A Simple Long-wavelength Fluorescent Probe for Simultaneous Discrimination of Cysteine/Homocysteine and Glutathione/Hydrogen Sulfide with Two Separated Fluorescence Emission Channels by Single Wavelength Excitation

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Small molecular biothiols, such as cysteine (Cys), homocysteine (Hcy), reduced glutathione (GSH), and hydrogen sulfide (H₂S), play crucial parts in regulating the redox balance of life activities, regulating normal physiological activities and preventing various diseases. Quantitative analysis of these important small molecular substances is very important for revealing their diverse physiological and pathological effects. Although many fluorescent probes have been reported to detect biothiols in cells, it is still not sufficiently advanced to detect biothiols with separated fluorescence emission peak by same wavelength excitation. In our work, we designed a simple conjugate of Nile red and NBD (7-nitro-1,2,3-benzoazidazole) as long-wavelength fluorescent probe NR-NBD for the simultaneous discrimination of these biothiols at single wavelength excitation. Probe NR-NBD could efficiently discriminate Cys/Hcy, GSH and H₂S by two separated fluorescence emission channels and absorption spectra. Importantly, probe NR-NBD has excellent specificity and sensitivity towards the monitoring of endogenous/exogenous Cys/Hcy and GSH/H₂S in living cells and zebrafish.

Keywords Fluorescent probe, two-channel imaging, biothiol, single wavelength excitation, two separated fluorescence emission

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Introduction

Biological thiols, such as cysteine (Cys), homocysteine (Hcy), reduced glutathione (GSH), and hydrogen sulfide (H₂S), play important parts in adjusting the redox state of biological systems. More and more attention has been paid to them in recent years.1−4 Cys is a precursor of glutathione and a precursor of many proteins, abnormal levels of Cys in the human body can cause hair discoloration, edema, drowsiness, liver injury, muscle relaxation, fat skin relaxation, and weakness.5−10 Hcy is converted from methionine. Hcy predicts not only cardiovascular and cerebrovascular disease, but also depression, miscarriage, birth defects and other conditions.11,12 Glutathione has many cellular functions, including maintenance of intracellular redox activity, xenobiosis, intracellular signal transduction, and gene regulation. Greater glutathione fluctuations are related with some diseases, for instance AIDS and cardiovascular diseases.13 H₂S has the physiological function of dilating blood vessels and lowering blood pressure, and has potential therapeutic value for neurodegenerative diseases such as Parkinson’s disease.14 In addition, some cancer drugs work based on differences in the concentration of thiols in and out of cancer cells.15,16 Therefore, quantitative analysis of these important small molecular substances is very important for revealing their diverse pathological effects and facilitating the early diagnosis and management of diseases.17,18 Fluorescence detection technology has attracted much attention for its advantages as follows: high spatial and temporal resolution, low cost and high sensitivity.19−32 It has been widely used in biological science, new drug development and clinical disease diagnosis. Many probes were designed and synthesized to detect biothiols through different chemical reaction mechanisms.33−48 However, two main design strategies have been widely used. One is to use the nucleophilicity, reductivity and metal ion complexation ability of the mercapto group in the biothiol molecule to react with the probe molecule. The other is to use the fluorescent probe to synergistically react with the mercapto group and amino group in the biothiol molecule. Significant advances have been made in selectivity to distinguish biothiols from other amino acids, and some fluorescent probes are specifically designed to detect one or two biological mercaptans.49−55 However, due to the similar structure and property of biothiols, the probe used to simultaneously detect biothiols still has the following defects. Firstly, the detection of biothiols by fluorescent probe requires two or more groups of excitation wavelengths, which complicates the analysis and increases the measurement error. Secondly, most probes for...
detecting biothiols have too close a range of emission peaks after reaction with biothiols. This caused great difficulties in spectral analysis and the distinction between biothiols. Thirdly, some probes have short excitation and making emission wavelengths, resulting in high phototoxicity, strong photo scattering and absorption by biomolecules, which is difficult to be applied to *in vivo* imaging (e.g. zebrafish). Finally, the synthesis of some probes is too complex with the low yield, which has some shortcomings in popularization and application. Hence, there is an urgent need for the development of a probe with single long wavelength excitation, long wavelength emission, and separated fluorescence emission peaks that can simultaneously discriminate of Cys/Hcy, GSH and H₂S.

