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A new turn-on fluorescent probe for selective detection of glutathione and cysteine in living cells†

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Mingjie Wei, Peng Yin, Youming Shen, Lingli Zhang, Jianhui Deng, Shanyan Xue, Haitao Li, Bin Guo, Youyu Zhang* and Shouzhuo Yao

A fluorescent probe (*N*-(4-methyl-2-oxo-2*H*-chromen-7-yl)-2,4-dinitrobenzenesulfonamide), which exhibits high selectivity to glutathione and cysteine among amino acids including sulphur-containing methionine and metal ions, was synthesized. The experiments demonstrate that the fluorescent probe is a reliable and specific probe for glutathione and cysteine in living cells.

The development of detection methods for thiols has attracted continuing interest because of their important roles in biological and pharmacological processes. There are several analytical methods reported for the analysis of thiols in biological samples, such as high performance liquid chromatography,[1] electrophoresis-based methods,[2] flow-injection analysis,[3] spectrophotometry,[4] voltammetry[5] as well as electrospray ionization-mass spectrometry.[6] However, these conventional methods are complex and expensive.

In recent years, some fluorescence detection methods have been reported.[7] Qu's group reported a facile, label-free, highly sensitive sensor for detection of biothiols based on silver metallization engineered conformational transition of the G-quadruplex.[8] Ma and colleagues developed a luminescent G-quadruplex switch-on probe for selective and tunable detection of cysteine (Cys) and glutathione (GSH).[9] Hepel and Xu reported a fluorescence turn-on "molecular beacon" for detection of GSH and Cys.[10] Moreover, the fluorescent probes have become an important diagnostic tool due to their versatile applications in chemical, biological and environmental fields.[11] However, most of the previously reported fluorescent sensors for GSH and Cys also have marked responses to homocysteine (Hcy) and have low selectivity to GSH and Cys. Some of them have a relatively low sensitivity. There is an urgent need to develop novel reliable analytical methods. Herein, we report a new and simple strategy for the determination of GSH and Cys both *in vivo* and *vitro*.

Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research (Ministry of Education), College of Chemistry and Chemical Engineering, Hunan Normal University, Changsha 410081, PR China.
E-mail: zhangyy@hunnu.edu.cn; Fax: +86 73188872531; Tel: +86 75188865515

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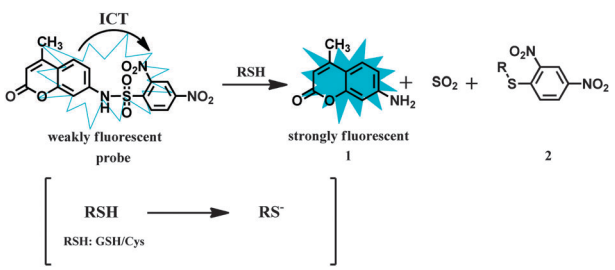
Initially, we proposed the optical probe (*N*-(4-methyl-2-oxo-2*H*-chromen-7-yl)-2,4-dinitrobenzenesulfonamide) (NDS), with the coumarin moiety and a strong electron-withdrawing group that could be applied for the detection of such a reactive nucleophile as GSH or Cys. The coumarin moiety is a fluorescence signaling unit, and the intramolecular charge-transfer (ICT) process occurs in NDS molecule because of the introduction of the strong electron-withdrawing group, 2,4-dinitrobenzenesulfonyl[12] (Scheme 1). When electrons are diverted from 7-amino-4-methylcoumarin to 2,4-dinitrobenzenesulfonyl, the fluorescence of NDS becomes very weak. However, upon removal of the electron-withdrawing 2,4-dinitrobenzenesulfonyl, the fluorescence is turned on. The sulphur anion (RS^-) is the nucleophilic center in biothiols (RSH). When compounds containing sulfhydryl such as GSH and Cys coexist with NDS under appropriate conditions, the removal of 2,4-dinitrobenzenesulfonyl from NDS will occur, resulting in a high fluorescent signal. NDS was synthesized by a nucleophilic reaction of aniline-appended coumarin with 2,4-dinitrobenzenesulfonyl chloride. The synthesis processes and structure characterization (using ^{1}H NMR, ^{13}C NMR, MS and IR) of NDS are provided in the ESI.†

The fluorescence of NDS in the absence and presence of GSH/Cys was investigated. As shown in Fig. 1, in the absence of GSH/Cys, the fluorescence of NDS was very weak (curve a in Fig. 1). The fluorescence intensity was dramatically enhanced in the presence of GSH/Cys (curve b in Fig. 1), resulting from the effective cleavage of the electron-withdrawing



Scheme 1 The principle for the detection of GSH/Cys with NDS.

