins can be divided into three categories: exogenous membrane proteins, also known as peripheral membrane proteins, and intrinsic membrane proteins, also known as integral membrane proteins and lipid-anchored proteins. Thereinto, integral membrane proteins bind fatty acid molecules covalently on cysteine residues on one side of the cytoplasmic matrix, inserted between lipid bilayer, a few proteins are covalently bound to glycolipids [6]. *N*-Ethylmalemide (NEM), as a masking agent for cysteine (Cys), in other words, a covalent modification reagent for Cys residues of protein, after using to incubate cells, Cys residues may form covalent binding with NEM, which breaks the structure of covalent binding polypeptide formed by Cys residues and fatty acid molecules on one side of the cytoplasmic matrix of integral membrane proteins, thus the original structure of the cell membrane is destroyed and then the permeability of the cell membrane is changes [7–9].

Cysteine (Cys), the only amino acid with the reducing group (-SH) in more than score of amino acids that make up proteins, plays a vital role in maintaining normal physiological and biological processes [10–18]. The total content of Cys in cells is 30–200 μM have been clear [19–21], elevating and reducing levels of Cys have been associated with various diseases, for example, rheumatoid arthritis, neurotoxicity, Alzheimer's disease, Parkinson's disease, slow growth in children and liver damage [22–31]. Therefore, it is of great value and significant important to detect intracellular even cytoplasm Cys in biochemistry and clinical applications area.

Previous work, we have designed and synthesized a series of probes based on coumarin fluorophore. Among them, there was a peculiar probe containing-carboxyl willfully stayed at cytomembrane when probe was incubated only with cells. However, subsequent addition of

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NEM, probe will exhibit detection effect for Cys in cytoplasm not in cytomembrane [32,33]. To discover this mystery, we obtained another sibling probe to carry out detailed research in this work. The probe showed very low background fluorescence and highly selectivity to Cys with a turn-on fluorescent response peaked with 62-fold fluorescent enhancement at 500 nm. It is well known that NEM is a trapping reagent of thiol species [34–36], which made probe **1** to reversibly detect Cys in our system. More important, via the regulation of NEM made probe permeate high efficiently and sequentially real-time detect the Cys in cytoplasm. It can be explained that the addition of NEM improved membrane permeability by NEM reacting with sulfhydryl of membrane protein.

2. Materials and methods

2.1. Synthesis of probe *1*

Probe **1**: compound **6** (0.10 mmol, 0.046 g) and 2-cyanoacetic acid (0.15 mmol, 0.013 g) were dissolved in EtOH (15 mL). 50 μL piperidine was added and the mixture was stirred at 78 °C refluxed for 4 h (TLC monitoring process). After cooling to 25 °C, reducing pressure, the red solid was purified by column chromatography (MeOH:DCM = 1:10), giving probe **1** ((*Z*)-2-cyano-3-(4-((E)-3-(7-(4-(ethoxycarbonyl) piperazin-1-yl)-2-oxo-2H-chromen-3-yl)-3-oxoprop-1-en-1-yl) phenyl) acrylic acid) as a red powder (0.0455 g, 68% yield) (synthetic route and relevant NMR data see supporting information). ^{1}H NMR data (600 MHz, DMSO d_6): δ 8.65 (s, 1H), 7.97 (t, J = 12.5 Hz, 4H), 7.85 (d, J = 8.2 Hz, 2H), 7.76 (d, J = 9.0 Hz, 1H), 7.71 (d, J = 15.7 Hz, 1H), 7.05 (dd, J = 9.0, 2.0 Hz, 1H), 6.90 (s, 1H), 4.08 (q, J = 7.1 Hz, 2H), 3.58–3.50 (m, 8H), 1.21 (t, J = 7.1 Hz, 3H). ^{13}C NMR data (151 MHz, DMSO): δ (ppm): 186.3, 160.0, 158.2, 155.3, 155.1, 148.8, 141.4, 132.5, 130.5, 129.3, 126.8, 117.9, 112.1, 109.7, 98.9, 61.4, 46.5, 15.0. ESI-MS: found: m/z 528.1767; Molecular formula: $[C_{29}H_{25}N_3O_7]^+$, Calc: 528.5405 (Fig. S5).

