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Sensitivity evaluation of NBD-SCN towards cysteine/homocysteine and its bioimaging applications

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ABSTRACT

A push–pull fluorogenic reagent, NBD-SCN, was applied for specific detection of cysteine (Cys) and homocysteine (Hcy). Replacing thiocyanato group with Cys/Hcy increased the push–pull characteristic of the probe and resulted in emission of fluorescence. The fluorescent response of the probe toward Cys/Hcy was significantly higher than toward glutathione and other amino acids. The probe showed a 470- and 745-fold fluorescence enhancement at 550 nm and detection limit of 2.99 and 1.43 nM for Cys and Hcy, respectively. Time-dependent fluorescence assays showed that the fluorescence intensity reached a plateau within 20 s after addition of Cys and within 10 min after addition of Hcy. Furthermore, the fluorescence images of Cys/Hcy in Raw 264.7 cells were obtained after adding this probe to the cells. These results indicate that NBD-SCN not only possesses good selectivity and sensitivity for Cys/Hcy but also can penetrate cells for Cys/Hcy bioimaging.

1. Introduction

Biological low molecular weight thiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play important roles in biological systems (Ball et al., 2006; Hong et al., 2006; Schulz et al., 2000; Seshadri et al., 2002; Wood et al., 2003; Zhang et al., 2004). Cys and Hcy are biologically essential molecules required for the growth of cells and tissues in living organisms (Wood et al., 2003). However, abnormal concentrations of Cys and Hcy are associated with various diseases such as growth retardation in children, hair depigmentation, liver damage, edema, muscle and fat loss, Alzheimer's disease, cardiovascular disease, neural tube defect and coronary heart disease (Heafield et al., 1990; Jacobsen, 1998; Nygard et al., 1997; Shahrokhian, 2001). Therefore, the development of probes for rapid detection of Cys/Hcy is important to investigate their functions in cells and disease diagnoses.

To date, several analytical methods such as high performance liquid chromatography (HPLC) (Chen et al., 2008; Guo et al., 2013; Ivanova et al., 2000; Nolina et al., 2007; Ogasawaraa et al., 2007; Wada et al., 2013), mass spectrometry (MacCoss et al., 1999; Rafii et al., 2007; Sass and Endres, 1997; Yana et al., 2007), and electrochemistry (Salimi and Hallaj, 2005; Salimi and Pourbeyram, 2003; Tanga et al., 2010) have been reported for the determination of thiols. Among these analytical methods, fluorimetry is particularly attractive due to its simplicity and sensitivity. Of importance, fluorescent method can be applied in intracellular detection. Various fluorescent probes for thiols have been reported in the past few years. These probes based on different detecting strategies, such as Michael addition (Chen et al., 2010; Huo et al., 2009, 2010; Jung et al., 2012; Lin et al., 2009; Wang et al., 2012a; Yang et al., 2011, 2013; Yi et al., 2009; Yin et al., 2013; Zhang et al., 2012a; Zhou et al., 2012), cyclization reaction (Das et al., 2012; Ma et al., 2011; Madhu et al., 2013; Sun et al., 2011; Zhang et al., 2010), cleavage reaction (Bouffard et al., 2008; Shao et al., 2011, 2012; Wang et al., 2012b; Zhang et al., 2012c), displacement of coordination by thiols (Chen et al., 2013; Han et al., 2009; Huang et al., 2013; Leung et al., 2013) and many other methods (Fujikawa et al., 2008; Lee et al., 2010; Murale et al., 2013; Niu et al., 2012; Tang et al., 2007; Zhang et al., 2007, 2012b) have been reported. However, many of them were reported to have a long response time under physiological condition and high background fluorescence.

A change in fluorescent emission profile of a fluorophore can be achieved by the alteration of the electronic features of the substituents,

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through either intramolecular charge transfer (ICT) or photo-induced electron transfer (PET) pathways. The ICT type fluorophore can provide high sensitivity due to its very low intrinsic fluorescence (Uchiyama et al., 1998). The 7-nitrobenz-2-oxa-1,3-diazolyl (NBD) moiety has been used as a building block for the design of ICT type of fluorogenic reagents. For example, the photoaffinity labeling tag, 7-azido-4-nitrobenz-2-oxa-1,3-diazole (NBD-azide), is a non-fluorescent compound because the two electron-withdrawing groups (7-azido and 4-nitro) that results in a pull–pull system and blocks the ICT process. The non-fluorescent azido fluorogen can be photoconverted to an amino form that becomes a push–pull system and results in a dramatic increase in fluorescence (Lord et al., 2010). In addition to the ICT characteristic, the NBD-based fluorophore also possesses some benefits such as ease of synthesis, rather long wavelength emission and good cell permeability.