Based on the above analysis, in this study, we synthesized probe NR-NBD by the reaction of NBD-Cl (4-chloro-7-nitro-

Table 1  Comparison of fluorescent probes for biothiols

| Probe                     | Selectivity   | λ<sub>ex</sub>/nm | λ<sub>em</sub>/nm | Imaging            | Reference                              |
|---------------------------|---------------|-------------------|-------------------|--------------------|----------------------------------------|
| Cys/Hcy + GSH             | 350           | 517/490           | Living cells      | Anal. Chem. 87 (2015), 3460 - 3466 |
| Cys/Hcy + GSH             | 458           | 621               | Living cells      | Anal. Chem. 88 (2016), 3638 - 3646 |
| Cys + Hcy + GSH + H₂S     | 376           | 473               | Living cells      | Anal. Chem. 91 (2019), 1904 - 1911 |
| Cys/Hcy + GSH + H₂S       | 376           | 480               | Living cells      | ACS Sens. 3 (2018), 2513 - 2517     |
| Cys/Hcy + GSH             | 390           | 535               | Living cells      | Chem. Commun. 51 (2015), 4245 - 4248 |
| Cys + GSH                 | 470           | 585               | /                 | Chem. Commun. 51 (2015), 9388 - 9390 |
| Cys + Hcy + GSH           | 360           | 457               | Living cells      | Angew. Chem. Int. Ed. 57 (2018), 4991 - 4994 |
| Cys/Hcy + GSH + H₂S       | 500           | 548               | Living cells and  | This work                             |
|                           |               | 652               | zebrafish         |                                        |
1,2,3-benzoxadiazole) with long-wavelength Nile red dye (Scheme 1). When we prepared the manuscript, similar structures were reported by Ye et al.\textsuperscript{56} and Ding et al.\textsuperscript{57} In our work, NR-NBD could simultaneously discriminate biothiols by single long wavelength excitation (500 nm). NR-NBD acts on Cys/Hcy to give two separated fluorescence emissions (green fluorescence λ\textsubscript{em} = 548 nm and red fluorescence λ\textsubscript{em} = 652 nm) with 104 nm emission peak displacement, and reacted with GSH/H\textsubscript{2}S to give red fluorescence (λ\textsubscript{em} = 652 nm). In addition, the absorption spectra of GSH and H\textsubscript{2}S with probe NR-NBD were significantly different. So, NR-NBD could effectively detect Cys/Hcy, GSH and H\textsubscript{2}S. Further in\textit{vivo} experiments were carried out, and to our delight, probe NR-NBD could visualize the fluctuations of endogenous/exogenous Cys/Hcy and GSH/H\textsubscript{2}S with satisfactory selectivity and sensitivity in living cells and zebrafish. This is very important and better than those reported (Table 1).

**Experimental**

**Materials and general methods**

Nile red dye (0.67 g, 2 mmol), NBD-Cl (0.48 g, 2.4 mmol) and Et\textsubscript{3}N (0.24 g, 2.4 mmol) were mixed in DMF (12 mL) and stirred at 25 °C for 5 h. The resulting solution was extracted with dichloromethane and a water system, and the product was then filtered and further purified by chromatographic column to obtain a black-purple solid probe NR-NBD (0.56 g, 68%).

\[ ^{1}H \text{ NMR (400 MHz, CDCl}\textsubscript{3}) \delta (ppm): 1.283 (t, J = 7.2 Hz, 6H), 3.475 – 3.528 (m, 4H), 6.460 (s, 1H), 6.520 (d, J = 2.0 Hz, 1H), 6.680 (d, J = 8.4 Hz, 1H), 6.714 (d, J = 2.4 Hz, 1H), 7.516 (dd, J = 2.4, 8.4 Hz, 1H), 7.560 (d, J = 9.2 Hz, 1H), 8.022 (s, 1H), 8.475 (d, J = 8.8 Hz, 1H), 8.513 (t, J = 2.0 Hz, 1H); ^{13}C \text{ NMR (100 MHz, CDCl}\textsubscript{3}) \delta (ppm): 12.59, 45.31, 96.39, 105.57, 108.55, 110.36, 115.39, 122.15, 125.35, 129.20, 130.29, 131.52, 133.18, 145.21, 147.18, 151.44, 152.58, 153.66, 155.22, 157.06, 162.55, 168.94, 182.09, 184.23. HRMS (ESI): Calcd for C\textsubscript{26}H\textsubscript{20}N\textsubscript{5}O\textsubscript{6} [M+H]+ 498.1414; Found, 498.1410.