2.2. Solutions preparation and UV–vis and fluorescence measurements

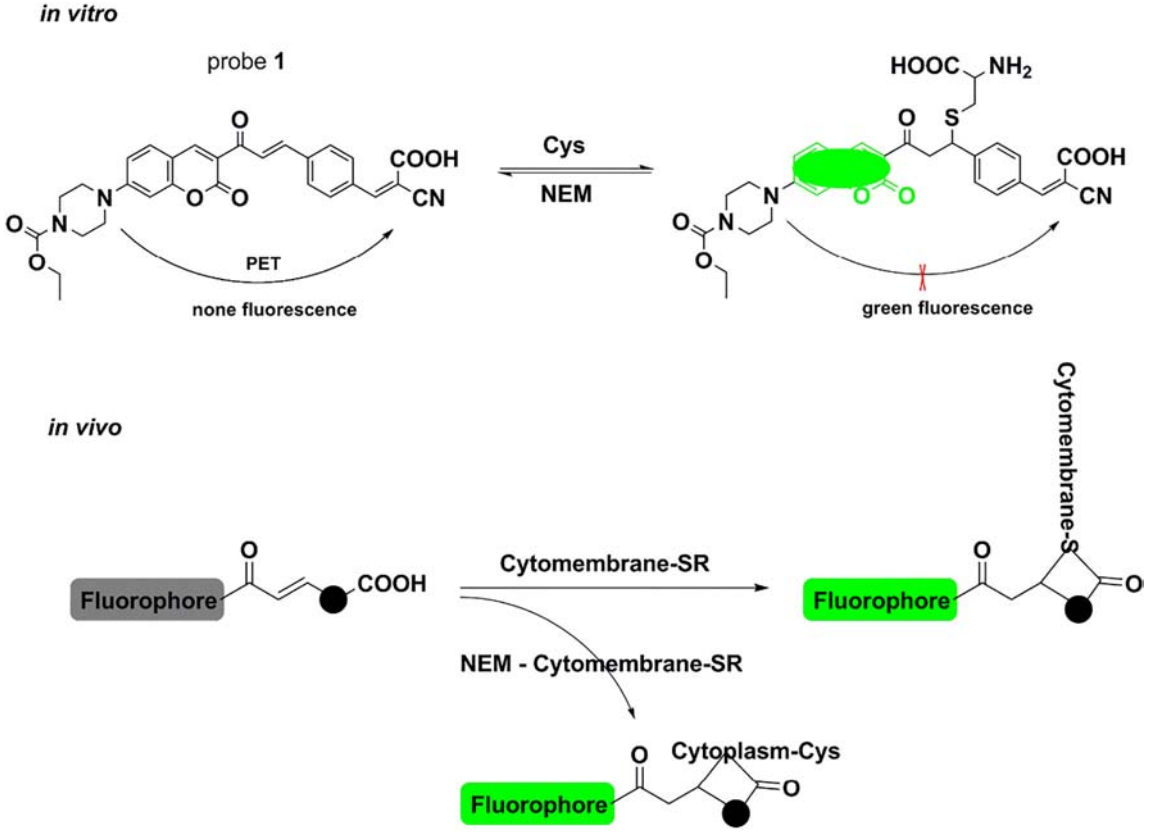
Probe **1** was prepared with 2 mL DMSO solution at a concentration of 2 mM. Cys and NEM stock solutions (2 mM) were as prepared in deionized water. Other analytes solutions (0.2 M) were prepared by using deionized water, which including amino acids, peptide, metal cations and anions. And fluorescence measurements were carried out with an excitation wavelength at 440 nm, emission wavelength at 500 nm and a slit width of 5 nm.

2.3. Cell imaging

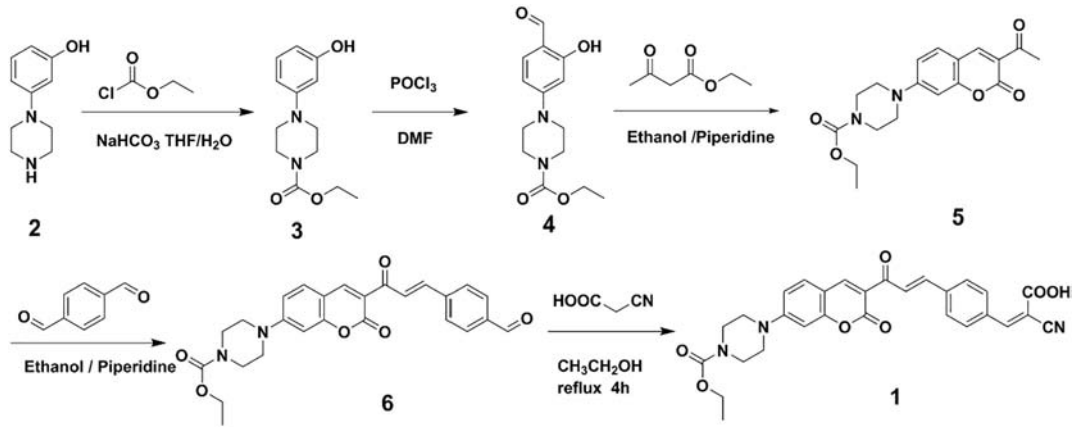
The cells were directly incubated with 10 μM probe **1** for 0.5 h. The cells were sequentially incubated with NEM (1 mM) and probe **1** (10 μM) for 0.5 h each. The cells were sequentially incubated with NEM (1 mM), Cys (200 μM), and probe **1** (10 μM) for 0.5 h each. The cells were sequentially incubated with NEM (1 mM), GSH (1 mM), and probe **1** (10 μM) for 0.5 h each. The cells were sequentially incubated with NEM (1 mM), Hcy (15 μM), and probe **1** (10 μM) for 0.5 h each. Before imaging, each step is performed first the cells were washed by PBS once. Fluorescence images were obtained with the emission was collected at 480–530 nm and the excitation wavelength is at 458 nm by a Zeiss LSM880 Airyscan confocal laser scanning microscope (Scheme 1).