NBD-SCN has been considered as a thiol-neutralizing agent and a potent inhibitor of nucleic acid synthesis in leucocytes (Whitehouse and Ghosh, 1968). Herein, we report NBD-SCN as a fluorescent probe for the specific detection of Cys and Hcy. Owing to the donor-π-acceptor architecture, the reaction with Cys and Hcy will promote the ICT property of NBD-SCN and initiate fluorescent emission. The specificity of NBD-SCN toward Cys/Hcy was evaluated by comparing the fluorescence intensities of NBD-SCN in the presence of various amino acids and anions. To study the detecting ability of NBD-SCN toward to Cys and Hcy, time- and concentration-dependent studies were investigated, respectively. The correlation between fluorescence intensity and Cys and Hcy concentration were also examined. The fluorescence intensities of NBD-SCN in the absence and presence of Cys and Hcy in various pH conditions were measured in order to study the stability of NBD-SCN. Furthermore, the applicability of intracellular Cys/Hcy fluorescence detection was evaluated in vitro using fluorescent imaging techniques.

2. Experimental section

2.1. Materials

4-Chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), histidine (His) isoleucine (Ile), proline (Pro), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), cystine, cysteine (Cys), glutathione (GSH), arginine (Arg), lysine (Lys), valine (Val), alanine (Ala), leucine (Leu), methionine (Met), glycine (Gly), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), sodium sulfate (Na_2SO_4), sodium thiocyanate (NaSCN), sodium hydrosulfide (NaHS) and tetrabutylammonium cyanide (TBACN) were purchased from Sigma-Aldrich. Serine (Ser) was purchased from Acros Organics. Sodium acetate (CH_3COONa) and sodium nitrate ($NaNO_3$) were purchased from Showa. Phenylalanine (Phe), homocysteine (Hcy) and *N*-ethylmaleimide (NEM) were purchased from Alfa Aesar. All chemicals were used directly without any further purification.

2.2. Apparatus

Flash column chromatography was performed on reverse phase C18 silica gel (25–40 μm). ^1H and ^{13}C NMR spectra were obtained on an Agilent 400-MR DD2 spectrometer at 400 and 100 MHz, and were referenced to the internal ^1H and ^{13}C solvent peaks. ESI-MS spectra were recorded on a Micromass Q-Tof mass spectrometer. Analyses of carbon, hydrogen, nitrogen and sulfur were obtained with an Elementar vario EL III (Heraeus) analyzer. UV–vis absorption spectra were recorded on a Hitachi U-3000 spectrophotometer. The absorption spectra of all samples were measured in a 10.0 mm path length quartz cuvette with a volume of 3.5 mL. Fluorescence spectroscopic studies were acquired on a Hitachi F-7000 spectrophotometer. The slit width was 5.0 nm for both excitation and emission and the photon multiplier voltage was 600 V.

2.3. Synthesis of 4-nitro-7-thiocyanatobenz-2-oxa-1,3-diazole (NBD-SCN)

A modification of the synthetic procedure of NBD-SCN (Ghosh, 1968) was employed. To a solution of 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl, 501.2 mg, 2.5 mmol) in methanol (25 mL), sodium thiocyanate (1015.5 mg, 12.5 mmol) was added, the mixture was stirred for 3.5 h at room temperature and monitored by TLC until the consumption of the starting materials. Then, the solvent was removed in vacuum and the residue was subjected to column chromatography for purification to obtain the targeting compound as a yellow powder (223.5 mg, 40.2%). IR (KBr, cm^{-1}) 2166.5 (SC≡N), 1533.6 and 1334.2 (NO_2). ^1H NMR (400 MHz, CD_3OD): δ 8.65 (d, J=8 Hz, 1H), 8.02 (d, J=7.6 Hz, 1H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ 150.14, 144.23, 131.86, 131.18, 126.03, 107.23 ppm; EI-MS, calcd for $C_7H_2N_4O_3S$: 221.9; found: m/z 221.9 (M^+). Anal. Calcd for $C_7H_2N_4O_3S$: N, 25.22; C, 37.84; H, 0.91; S, 14.43; found: N, 25.90; C, 38.25; H, 0.88; S, 14.53.

2.4. Reaction of NBD-SCN with Cys/Hcy

A solution of cysteine (39.5 mg, 0.225 mmol) in 1 mL H_2O was added into a solution of NBD-SCN (50 mg, 0.225 mmol) in 10 mM HEPES with 1% CH_3OH. The mixture was stirred for 1 h at room temperature and monitored by TLC until the consumption of the starting materials. Then, the solution was subjected to column chromatography for purification to obtain the product. The product was characterized by ^1H NMR and ESI-MS. ^1H NMR (400 MHz, CD_3OD): δ 8.58 (d, J=8 Hz, 1H), 7.67 (d, J=8 Hz, 1H), 4.45 (t, J=6 Hz, 1H), 4.05–3.88 (m, 2H) ppm; ESI-MS, calcd for $C_9H_8N_4O_5S$: 284.02; found: 285.1.