**Mechanism Studies**

The probe NR-NBD we designed is almost non-fluorescent. When Cys/Hcy/GSH/H\textsubscript{2}S reacted with probe NR-NBD, mercapto nucleophiles act on NR-NBD to form long-wavelength Nile red fluorescent dyes and intermediates with nearly non-fluorescence (NBD-S-Cys/Hcy/GSH/H\textsubscript{2}S) by the thiolysis. NBD-S-Cys/Hcy rapidly conducted intramolecular rearrangement to generate NBD-N-Cys/Hcy, and an apparent emission peak at 548 nm. NBD-S-GSH cannot achieve the above conversion. Especially, NBD-SH displayed a different absorption spectrum from NBD-GSH due to the formation of NBD-S with strong intramolecular charge transfer (ICT) structure. The corresponding HRMS were also achieved (Fig. S1, Supporting Information), respectively. Drawing on existing literature,\textsuperscript{56–58} the course of NR-NBD for effectively discriminating biothiols is explained in Scheme 2.

**Results and Discussion**

**Spectroscopic analysis of NR-NBD**

NR-NBD is almost non-fluorescent at 548 and 652 nm with the excitation of 500 nm in phosphate buffered solution (Fig. 1a). In addition, the quantum yield of the probe and the
Nile red dye were estimated (probe NR-NBD: \( \Phi = 0.001 \); Nile red dye: \( \Phi = 0.284 \)) to further demonstrate the design of the probe was reasonable. NR-NBD acts on Cys/Hcy to show a significant fluorescence signal at 548 and 652 nm. NR-NBD acts on GSH/H\(_2\)S to exhibit only a fluorescence signal at 652 nm. In addition, the absorption spectra of NR-NBD on Cys, Hcy and GSH were significantly lower than before, while NR-NBD on H\(_2\)S exhibited a strong blue-shifted absorption compared with Cys, Hcy and GSH (Fig. 1b). Combined with fluorescence and absorption spectrometry, NR-NBD be able to effectively discriminate Cys/Hcy, GSH and H\(_2\)S. Moreover, we further tested the absorption spectra of Nile red and reaction products.

Fig. 2  Left column from top to bottom: quantitative relationship of NR-NBD in response to Cys, Hcy, GSH, and H\(_2\)S. Right column: linear relationship between fluorescence intensity and biothiols in low concentration range.
of NBD-Cl with the corresponding biothiol to indicate single-wavelength excitation (Fig. S2, SI).

Quantification of biothiols

Sensing properties of NR-NBD towards Cys, Hcy, GSH, and H2S were investigated carefully (Fig. 2). When Cys and Hcy were singly added to NR-NBD (5 μM) solution, the fluorescence significantly enhanced at 548 and 652 nm. In contrast, the fluorescence significantly enhanced at only 652 nm when GSH and H2S were singly dropped into NR-NBD solution. Moreover, the fluorescence gradually enhanced with the increase of biothiol concentrations, and a good linear relationship was satisfied within a certain concentration range of biothiols (A2-D2 in Fig. 2). The detection limits (3σ/k) of Cys, Hcy, GSH and H2S were calculated to be 6.8×10⁻², 3.8×10⁻², 6.6×10⁻², and 6.6×10⁻² μM. Therefore, it could be concluded that probe NR-NBD has quantitative detection ability for Cys, Hcy, GSH and H2S with excellent sensitivity.

Specificity studies

The selectivity of probe NR-NBD in response to biothiols was analyzed by fluorescence spectrometry. Probe NR-NBD showed high selectivity towards Cys, Hcy, GSH, and H2S over other analytes, such as Ala, Gly, Leu, Ser, K⁺, Ca²⁺, Na⁺, Mg²⁺, SO₃²⁻, NO₃⁻, CO₃²⁻, Cl⁻, SO₄²⁻, PO₄³⁻, HSO₃⁻, and H₂O₂. Cys/Hcy and GSH/H₂S produced considerable fluorescence changes, and almost no fluorescence changes were caused by other potential interfering substances (Fig. 3). These data demonstrated that NR-NBD could selectively detect Cys/Hcy and GSH/H₂S. Moreover, the above analytes were used to test the anti-interference performance of NR-NBD for detecting biothiols (Figs. S3 and S4, SI). The results showed that the probe had good anti-interference ability. Further, the effects of pH on the detection of biothiols have been investigated carefully. The obtained results indicated that the probe has satisfactory stability and could work well on Cys, Hcy, GSH, and H₂S in physiological pH ranges. Detailed data are presented in Figs. S5 and S6 (Supporting Information).