2.4. Cell viability

Cytotoxicity was assessed by performing Cell Counting Kit-8 (CCK-8) assay with the HepG-2 cells. The HepG-2 cells were seeded on 96-well plates and incubated for 24 h at 37 °C (5% CO_2). Different concentrations of probes 1 (0, 1, 2, 3, 5, 7, 10 and 20 μM) were then added to the wells in sequence with 6 replicates per concentration. After incubation for 5 or 10 h, CCK-8 (100 μL, 10% in serum free culture medium) was added to each well, and then the plates continue to be incubated



Scheme 1. Possible response mechanism of probe **1** toward Cys: above) the probe **1** reversibility response to Cys in vitro; bottom) the probe **1** response to Cys in cell and in vivo environment.



Scheme 2. Synthesis of probe **1**.

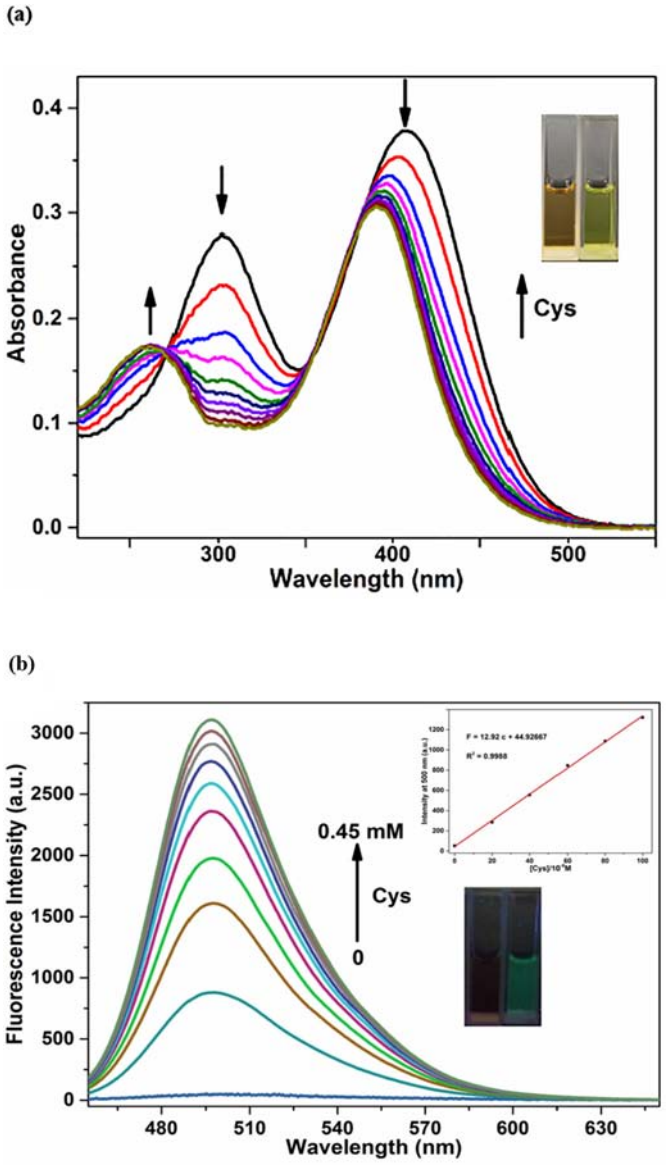


Fig. 1. (a) Absorption spectra of probe **1** (10 μM) upon addition of Cys (0–450 μM) in PBS–DMSO buffer (10 mM, pH 7.4, 4:1, v/v) system. (b) Fluorescence spectra of probe **1** (10 μM) upon addition of Cys (0–450 μM) in PBS–DMSO buffer (10 mM, pH 7.4, 4:1, v/v) system. Working curve of probe **1** to detect Cys obtained by addition of various concentrations of Cys (0–100 μM) to probe **1** (10 μM). Each spectrum was obtained 3 min after addition. (λ_{em} = 500 nm, λ_{ex} = 440 nm, slit: 5 nm/5 nm).