A similar procedure was performed in the reaction of NBD-SCN with Hcy and GSH. For the reaction product of NBD-SCN and Hcy: ^1H NMR (400 MHz, CD_3OD): δ 8.56 (d, J=8 Hz, 1H), 7.52 (d, J=8 Hz, 1H), 4.12 (t, J=6.4 Hz, 1H), 3.64–3.46 (m, 2H), 2.52–2.31 (m, 2H) ppm; ESI-MS, calcd for $C_{10}H_{10}N_4O_5S$: 298.04; found: 299.1. For the reaction product of NBD-SCN and GSH: ^1H NMR (400 MHz, CD_3OD): δ 8.54 (d, J=7.9 Hz, 1H), 7.62 (d, J=7.9 Hz, 1H), 4.87 (t, J=7.2 Hz 1H), 4.01–3.92 (m, 3H), 3.88 (dd, J=13.9, 5.4 Hz, 1H), 3.58 (dd, J=13.9, 8.5 Hz, 1H), 2.56 (t, J=7.2, 2H), 2.21 (dt, J=15.3, 7.6 Hz, 2H) ppm; ESI-MS, calcd for $C_{10}H_{10}N_4O_5S$: 470.09; found: 471.2.

2.5. General procedure of UV–vis and fluorescence spectra measurement

For the time-dependent fluorescence response study, the Cys and Hcy aqueous solutions (1.0 mM, 30 μL) were added into the NBD-SCN solutions (5 μM, 3 mL) and were measured the fluorescence intensity under the time scan mode immediately. All of the fluorescence spectra were measured at 550 nm with excitation wavelength at 476 nm.

For the concentration-dependent fluorescence response study, appropriate volumes and concentrations of Cys and Hcy aqueous solution were added into the NBD-SCN solution and then the fluorescence intensities were measured after 30 min mixing.

2.6. Selectivity

Stock solutions of amino acids and anions (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, GSH, Hcy, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tys, Val, Cystine, CH_3COO^-, SO_4^{2-}, CO_3^{2-}, HCO_3^-, NO_3^-, SCN^-, HS^- and CN^-) (1.0 mM) were prepared. Stock solutions of NBD-SCN (0.5 mM) were also prepared in methanol. In the selectivity experiment, test solutions were prepared by diluting 30 μL NBD-SCN stock solution with HEPES buffer (10 mM, pH 7.4) in cuvettes, and adding 2 equivalents of Cys/Hcy or 100 equivalents of other biologically relevant analytes. The absorption and emission spectra of test solutions were measured after the addition of analyte for 30 min, respectively.

2.7. Detection limit

The limit of detection (LOD) was determined based on the fluorescence titration and was calculated by the following equation (Joshi et al., 2009):

$$\mathrm{LOD} = 3S_{\mathrm{bi}}/a$$

where S_{bi} is the standard deviation of blank measurements and a is the slope between fluorescence intensity versus sample concentration. To determine the standard deviation of blank, the fluorescence intensity of NBD-SCN was measured thrice.

2.8. pH stability

Solutions of 5 μM NBD-SCN in HEPES buffer (1% CH_3OH) in the pH range of 6.0–8.3 were adjusted by HCl and NaOH solutions. The fluorescence spectra of each solution were collected in a 3 mL cuvette. After mixing with 2 equivalents of Cys and Hcy for 30 min, the fluorescence spectra were measured again, respectively. The fluorescence intensity was monitored at 550 nm.

2.9. Cell culture and fluorescence imaging

Raw 264.7 murine macrophage is organized from tumor ascites induced by intraperitoneal injection of Abselon Leukemia Virus (A-MuLV) in male mouse. Raw 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate and 1% MEM nonessential amino acids at 37 °C in a humidified 5% CO_2 atmosphere. For imaging studies, Raw 264.7 murine macrophages were plated into poly-D-lysine coated plates containing 2 mL of DMEM, and incubated at 37 °C with 5% CO_2. The cells were incubated with 5 μM NBD-SCN for 30 min and then subjected to fluorescence microscope. For biothiols removed study, Raw 264.7 macrophages were pre-treated with 12.5 μM *N*-ethylmaleimide (NEM) for 30 min at 37 °C to remove intracellular thiols. After washing, the cells were incubated with 5 μM NBD-SCN for 30 min and then subjected to fluorescence microscope. Bright-field and fluorescence images were recorded on a fluorescence microscope (IX71, Olympus) equipped with a 100 W mercury lamp, excitation filter: 442.5–512.5 nm; emission filter: 490–570 nm, and a color CCD camera system.

