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A cysteine-selective fluorescent probe for monitoring stress response cysteine fluctuations†

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Rare studies provided evidence for the real-time monitoring of stress response cysteine fluctuations. Here, we have successfully designed and synthesized a cysteine-selective fluorescent probe 1 to monitor stress response Cys fluctuations, providing visual evidence of Hg²⁺ regulated cysteine fluctuations for the first time, which may open a new way to help researchers to reveal the mechanism of heavy metal ion poisoning.

Biological thiols, indispensable reactive sulfur species (RSS) in the body, are present in the form of low molecular weight thiols or protein thiols, mainly including cysteine (Cys), glutathione (GSH), homocysteine (Hcy) and cysteine residues in proteins. And they can transform into different oxidative intermediates when suffering from intracellular or external environmental changes. They play important roles in various physiological processes, such as maintaining redox balance, protein synthesis and detoxification.¹ Many studies have suggested that the endogenous concentration changes of these thiols were associated with the functional status of the corresponding enzymes and proteins. Their abnormal levels are related to diseases.^{2–5} Hcy can be catalyzed by cystathionine β -synthase (CBS) to condense with serine to form cystathionine, which is then hydrolyzed to Cys by cystathionine γ -lyase (CSE). Cysteine is one of the natural components that make up glutathione (GSH).⁶ It is a non-essential amino acid for the human body and mainly exists in proteins. Abnormal levels of cysteine

caused many diseases, such as slow growth in children, liver damage, ischemic stroke, hair pigmentation, edema, lethargy, skin lesions and weakness, muscle and fat loss, *etc.*⁷ Due to a similar structure and sensitivity to the environment of biological thiols, it remains a great challenge to develop novel tools for their discriminative and dynamic detection.

The traditional detection methods adopted for Cys analysis mainly include high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). These methods can provide super resolution for Cys detection but required high-cost instruments, complicated pre-processing, long inspection time and other limitations.⁸ A critical fact is that the aforementioned methods cannot accomplish the real-time monitoring of species in living systems, which is especially important for environmentally sensitive species. Fluorescence imaging technology has gradually entered into the field of view of researchers due to its advantages of non-invasiveness, high specificity, visualization and real-time dynamic monitoring. And numerous fluorescent probes have been widely used in the imaging of biologically reactive species to reveal life phenomena in cells or *in vivo* in recent years,^{6–11} including related research on cysteine monitoring. A series of receptors were selected and used as recognition groups for probe design, such as 2,4-dinitrobenzenesulfonyl,^{12–16} acryloyl,^{17–23} α,β -unsaturated ketone,²⁴ nitrobenzodioxazole (NBD) and others,^{25–27} and we have summarized and compared the differences among them (Fig. 1A and Table S1, ESI†). Although so many fluorescent probes have been developed for the detection of Cys, the current probes suffered from one way or another inadequacy (biocompatibility, selectivity and sensitivity), which greatly limit their application during the practical process. Furthermore, most of studies focus on novel probe design, rare researches have considered used them for real-time monitoring the stress response cysteine fluctuations to reveal its roles in living system. Therefore, the development of a novel imaging strategy to provide visual evidence for the stress response Cys fluctuations is needed.

Taking all these factors into account, we intend to design and synthesise a cysteine-selective fluorescent probe to monitor Cys fluctuations and provide visual evidence for the stress

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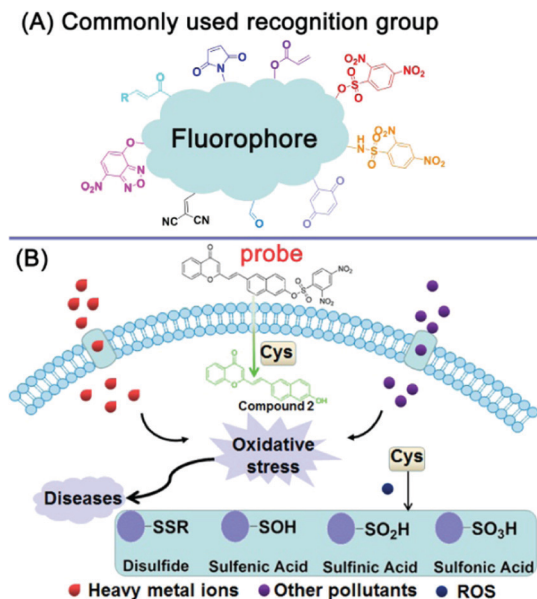


Fig. 1 (A) Commonly used recognition group for probe design in previous work. (B) The designed probe for monitoring the stress response cysteine fluctuations.