for another 1 h. The optical densities of each well at 450 nm were then measured by a microplate reader (Scheme 2).

2.5. Mice imaging

The bal. a/c mice were fed commercial mice chow in individual stainless steel cages and left freely wandering in their housing for 2 weeks with 12 h dark/light cycles for acclimatization before the experiment. The mice were in anesthesia by Pentobarbital sodium (100 μL, 0.5 mL/0.03%) subcutaneously injection. The mice were injected in the subcutaneous with 100 μM of probe 1, 2 equal of Cys was slowly injected to the same location. Then, the fluorescence images were performed at Equal difference period of time (0.5, 5, 10, 20, 25, 30 min). Images were carried out on using an excitation laser of 474 nm and emission was collected 520 ± 5 nm filter.

3. Results and discussion

3.1. The selective response of probe *1* to Cys

The level of probe activity (simple mixture, plasma, in vitro environment or organism) was depended on the selectivity of the

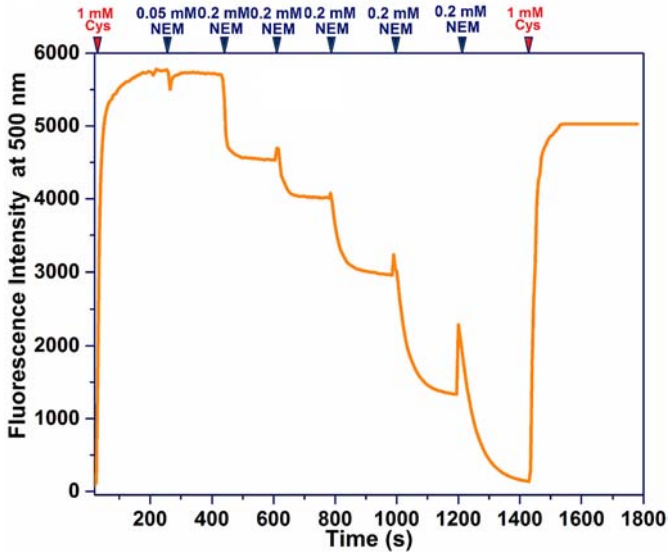


Fig. 2. Fluorescent (500 nm) of probe **1** (10 μM) upon addition of Cys (20 mM) and NEM (20 mM). Solvent: 10 mmol/L sodium phosphate buffer, pH 7.4. Cys and NEM concentrations in the figure are the final concentrations in the cuvette (λ_{em} = 500 nm, λ_{ex} = 440 nm, slit: 5 nm/5 nm).

probe. So for a probe, the specific selectivity for a substance is extremely important. Subsequent the selective experiment of probe toward Cys was implemented, the fluorescence response of the probes to various analytes including amino acids and some of ions in PBS–DMSO buffer (10 mM, pH 7.4, 4:1, v/v) was determined (Fig.S6). 0.2 mol/L various analytes including amino acids, peptide, metal cations and anions. Furthermore, analysis of fluorescence enhancement at 500 nm (λ_{ex} = 440 nm) of probe **1** with other amino acids and some of ions illustrated discriminate ability in the following order: Cys > Hcy > GSH ≈ other amino acids ≈ metal cations and anions. Thus, probe **1** appears to be a relatively high selectivity probe for Cys.

3.2. Spectral properties of probe *1* toward Cys

Moreover, pH-effect of probe **1** and the probe **1**–Cys system was measured with pH change from 2 to 11 (Fig. S7). The probes itself displayed inappreciable intensity boost in pace with the pH of the detection system changing. In the range of pH from 5 to 9, the spectra of the system indicated strong fluorescent emission at 500 nm (λ_{ex} = 440 nm). The results illustrated that probe **1** could detect Cys could be realized over normal physiological conditions (pH = 7.4).