2.10. Confocal fluorescence imaging

For confocal fluorescence images study, Raw 264.7 macrophages were seeded at a density of 2×10^5 cells/well on cover glasses ($24 \times 24\ \mathrm{mm}^2$) and grown for 24 h. The cells with or without 5 μM NBD-SCN incubation and 12.5 μM NEM treatment were fixed with 4% formaldehyde solution for 20 min at room temperature. Cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). Cover glasses containing fixed cells were mounted in a mixture of PBS and glycerol (1:1) on a microscope slide. The cells were observed using a laser scanning confocal imaging system (Olympus Fluoview 300) consisting of Olympus BX51 microscope and a 20 mW-output argon ion laser.

3. Results and discussion

3.1. Design and synthesis of the probe

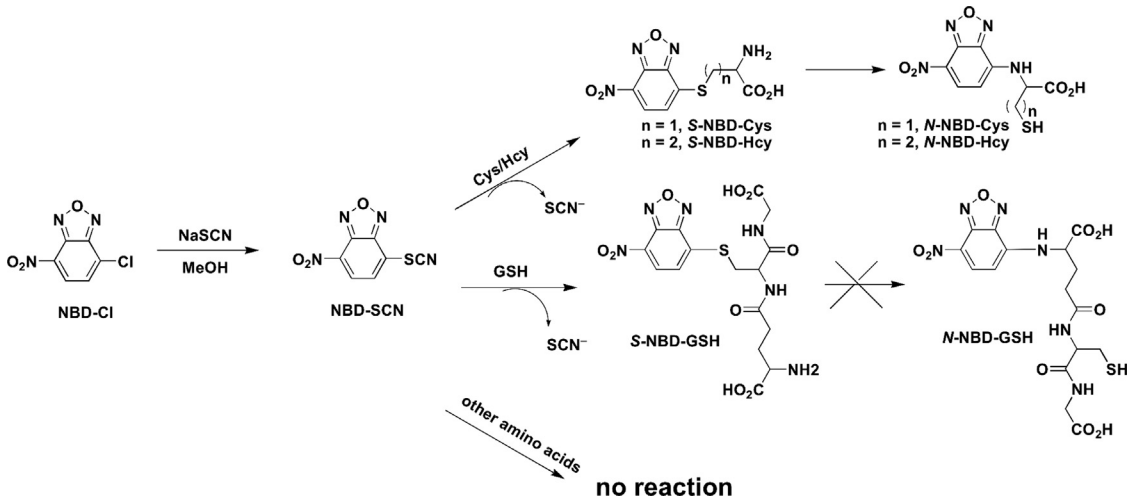
The NBD-SCN was designed based on the electron withdrawing ability of the groups attached. NBD-based molecules are common ICT type fluorophores for the design of sensing molecules. For example, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) can act as a fluorogenic reagent for amino acids and other amines (Ghosh and Whitehouse, 1968). The reactivity of NBD-Cl and NBD-SCN toward the $-NH_2$ group of amino acids are related to the electron-withdrawing characteristics of –Cl and –SCN groups. Chloride, possessing the more electron-withdrawing characteristic, can react with both amino and thiol groups of amino acids whereas SCN, with the less electron-withdrawing characteristic, can react towards the thiol groups. Hence, NBD-SCN was designed in such a way that the more electron-withdrawing −Cl group was replaced by a less electron-withdrawing group −SCN group. The probe was obtained by reacting NBD-Cl with sodium thiocyanate in methanol with 40.2% yield.

3.2. Proposed mechanism

As shown in Scheme 1, NBD-SCN can be activated by the 4-nitro group and react with biothiol through the nucleophilic aromatic substitution to generate less fluorescent *S*-NBD-biothiol derivative (Whitehouse and Ghosh, 1968). The 7-*S*-cysteinyl-4-nitrobenz-2-oxa-1,3-diazole (*S*-NBD-Cys) and 7-*S*-homocysteinyl-4-nitrobenz-2-oxa-1,3-diazole (*S*-NBD-Hcy) further convert to highly fluorescent *N*-NBD-biothiol derivatives, 7-*N*-cysteinyl-4-nitrobenz-2-oxa-1,3-diazole (*N*-NBD-Cys) and 7-*N*-homocysteinyl-4-nitrobenz-2-oxa-1,3-diazole (*N*-NBD-Hcy), whereas glutathione ends with the formation of *S*-derivative, *S*-NBD-GSH. The intramolecular S→N migration occurred in a five-membered/six-membered ring in cysteine/homocysteine case whereas in glutathione migration would involve an unfavorable ten-membered ring. (Birkett et al., 1970; Persson and Wilson, 1978; Price and Radda, 1972). The corresponding products by the reaction of NBD-SCN with Cys, Hcy, and GSH were synthesized, respectively. NMR and mass spectroscopic analysis of products supported the formation of NBD-biothiol derivatives. In order to confirm whether the products are either *S*-NBD-biothiol or *N*-NBD-biothiol, the compounds were subjected for thiol-disulfide exchange reaction to prove the presence of sulfhydryl group in the NBD-biothiol derivatives by using ESI-MS spectrometry. Addition of 2,2′-dipyridyl sulfide in the NBD-Cys and NBD-Hcy solutions resulted in peaks at *m*/*z* 393.9 and 407.9 which correspond to [*N*-NBD-Cys-*S*-Py+H^+] and [*N*-NBD-Hcy-*S*-Py+H^+], respectively (corresponding data were shown in Supporting information Scheme S1, Figs. S1 and S2). However, the corresponding peak for disulfide product in the NBD-GSH solutions was not found. These results revealed that the corresponding products by the reaction of NBD-SCN with Cys, Hcy, and GSH were *N*-NBD-Cys, *N*-NBD-Hcy, and *S*-NBD-GSH, respectively.