response Cys fluctuations in living systems. In recent years, our lab has been endeavoring to develop a series of fluorescent probes for the detection of endogenous biological species.^{28–31} We have developed a chemical covalent tactic for bio-thiol sensing using compounds containing a methyl sulfoxide moiety and accomplished protein thiol labeling.³² Also, we have successfully evaluated the protective effects of mitochondrial GSH during cerebral ischemia/reperfusion injury using a near-infrared GSH fluorescent probe.³³ In this work, we selected compound 2 (Fig. 1B) as a fluorophore because its skeleton is suitable for ratiometric fluorescent probe design, which was already discovered in our previous work.³² 2,4-Dinitrobenzenesulfonyl was selected as a recognition group because of its two characteristics: one is that it is a strong electron-deficient group and can effectively quench the fluorescence of fluorophores; the other is that it can be replaced by a strong nucleophile, which may provide a potential possibility of discriminatively detecting biological thiols relaying on their ionization constants (pK_a , Cys, 8.3; GSH, 8.8; Hcy, 10.0). Thus, as expected, we have successfully designed and synthesized fluorescent probe 1 to detect Cys with high sensitivity and selectivity over GSH and Hcy. It can be used for imaging endogenous and exogenous cysteine in living cells. More importantly, we also successfully monitored Cys fluctuations and confirmed the relationship between the intracellular Cys level and oxidative stress through Hg^{2+} and H_2O_2 stimulations (Fig. 1B).

The probe was synthesized in two steps and the detailed route is shown in Scheme S1 (ESI[†]). Firstly, the fluorophore (compound 2) was obtained by the coupled reaction of methyl and aldehyde groups according to our previously reported method.³³ Subsequently, it reacted with 2,4-dinitrobenzenesulfonyl chloride under

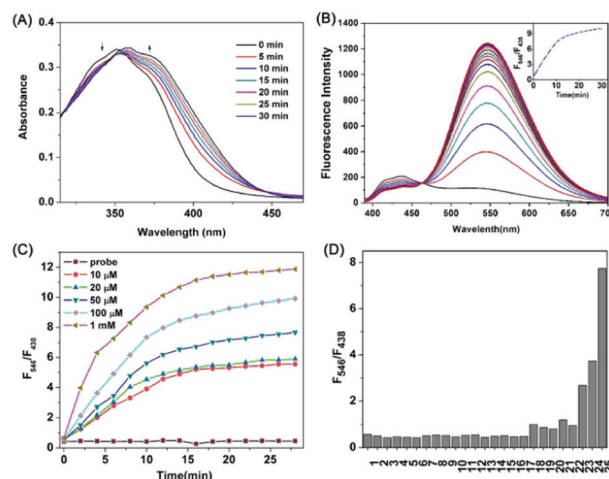


Fig. 2 The spectral studies of the probe in HEPES–EtOH buffer (3/2, v/v, pH = 7.40). (A) Time-dependent absorption spectra of probe 1 (10 μ M) towards Cys (100 μ M), (B) time-dependent fluorescence spectra of probe 1 (10 μ M) towards Cys (100 μ M), (C) time- and dose-dependent fluorescent response of probe 1 (10 μ M) towards Cys (0–1 mM), and (D) probe 1 in the presence of various analytes (100 μ M). 1–25 represent probe, Mg^{2+} , Na^+ , K^+ , Ca^{2+} , NO_3^- , Tyr, L-ascorbic acid, Asp, His, Val, Pro, Ala, Phe, Ser, Gly, Thr, GSSG, ClO^- , cystine, Hg^{2+} , H_2O_2 (500 μ M), Hcy, GSH, and Cys.

organic bases to give probe 1. All compounds were characterized using NMR and MS spectra in Fig. S1–S6 (ESI[†]). With the probe in hand, we set out to determine its spectral properties. Probe 1 (10 μ M) and Cys (100 μ M) were measured in HEPES–EtOH buffer (3/2, v/v, pH = 7.40). As can be seen from Fig. 2A, probe 1 showed the main absorption band at 350 nm, and then shifted to 375 nm after Cys was added to the solution as time increased. In fluorescence spectra (Fig. 2B), probe 1 has weak blue fluorescence and the maximum emission wavelength center at 438 nm. When Cys was added into the solution of probe 1, the fluorescence at 438 nm gradually decreased, meanwhile the fluorescence at 546 nm increased rapidly within 10 min, and then saturated slowly. The time- and dose-dependent fluorescent response of probe 1 towards Cys was also investigated (Fig. 2C). We further determined that the probe has a superior detection limit of up to 2.89 nM ($3\sigma/k$) for Cys (Fig. S7, ESI[†]),³⁴ which is lower than those of many reported probes (Table S1, ESI[†]). The response mechanism was confirmed by HPLC analysis and HRMS (Fig. S8, S9 and Scheme S2, ESI[†]). And the fluorophore displayed aggregation-induced emission character (Fig. S10, ESI[†]).³⁵ The biological environment is commonly complex, so it is important and necessary for the probe to have good selectivity. In order to confirm the selectivity and specificity of probe 1 for Cys, the potential interferent was investigated, including some biological thiols (GSH and Hcy), anions, cations (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , and Hg^{2+}), some amino acids (Tyr, L-ascorbic acid, Asp, His, Val, Pro, Ala, Phe, Ser, Gly, and Thr), and ROS (H_2O_2 and ClO^-). As shown in Fig. 2D, both GSH and Hcy elicited a certain enhancement much less than that of Cys at a F_{546}/F_{438} ratio, while others did not cause any fluorescence changes. Considering high concentrations of Na^+ and K^+ , we investigated the fluorescent response of the probe towards them. As shown in Fig. S11 (ESI[†]), they did not affect Cys detection.