Detailed spectral titrations for Cys were carried out. The UV–Visible spectrum response of probe **1** (10 μM) toward Cys (0–450 μM) was studied in PBS-DMSO (10 mM, pH 7.4, 4:1, v/v) system. Probe **1** (10 μM) itself

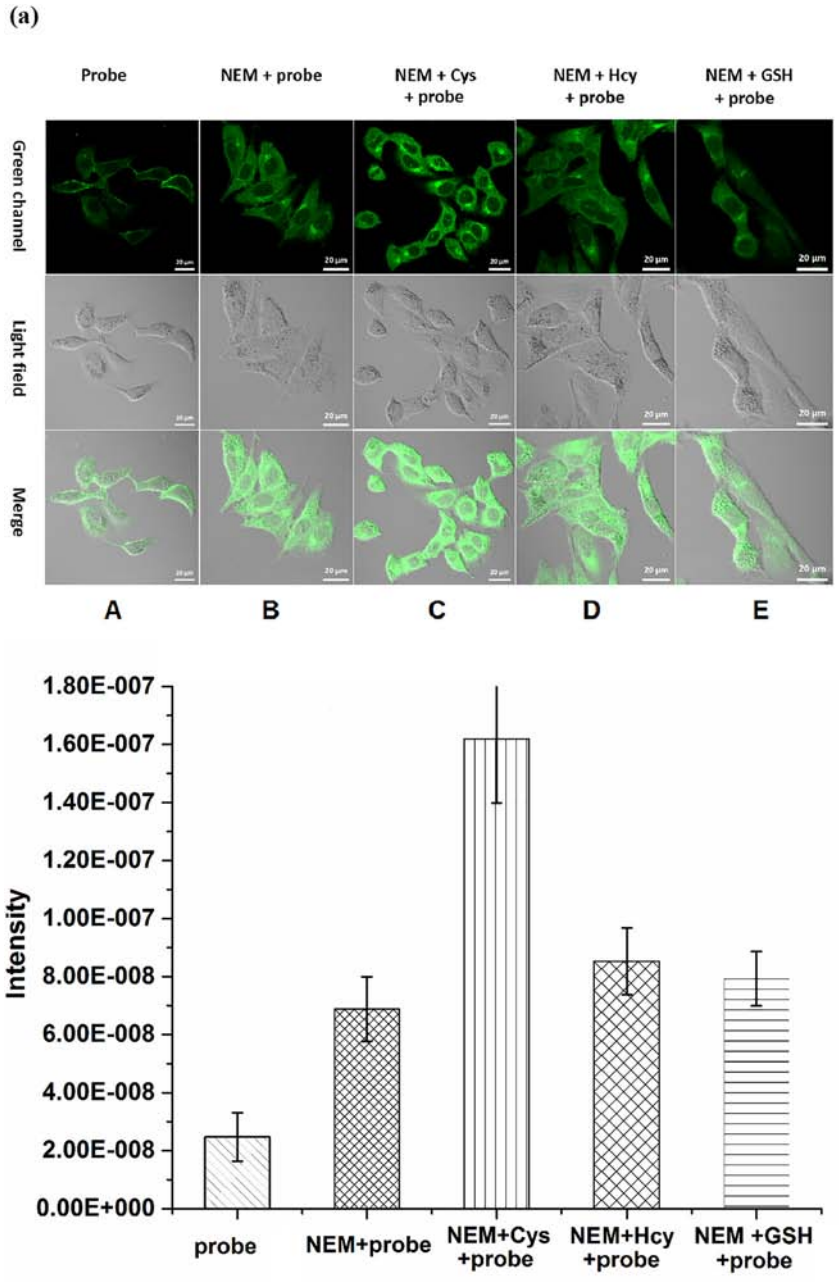


Fig. 3. (a) Top: Cell images of HepG-2 cells lines. A: HepG-2 cells incubated with probe **1** (10 μM) for 0.5 h; B: HepG-2 cells pre-treated with 1 mM NEM for 0.5 h, then incubated with probe **1** (10 μM); C, D, E: Cells were pre-treated with 1 mM NEM for 0.5 h, then incubated with 200 μM Cys/15 μM Hcy/1 mM GSH for 0.5 h, then incubated with probe **1**(10 μM). Green channel: λ_{em} = 480–530 nm (λ_{ex} = 458 nm) Scale bar: 20 μm. Bottom: Fluorescence intensity per cells. (b) Cysteine visualization distribution analysis on cytomembrane of cell imaging by probe **1** incubation (from cytomembrane to cytoplasm to cytomembrane or from cytomembrane to cytoplasm to nucleus to cytoplasm to cytomembrane). (c) Time-dependent confocal images of endogenous Cys in A549 cells with probe 1 (10 μM). Green channel: λ_{em} = 480–530 nm (λ_{ex} = 458 nm) Scale bar: 20 μm.