3.3. Fluorescence emission and UV–vis absorption of the probe

We proposed that the probe, NBD-SCN, will be non-fluorescent due to the pull–pull nature of the two electron withdrawing



Scheme 1. Schematic illustration of the reaction of NBD-SCN with Cys, Hcy, and GSH.

groups, $-NO_2$ and $-SCN$, against the ICT process. After reacting to thiol groups, the reaction products, *N*-NBD-Cys/*N*-NBD-Hcy, will be fluorescent because the derivatives becomes a push–pull system. The fluorescence spectra of the probe in the absence and presence of Cys/Hcy are shown in Fig. 1a. Almost no fluorescence was observed in the NBD-SCN solution, however, a significant fluorescence was recorded in the presence of Cys/Hcy. In addition, the difference in UV–vis absorption spectra in the absence and presence of Cys/Hcy were also noted. NBD-SCN showed an absorption spectrum at 358 nm and no absorption between 450 nm and 500 nm, whereas absorption spectra at 476 nm and at 478 nm were observed in the presence of Cys and Hcy, respectively. The formation of the new charge transfer band was attributed to the replacement of –SCN group by Cys/Hcy and thus reinstatement of ICT process (Fig. 1b).

3.4. Selectivity

After confirming the fluorescence OFF–ON switching, we evaluated the specificity of this probe toward Cys and Hcy. To investigate the selectivity of this probe, the absorption and emission spectra of NBD-SCN with the addition of Cys/Hcy, other amino acids and anions were monitored. As shown in Fig. 2, introduction of 2 equivalents of Cys and Hcy to a solution of NBD-SCN resulted in an increase in fluorescence intensity, with 470- and 745-fold enhancements for Cys and Hcy, respectively. In contrast, the fluorescent response toward either 2 equivalents or 100 equivalents of GSH was much lower than those of Cys and Hcy. Furthermore, no visible change in fluorescent response was observed when 100 equivalents of other biologically relevant species were added into the NBD-SCN solution. In order to evaluate the utility of NBD-SCN as a fluorescent probe for the detection of Cys/Hcy in complicated biological samples, the fluorescence response of NBD-SCN toward Cys/Hcy in the presence of other amino acids and anions were also measured, respectively (Fig. S3). The results indicated that the other amino acids and anions had no influence on the fluorescence detection of Cys and Hcy. Furthermore, in the presence of HS^-, no significant fluorescence was observed after the addition of Cys/Hcy to NBD-SCN. This phenomenon might be due to the formation of non-fluorescent product, 4-thio-7-nitrobenzofurzan (NBD-SH), by the nucleophilic aromatic substitution reaction of HS^- with NBD-SCN (Montoya et al., 2013).

In agreement with the shift in the emission spectra, introduction of Cys/Hcy/GSH to a NBD-SCN solution resulted in a formation of a new redshift absorption peak. In contrast, other biological relevant species except NaHS elicited no noticeable change in the absorption spectra (Fig. S4). Although the addition of HS^- caused a new redshift absorption peak, HS^- did not enhance the fluorescence intensity of NBD-SCN. These results indicate that NBD-SCN possesses high selectivity toward Cys and Hcy.