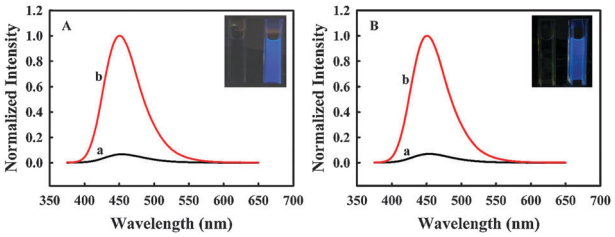


Fig. 1 Fluorescence emission spectra (λ_{ex} = 353 nm) of NDS before (a) and after the addition of GSH (A) or Cys (B) in phosphate buffer (pH 9.0, 0.01 M). The concentrations of NDS and GSH/Cys were 20 μM and 30 μM, respectively. The insets show the photos of samples illuminated by UV light of 365 nm.

2,4-dinitrobenzenesulfonyl moiety from the weakly fluorescent probe NDS.[13] The reaction of GSH with NDS was investigated and described in the ESI.† As expected, the electron-withdrawing 2,4-dinitrobenzene-sulfonyl moiety was effectively dropped from DNS after the reaction, and compound **1** and compound **2** were generated, which were confirmed using NMR (Fig. S5–S8 in ESI†).[14] As shown in the inset of Fig. 1, the probe emitted bright blue light under UV light excitation in the presence of GSH and Cys, suggesting that the fluorescence product was generated in the test system.

Considering the factors affecting the reaction of NDS with GSH/Cys, pH, incubation temperature and time were optimized (Fig. S9, ESI†). As shown in Fig. S9A (ESI†), the fluorescence of NDS increased with increasing pH value and reached the maximum value at pH 9.0. This fact reflects the influence of the pK_a value of aliphatic thiols on the reaction.[15] In a reaction medium, GSH/Cys can be ionized to a reactive nucleophilic neutral form (RS^-). The degree of dissociation of the thiol group in GSH/Cys depends on the pH of the medium. The concentration of RS^- increases with the increasing in the pH value. As expected, a dramatic enhancement of the fluorescence intensity was observed as a result of the effective cleavage of the electron-withdrawing 2,4-dinitrobenzene-sulfonyl moiety from the weakly fluorescent probe NDS by GSH/Cys in an alkaline solution. Therefore, a pH 9.0 phosphate buffer was selected as the working buffer in the following experiments. Furthermore, the effects of incubation temperature and time were investigated. The experimental result suggested that the optimum temperature was 45 °C (Fig. S9B in ESI†). Therefore, 45 °C was chosen as the favorable incubation temperature for the detection of GSH/Cys. As shown in Fig. S9C (ESI†), the fluorescence intensity at 450 nm increased sharply upon increasing the incubation time from 0 h to 2 h and then reached a constant value after 2 h. This demonstrates that the interaction of NDS with GSH/Cys reached equilibrium within 2 h. Thus, the incubation time was controlled at 2 h. Furthermore, no apparent hydrolysis of NDS was detected in the above experimental conditions.

Under the optimal conditions, we evaluated the capability of the proposed method for quantitative detection of GSH/Cys. The fluorescence responses of NDS with different concentrations of GSH and Cys are shown in Fig. 2. A linear relationship between the fluorescence intensity and the GSH/Cys concentration ranging from 30 nM to 60 μM was obtained. And, a linear

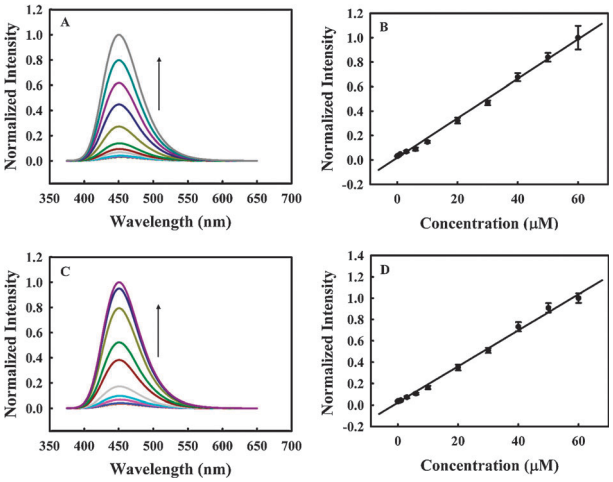


Fig. 2 Fluorescence spectra of NDS in the presence of various concentrations of GSH (A) and Cys (C). A linear relationship of the fluorescence intensity *vs.* the concentrations of GSH (B) and Cys (D). The concentrations of GSH and Cys were from 30 nM to 60 μM. The error bars represent the standard deviation of three independent measurements.

regression equation for GSH, $F = 0.0189 + 0.0161x$, was obtained, where F refers to the measured fluorescence intensity and x refers to the concentration of GSH. The linear correlation coefficient was 0.9966. For Cys detection, the linear regression equation was $F = 0.0225 + 0.0169x$, with a linear correlation coefficient of 0.9956.