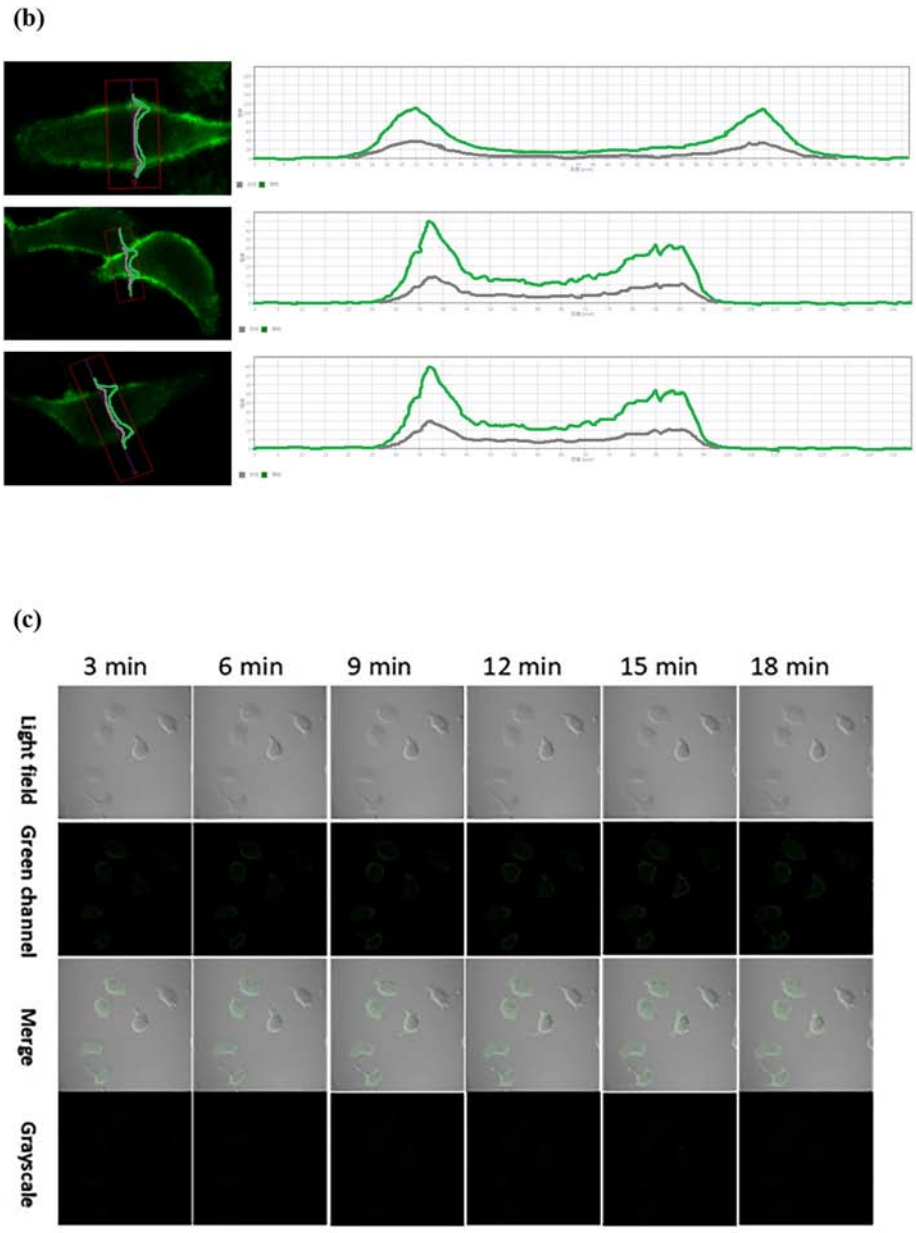


Fig. 3 (*continued*).

featured two absorption peaks at 312 nm and 403 nm in an aqueous buffer solution of PBS-DMSO (10 mM, pH 7.4, 4:1, v/v) (Fig. 1a). With adding Cys (450 μM), the both absorption peak decreased and an obvious new band at 262 nm appeared with isosbestic point at 270 nm forming, which implied new compounds generated in the system. Fig. 1b showed the fluorescence spectra change of probe **1** (10 μM) upon adding Cys (0–450 μM) in PBS-DMSO (10 mM, pH 7.4, 4:1, v/v).