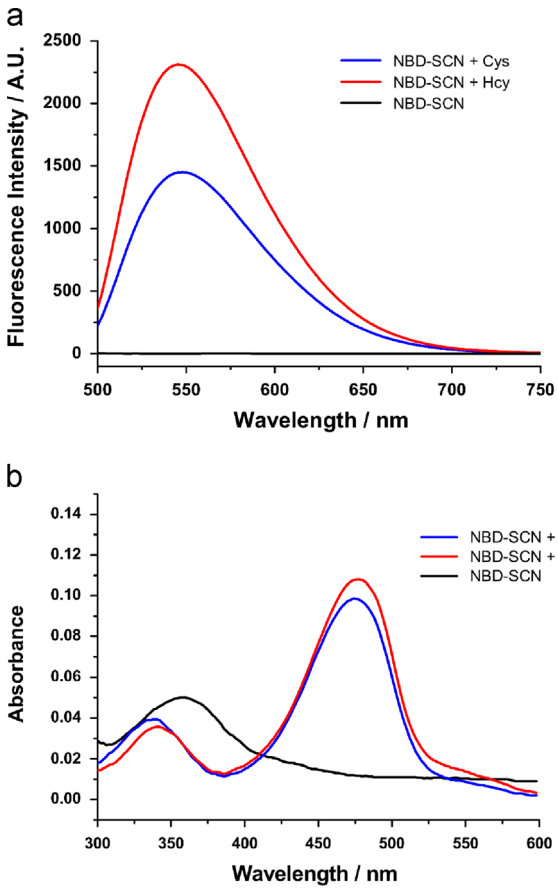


Fig. 1. (a) Fluorescence emission and (b) UV–vis absorption spectra of 5 μM NBD-SCN (in 10 mM HEPES with 1% CH_3OH at pH 7.4) in the absence and presence of 2.0 equivalents of Cys and Hcy.

3.5. Time-dependent fluorescence measurements

To compare the reactivity of NBD-SCN toward Cys and Hcy, time-dependent fluorescence responses were obtained by monitoring the

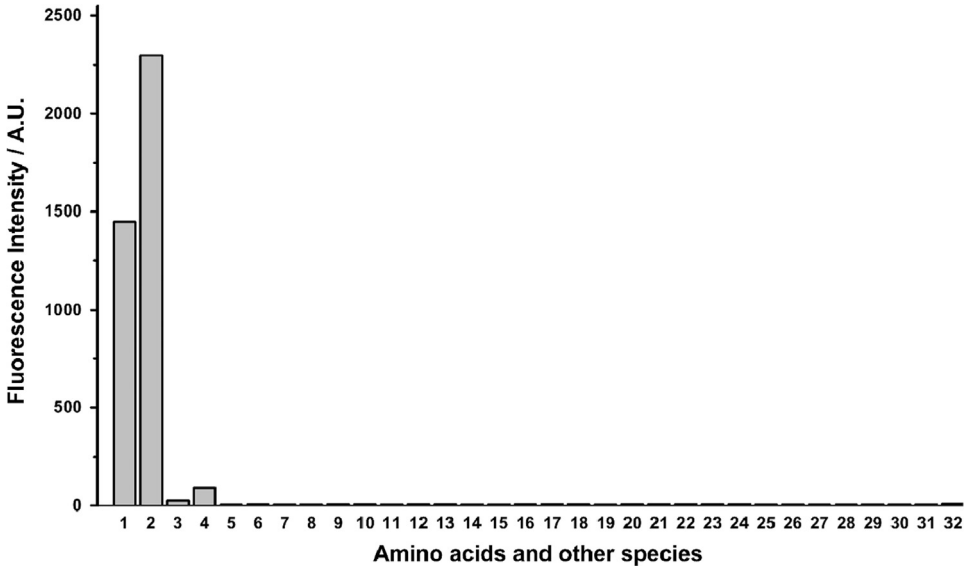


Fig. 2. Specificity of NBD-SCN for Cys/Hcy over other amino acids and other species. Fluorescence intensities of 5 μM NBD-SCN (in 10 mM HEPES with 1% CH_3OH at pH 7.4) at λ_{em}=550 nm was plotted versus substances: 2 equivalents of (1) Cys, (2) Hcy, (3) GSH; 100 equivalents of (4) GSH, (5) Ala, (6) Arg, (7) Asn, (8) Asp, (9) Gln, (10) Glu, (11) Gly, (12) His, (13) Ile, (14) Leu, (15) Lys, (16) Met, (17) Phe, (18) Pro, (19) Ser, (20) Thr, (21) Trp, (22) Tys, (23) Val, (24) cystine, (25) CH_3COONa, (26) Na_2SO_4, (27) Na_2CO_3, (28) $NaHCO_3$, (29) $NaNO_3$, (30) NaSCN, (31) NaHS, (32) TBACN. All data were measured after 30 min addition substances at 25.0 ± 0.1 °C.

fluorescence changes of the mixture of NBD-SCN and 2 equivalents of Cys and Hcy, respectively. In the case of Cys, the reaction rate was fast and the reaction completed within 20 s whereas in the case of Hcy, it took 10 min for its fluorescence intensity to reach a plateau (Fig. 3). This may be attributed to the diverse *S* to *N* transfer rate of the reaction products. The conversion of *S*-NBD-Cys to *N*-NBS-Cys was very fast due to the formation of a favorable five-membered ring during *S* to *N* transfer whereas the conversion of *S*-NBD-Hcy to *N*-NBS-Hcy was expected to be very slow during *S* to *N* transfer and hence the reaction rate with Hcy is found to be slow (Birkett et al., 1970; Persson and Wilson, 1978; Price and Radda, 1972).