To evaluate the selectivity of the developed method for GSH/Cys, a series of metal ions, amino acids and bovine serum albumin (BSA) were detected. The results are shown in Fig. 3. Only GSH and Cys can cause remarkable fluorescence responses. Because no thiol anion was produced in this test system, other amino acids including sulfur-containing methionine and metal ions had no apparent interference in the detection. 50 μM Hcy only caused a little fluorescence response. Under the experimental conditions, the capability of GSH, Cys and Hcy to form RS^- is of the order GSH > Cys > Hcy (their corresponding pK_a are 9.12, 10.29 and 10.86, respectively).[16] Theoretically, the response of GSH should be greater than Cys. But due to the greater steric hindrance of GSH, the response of GSH was in

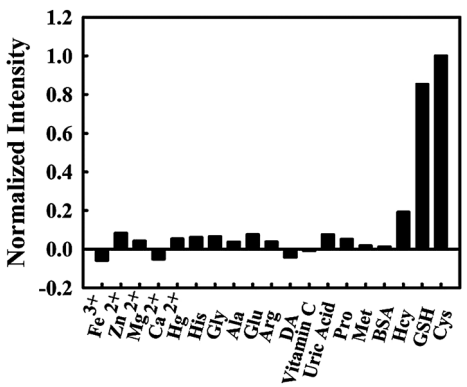


Fig. 3 Fluorescence responses of NDS to different analytes. The concentration of NDS was 20 μM. GSH, Cys and BSA were used at 20 μM, Hcy at 50 μM and other analytes were used at 1 mM.

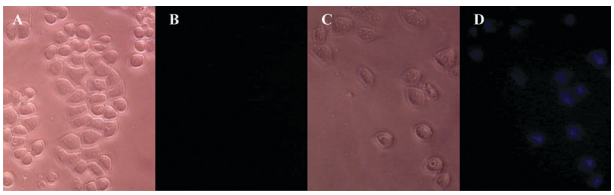


Fig. 4 Fluorescence microscopy images of Hela cells. (b) Images of cells pretreated with *N*-methylmaleimide (0.5 mM) for 1 h at 37 °C and then incubated with NDS (30 μM) overnight at 37 °C. (d) Images of cells incubated with NDS (30 μM) overnight at 37 °C. (A) and (C) represent the bright-field images of (B) and (D), respectively.

fact slightly smaller than Cys. Hcy should be the least negatively charged among the three analytes because of its highest pK_a value, so the response of Hcy is much lower than GSH/Cys. The reaction of NDS with BSA did not show any increase in the fluorescence intensity. This can be explained by the fact that most of the cysteines in BSA participate in disulfide bonds and BSA has a large steric hindrance.[17] These results clearly demonstrate that our proposed sensing system is specific for GSH and Cys.

In order to evaluate the function of NDS for biological applications, and clarify whether it is sensitive enough to detect the physiologically relevant level of GSH/Cys in living cells, we performed an assay to detect intracellular GSH/Cys in living cells. Hela cells were incubated with a solution of NDS (30 μM, 1 : 99 DMSO–PBS v/v, pH 7.4) overnight at 37 °C. The results of fluorescence microscopic observations are shown in Fig. 4. It was found that NDS was cell-permeable and could react with intracellular thiols, resulting in strong fluorescence emission (Fig. 4D). The results clearly demonstrate that NDS was able to sense the thiols in living cells. In a control experiment, cells were pretreated with an excess of the thiol-reactive *N*-methylmaleimide to consume all of the free thiols within the cell, and then incubated with NDS (the fluorescence measurement results shown in Fig. S10 (ESI†) indicate that an excess of *N*-methylmaleimide could consume all of the free thiols). As shown in Fig. 4B, the fluorescence microscopy studies did not show a significant fluorescence signal after incubating with NDS.

The results confirm that the fluorescence changes in the Hela cells really resulted from the change of the intracellular thiol level. The bright-field images (Fig. 4A and C) confirm that the cells were viable throughout the imaging experiments. These results demonstrate that NDS is a specific probe for detection of thiols in living cells. The method described here might provide a simple way to monitor alterations of thiol concentration in living cells.

In summary, we synthesized a novel fluorescent probe NDS for selective detection of GSH/Cys. The new synthesized probe, NDS, is a reactive probe of incision-type and can be used to detect GSH and Cys both *in vivo* and *in vitro*. The probe has a low background signal and good sensitivity to GSH and Cys. The developed method exhibits a good selectivity for GSH/Cys over a series of amino acids, metal ions and protein. The application of the probe for the detection of GSH/Cys in living cells has been well-demonstrated, which shows potential applications in disease diagnosis.

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