Kinetic studies

The time-course of NR-NBD for detecting Cys Hcy, GSH, H₂S was carried out, respectively (Fig. 4). When Cys/Hcy was singly added into NR-NBD solution, the emission spectra at 548 and 652 nm increased with time. The addition of Cys attained a stable condition within 18 min, and the addition of Hcy reached a stable state within 20 min. After adding GSH/H₂S,
emission spectra at 652 nm increased with time, and the fluorescence intensities of GSH and H2S increased to the maximum within 15 min. The results of these experiments demonstrated probe NR-NBD could provide a rapid detection method for these biothiols in the biological systems.

Cell imaging experiments

In order to study the biological application ability of NR-NBD, we performed imaging experiments with RAW 264.7 macrophages. Firstly, NR-NBD has been confirmed to have low cytotoxicity (Fig. S7, SI). In addition, we tested the stability of the probe by simulating the conditions of biological buffers (10 mM PBS, pH = 7.4, 150 mM NaCl), and the good stability ensures the reliability of biological imaging (Fig. S8, SI). Then the fluorescence imaging experiments were carried out. The cells treated with probe NR-NBD displayed green fluorescence and relatively strong red fluorescence (Fig. 5A), which was attributed to native intracellular biothiols. The cells pretreated with N-ethylmaleimide (NEM, a scavenger of biothiols) were further treated with NR-NBD, the fluorescence was almost not observed in the two channels (Fig. 5B). The cells precultured with NEM and Cys/Hcy were treated with NR-NBD, significant fluorescence appeared in the green and red channels (Figs. 5C and 5D). Moreover, the cells precultured with NEM and GSH/H2S were treated with NR-NBD, and red fluorescence was observed in the cells (Figs. 5E and 5F). The above experiments showed that probe NR-NBD could selectively and sensitively monitor the fluctuations of endogenous/exogenous Cys/Hcy and GSH/H2S in living cells.

Zebrafish imaging experiments

Then, we used zebrafish to study the detection properties of NR-NBD for biothiols. Zebrafish treated with NR-NBD showed green and obvious red fluorescence signals (Fig. 6A), which was ascribed to basal intracellular biothiols. After NEM incubation, zebrafish were further cultured with NR-NBD, and the fluorescence was hardly observed in the green and red channels (Fig. 6B). In contrast, NEM-preconditioned zebrafish was further incubated with Cys/Hcy and probe NR-NBD, and bright fluorescence appeared in both green and red channels (Figs. 6C and 6D). Moreover, NEM-preconditioned zebrafish were further incubated with GSH/H2S and probe NR-NBD, and only red fluorescence was obtained in zebrafish (Figs. 6E and 6F). These results convincingly showed that probe NR-NBD could selectively track Cys/Hcy and GSH/H2S levels with excellent sensitivity in zebrafish.

Conclusions

To sum up, we developed a dual-color high-resolution imaging probe for the differential monitoring of Cys/Hcy and GSH/H2S with two separated emission channels by same wavelength excitation in the biological systems. NR-NBD reacting with Cys/Hcy presented green and red fluorescence, while the probe reacting with GSH/H2S only exhibited red fluorescence. Combined with absorption spectrum, this probe could achieve the differential monitoring of Cys/Hcy, GSH and H2S. Additionally, the probe had negligible cytotoxicity and good
tissue penetration ability. The obtained biological imaging data displayed that the probe could visualize the fluctuations of endogenous/exogenous Cys/Hcy and GSH/H₂S levels with satisfactory selectivity and sensitivity in living cells and zebrafish. The NR-NBD probe is expected to be a promising tool for revealing different physiological and pathological functions of biothiols and facilitating the early diagnosis and management of biothiols-related diseases.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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