Probe **1** itself had non-fluorescent emission, until the addition of Cys (450 μM) induced a turn-on emission at peaked 500 nm with a 61-fold enhancement in above system. The fluorescence intensity was in good agreement with Cys concentration in the linear range ($R^2 = 0.9988$). Based on the detection limit defined by IUPAC (CDL = 3Sb/m), the corresponding of probe **1** for Cys was 1.22 μM from 10 blank solutions.

3.3. Time-dependence in the detection process of Cys

The kinetic study experiment of probe **1** to Cys was implemented that monitored fluorescence spectra of the probe system was in the presence of 10 eq. of Cys. The fluorescent intensity of the above system was balanced and kept within 200 s (Fig. S8), indicating that this probe has the potential for real-time detecting Cys in vivo.

3.4. Reversibility test in vitro

In order to further test probe **1** whether has the potential for real-time detecting Cys in vivo and whether NEM enable the probe to reversibly detect Cys in vitro, the reversibility test of probe **1**-Cys system was carried out: the fluorescence emission intensity at 500 nm (λ_{ex} = 440 nm) were monitored upon addition of Cys followed by addition of NEM (Fig. S9). The result displayed that the intensity at 500 nm of the system enhanced immediately after addition of Cys, and addition of NEM subsequently 6 times resulted in decreased rapidly and step by step of the fluorescence intensity, then restored by adding Cys, confirming reversibility. The reversible response was estimated by the fluorescence response of the probe **1**-Cys system under the action of NEM. NEM could effectively terminate the fluorescent emission of probe **1**-Cys system and which could be regained by subsequent

addition of Cys (Fig. 2). The reversible cycling tests–that is to say, three times addition of Cys and subsequent NEM added all induced consistent fluorescent signal changes, which promoted probe **1** as a potential effective tool for real-time determination of Cys content in vitro. It was verified that NEM reduced the fluorescence intensity of the probe **1**-Cys system by consuming Cys.

3.5. Proposed mechanism

^{1}H NMR and ESI-MS experiments were performed to clear the sensing mechanism of probe **1** toward Cys. The ^{1}H NMR data displayed that the bimodal signals at 8.00, and 7.71 part to α, β-unsaturated ketone double bonds disappeared, and new signals appeared at 4.50/3.70/3.40 (Fig. S10). To further testify that the response site of Cys with probe **1** is the double bond of α, β-unsaturated ketone, the ^{1}H NMR titration experiment of 2-mercaptoethanol (a Cys analogue) with probe **1** was implement (Fig. S11). The results showed that the site of the reaction with 2-mercaptoethanol is α, β-unsaturated ketone double bonds (the bimodal peaks of 8.00 and 7.71 are weakened), the proton e and f of probe **1** shift from 8.00, 7.71 to 4.49 and 3.63. Additionally, ESI-MS (Fig. S12) data of the reaction mixture of probe **1** with Cys displayed signal at 649.1962 which matched the structure [**1**-Cys + H]$^+$ and other fragments (m/z = 671.1781, [**1**-Cys + Na]$^+$). Further, the fluorescent emission of the reaction mixture of probe **1** with 2-mercaptoethanol matched the Cys added probe **1** system very well (Fig. S13). These results provided strong support, wherein fluorescence signal enhancement of probe **1** responses Cys by nucleophilic addition reaction of –SH of Cys with α, β-unsaturated ketone double bonds of the probe caused.

3.6. Fluorescence imaging in living cells

To evaluate the capability of NEM that regulate probe **1** to come true high efficient detect Cys in cytoplasm. Herein, HepG-2 cells were chosen for the confocal fluorescence imaging. Cell Counting Kit-8 (CCK-8) was carried out to evaluate the cytotoxicity of probe **1**. The result revealed that low cytotoxicity (83.9% viability) and good biocompatibility of probe **1** (20 μM) to HepG-2 cells at 37 °C for 5/10 h (Fig. S14), implied the probe has the potential to be used on living cells and mouse.

HepG-2 cells incubated with probe **1** showed obvious green fluorescence on cytomembrane (Fig. 3a (A) and Fig. 3b), indicating that probe **1** could answer Cys on the cytomembrane. While the cells were sequentially incubated with NEM and probe **1**, the obvious strong fluorescent in HepG-2 cells cytoplasm or not on cytomembrane or none-fluorescence in cells cytoplasm (Fig. 3a), indicating the role of NEM here was not to eliminate thiols in the cytoplasm as usual, and Cys content in cytoplasm is much higher than that of Cys on cytomembrane. Besides, the exogenous Cys result in that a more obvious fluorescence increase in green channel. The above imaging results of this probe were consistent with the imaging results of that probe in the previous work. Comparing fluorescence intensity in the Green Channel of four images of B/C/D/E in Fig. 3(a), showed that probe **1** can specific detect Cys, this was consistent with spectral data results, also verified the following conjecture: probably, NEM does not eliminate the thiols (GSH/Hcy/Cys) in the cytoplasm, but only improved membrane permeability by NEM reacting with sulfydryl of membrane protein which made probe permeate high efficiently and detect the Cys in cytoplasm. It is the first time improved membrane permeability by NEM reacting with sulfydryl of membrane protein which made probe permeate high efficiently and detect the Cys in cytoplasm. It is the first time noted that NEM can regulate Cys probe containing-carboxyl for high efficient detection in cytoplasm. Further experiments to reveal the above conclusion are on-going in our lab.