3.6. Concentration-dependence of NBD-SCN with cysteine and homocysteine

The sensitivity of NBD-SCN was then studied by fluorescence response of the probe toward Cys and Hcy. Different equivalents of Cys and Hcy were added to the NBD-SCN solutions (5 μM, 10 mM HEPES, pH 7.4) and the fluorescence spectra of the mixed solutions were measured (Fig. 4). In the absence of Cys, NBD-SCN showed almost no fluorescence in HEPES buffer. Upon addition of different amounts of Cys, the fluorescence intensity at 550 nm increased with increasing equivalents of Cys (Fig. 4a). When the fluorescence intensity of the mixture at 550 nm was plotted against 0–1.25 equivalents of Cys, a calibration curve with a good linearity was obtained (R^2=0.9955; Fig. 4a, inset). A similar fluorescence response was observed when different amounts of Hcy were added into NBD-SCN solution. The concentration-dependent fluorescence enhancement showed a good linearity (R^2=0.9945; Fig. 4b, inset) in the range of 0–1.0 equivalents of Hcy.

The detection limits of Cys and Hcy obtained from the calibration curve were 2.99 and 1.43 nM, respectively, which was comparable with other reported thiols probes (Table S1) (Leung et al., 2013; Liu et al., 2013; Wang et al., 2012b; Zhang et al., 2012c; Zhou et al., 2012). These results indicate that NBD-SCN is a sensitive probe for Cys and Hcy.

3.7. Stability of NBD-SCN in various pH

To be useful in biological applications, the probe should be functional at physiological pH values. Therefore, the fluorescence

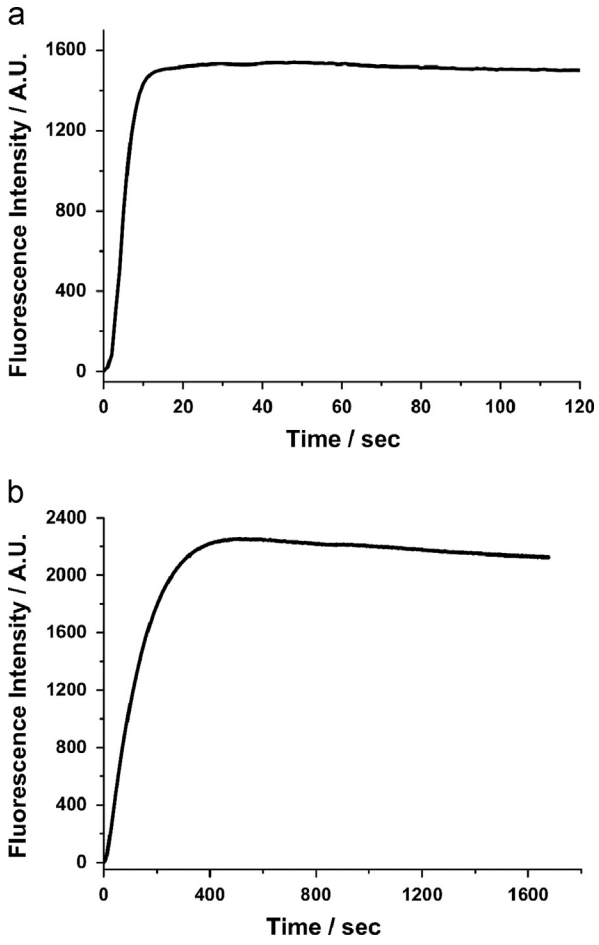


Fig. 3. Time-dependent fluorescence response of 5 μM NBD-SCN (in 10 mM HEPES with 1% CH_3OH at pH 7.4) at 550 nm in the presence of 2 equivalents of (a) Cys and (b) Hcy.

intensities of NBD-SCN in the absence and presence of Cys and Hcy were measured over various pH values. In the absence of Cys/Hcy, almost no change in fluorescence intensity for NBD-SCN was