These results proved that our probe 1 has high sensitivity and selectivity for the detection of Cys.

In light of the above excellent spectral studies, we wonder whether probe 1 has practical application in imaging in living cells. Before the imaging experiments, the MTT assays were carried out using Hep G2 cells. The cells have a high survival rate (more than 90%) in the presence of a probe (0–50 μM , Fig. S12, ESI[†]). It is worth mentioning that the survival rate of the cells was more than 99% when the concentration of probe 1 was 10 μM , suggesting that the probe has not caused great damage to the cells with low toxicity, and can be applied in cell experiments.

To evaluate the ability of the probe to monitor Cys in living cells, we applied it in the imaging of endogenous Cys using Hep G2 cells. The cells were incubated with the probe and observed for 5, 15, 20, and 30 min, respectively. As shown in Fig. 3, green fluorescence signals gradually appeared and became strong with time. The probe is mainly distributed in the cytoplasm but the nucleus (Fig. S13, ESI[†]). The probe was also successfully applied in imaging Cys in other cell lines, including A549, SH-SY5Y and HL-7702 (Fig. S14, ESI[†]). The results indicated that our probe can be used for the detection of endogenous Cys in living cells.

In order to further prove that our probe can be used for monitoring endogenous Cys fluctuations, we have performed a series of imaging experiments. Exogenous Cys was introduced into living cells by pretreatment with Cys for 30 min, and the cells were further incubated with the probe for another 30 min. As shown in Fig. 4f and g, the green fluorescence signals obviously become stronger due to elevation of the Cys level by exogenous addition. To impoverish the amount of Cys, a thiol scavenger *N*-ethylmaleimide (NEM) was selected for blocking them. The Hep G2 cells were incubated with NEM for 30 min and then with the probe for 30 min. Confocal imaging results showed that green fluorescence was significantly weakened and nearly disappeared after NEM treatment (Fig. 4h). The fluorescence can be restored by the subsequent exogenous addition of cysteine (Fig. 4i). These results firmly confirmed that our probe is a practical tool for monitoring Cys fluctuations. In a living system, cysteine existed in the form of an independent individual or protein cysteine (including RSH and RSSR').³⁶ Under oxidative stress, RSH can be oxidized and transformed into disulfide. In order to understand protein cysteine fluctuations, we used NEM to block thiols and then treated with tris(2-carboxyethyl)-phosphine (TCEP, a reducing agent commonly

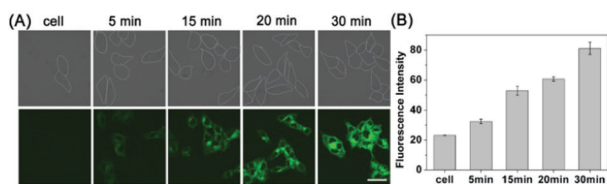


Fig. 3 (A) Time-dependent imaging of endogenous cysteine in Hep G2 cells using probe 1 (10 μM), scale bars: 20 μm . (B) Fluorescence intensity was quantified by ImageJ.

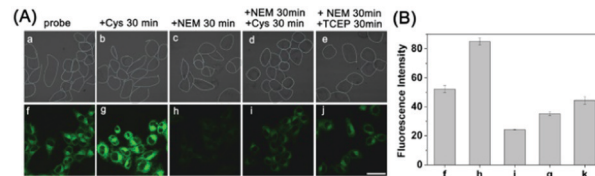


Fig. 4 (A) Imaging of Cys fluctuations in living cells using probe 1. The cells were incubated with probe 1 for 30 min (a and f). The cells were pretreated with exogenous Cys (100 μM) before incubation with the probe (10 μM) (b and g). The cells were pretreated with NEM (100 μM) before incubation with the probe (10 μM) (c and h). The cells were sequentially incubated with NEM, Cys and the probe (d and i). The cells were sequentially incubated with NEM, TCEP and the probe (e and j). λ_{ex} = 440 nm, λ_{em} = 500–600 nm. Scale bars: 20 μm . (B) Fluorescence intensity was quantified by ImageJ.

used for cleavage of disulfide in a biological system, 1 mM) for 30 min. After the cells were incubated with the probe, green fluorescence was observed in the channels (Fig. 4j). In this part, we have successfully performed imaging experiments to regulate cysteine level by using NEM and TCEP for evaluation the practical application of probe in monitoring Cys fluctuations.