3.7. Fluorescence imaging in mice

Next, we investigated the utility of probe **1** in mice. The mice were subcutaneously injected with probe **1** (20 μL 0.1 mM) and succedent Cys (20 μL, 0.2 mM) solution at different times after subcutaneous injection at the right rear side. The fluorescent intensity of the mouse enhances inch by inch over time and reaches maximum value in 20 min, was maintained by the body later (Fig. 4c-h).

4. Conclusions

In summary, probe **1** can be recycled three times or more in the presence of NEM in vitro, indicating that this probe could reflect Cys dynamics in the detection system on line. In this work, we found that probe containing-carboxyl just stagnated in cytomembrane due to carboxyl

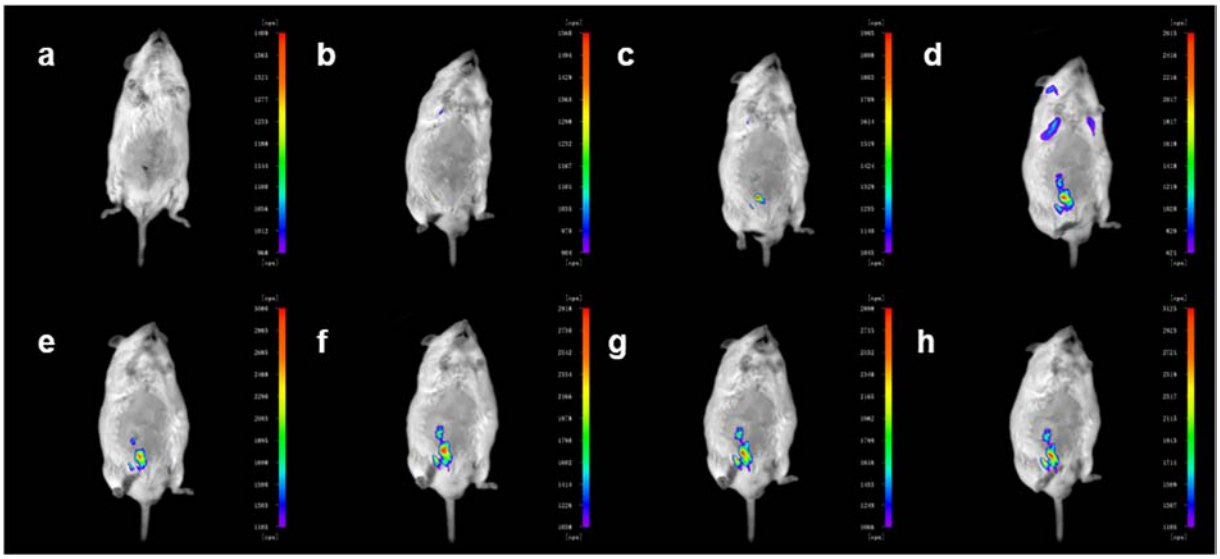


Fig. 4. Fluorescence images of probe **1** responding to exogenous Cys in mice. (a) Mice without any treatment, (b) 100 μM of probe **1** was subcutaneously injected only, (c-h) Representative fluorescence images (pseudocolor) of living mice treated with probe **1** (20 μL, 0.2 mM) and succedent Cys (20 μL, 0.2 mM) solution at different times (0, 0.5, 5, 10, 20, 25, 30 min) after subcutaneous injection. Images were taken using an excitation laser of 474 nm and an emission filter of 515–525 nm.

of probe and amino group of membrane protein forming peptide chains, nevertheless, the addition of NEM, improved membrane permeability by NEM reacting with sulfydryl of membrane protein, which made probe permeate high efficiently and sequentially real-time detect the Cys in cytoplasm. It is the first time noted that NEM can regulate Cys probe containing-carboxyl for high efficient detection in cytoplasm. Additionally, probe was successfully applied to image Cys in mouse.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.117517.

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