Heavy metal ions have attracted wide interest due to their deleterious effects on environmental security and public health, such as Hg^{2+} and Cd^{2+} .³⁷ These metal ions easily accumulated in the body through the food chain and eventually lead to a number of serious diseases. However, there are rare research studies providing visual evidence of how heavy metal ions affect endogenous biological molecule fluctuations. Considering the high affinity of Hg^{2+} to sulfur, we intend to explore the relationship between Hg^{2+} and cysteine levels. As shown in Fig. 5A, the low concentration of Hg^{2+} did not cause obvious fluorescence change (less than 20 μM). The fluorescence was greatly quenched when the concentration of Hg^{2+} reached up to 50 μM , suggesting that the cysteine level was regulated by Hg^{2+} . These results are mainly due to two factors, one is that the coordination of Hg^{2+} with cysteine (including the protein

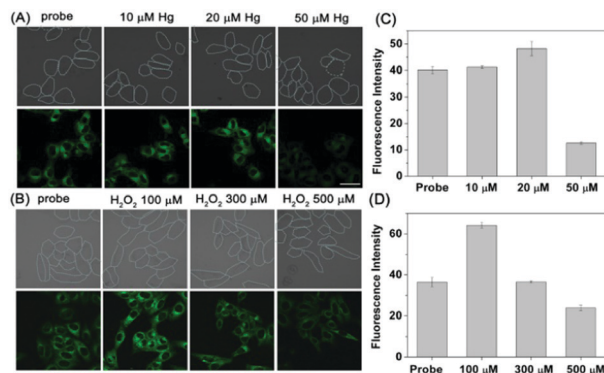


Fig. 5 Imaging of the stress response cysteine fluctuations using the probe. (A) The Hep G2 cells were incubated with different concentrations of Hg^{2+} (0, 10, 20, and 50 μM) for 15 min before the addition of the probe. (B) The Hep G2 cells were incubated with different concentrations of H_2O_2 (0, 100, 300, and 500 μM) for 30 min before the addition of the probe. Scale bars: 20 μm . (C and D) Fluorescence intensity was quantified by ImageJ.

cysteine residue) directly decreased the cysteine concentration; the other is that oxidative stress burst caused by coordination of Hg^{2+} reduces the antioxidant capacity of the system, which further decreased the cysteine concentration. Hg^{2+} could induce oxidative stress burst which has been confirmed in our previous work.^{38,39} To clarify the Hg^{2+} regulated cysteine level, we carried out imaging experiments to monitor cysteine fluctuations by pretreatment with different concentrations of H_2O_2 . As shown in Fig. 5B, the fluorescence enhanced slightly when the cells were incubated with 100 μM H_2O_2 . It is not surprising that the cells will promote the biosynthesis of cysteine against oxidative stress when within the regulatory capacity of biological antioxidant systems. When the concentrations of H_2O_2 increased (more than 300 μM) and exceed self-regulation, the cysteine will be consumed leading to fluorescence decay. The fluorescent change owed to the regulated Cys fluctuation rather than cell apoptosis and confirmed *via* cytometric apoptosis assay using flow cytometry analysis, Hoechst 33342 and CCK-8 kit (Fig. S15–S17, ESI†). These results indicated that the probe can be used to monitor Hg^{2+} induced cysteine fluctuations in living cells, providing visual evidence of Hg^{2+} regulated cysteine fluctuations for the first time, which may open a new way to help researchers to reveal the mechanism of heavy metal ion poisoning.

In summary, we have successfully designed and synthesized a cysteine-selective fluorescent probe 1 to monitor Cys fluctuations. The solid spectral and imaging results demonstrated that probe 1 has excellent performance in monitoring endogenous and exogenous cysteine. The detection limit of the probe for Cys can reach 2.89 nM. Finally, we used the probe to monitor Hg^{2+} induced cysteine fluctuations in living cells, providing visual evidence of Hg^{2+} regulated cysteine fluctuations for the first time, confirming the relationship between the intracellular Cys level and oxidative stress caused by Hg^{2+} . This work may open a new way to help researchers to reveal the mechanism of heavy metal ion poisoning and provide insights into the roles of Cys in future research.

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Conflicts of interest

There are no conflicts to declare.

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