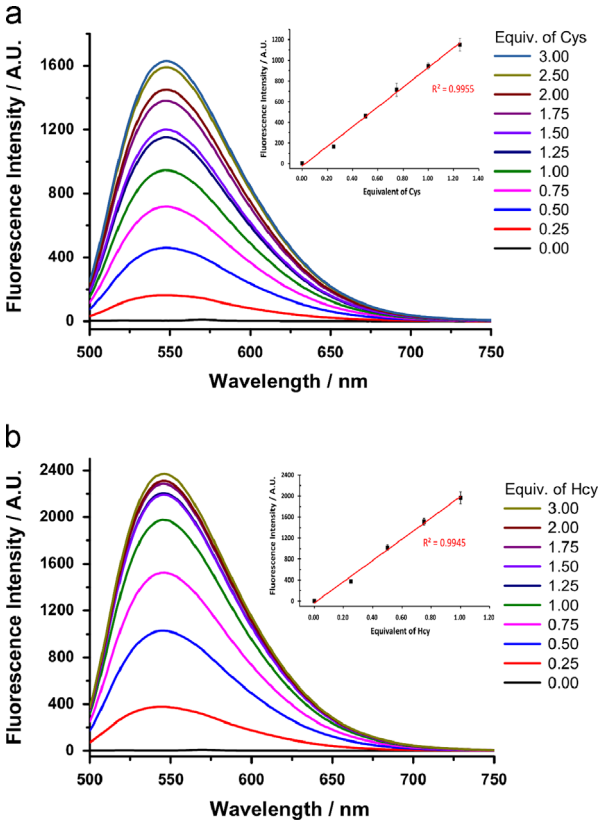


Fig. 4. Fluorescence response spectra of 5 μM NBD-SCN (in 10 mM HEPES with 1% CH_3OH at pH 7.4) upon reacting with different equivalents of (a) Cys and (b) Hcy. Excitation wavelength is 476 nm. Inset: plot of fluorescence intensity at 550 nm versus Cys/Hcy concentration.

observed in the pH 6.0–8.3 range. When Cys/Hcy was added to NBD-SCN, strong fluorescence was detected in this pH range. The results indicate that NBD-SCN responded to Cys/Hcy under physiological conditions (Fig. S5).

3.8. *Fluorescence images of intracellular biothiols*

To test the practical utility of NBD-SCN for cell imaging, Raw 264.7 macrophages was used (Fig. 5). Significant fluorescence was observed when Raw 264.7 cells were incubated with NBD-SCN (5 μM) for 30 min at 37 °C (Fig. 5d). When Raw 264.7 cells were pre-treated with *N*-ethylmaleimide (NEM, a thiol trapping reagent, 12.5 μM) for 30 min and then treated with NBD-SCN (5 μM, for 30 min) the fluorescence intensity in Raw 264.7 cells decreased dramatically because intracellular concentration of thiols is reduced by NEM (Fig. 5f). The cell permeability of NBD-SCN was further confirmed by confocal fluorescent images (Fig. S6). These results suggest that NBD-SCN could be applied in intracellular biothiols detection.

4. Conclusions

In summary, a fluorescent probe, NBD-SCN, with a one-step synthetic procedure for detection of Cys and Hcy is reported in this study. This probe exhibits high sensitivity, high selectivity, and low detection limit to Cys and Hcy. A great linearity relation between the fluorescence intensities and Cys/Hcy concentrations may contribute quantitative measurement of Cys/Hcy through fluorescent method. We also proved that this probe could be applied to detect Cys/Hcy in live cells. The success of intracellular imaging indicates that this probe could be employed in further biological applications.

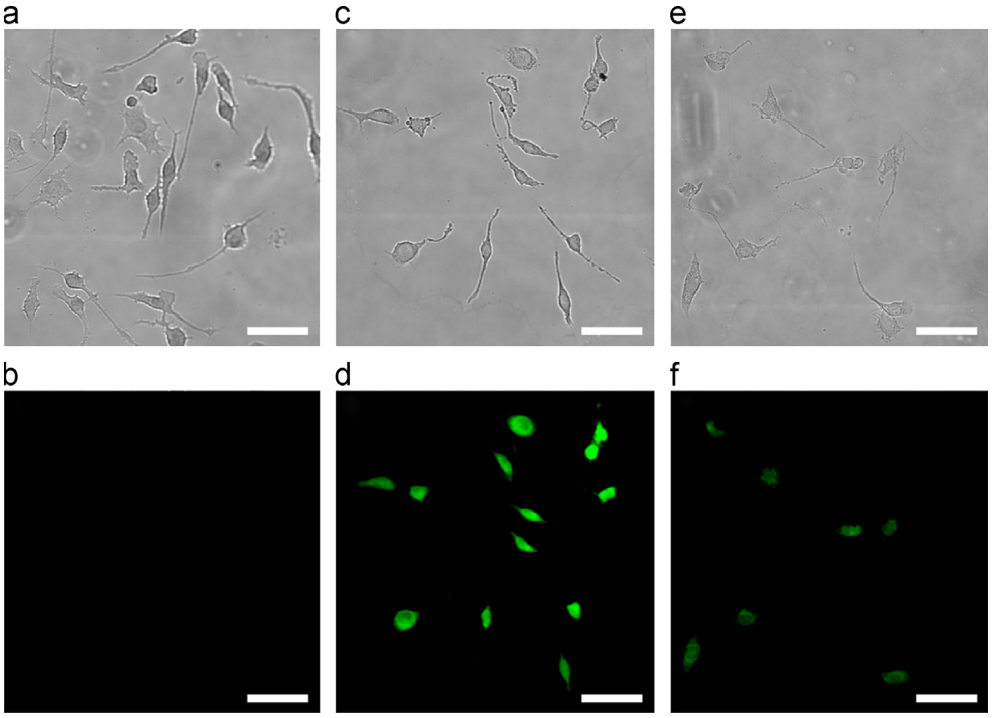


Fig. 5. Bright-field (top) and fluorescence (bottom) images of Raw 264.7 macrophages in the absence (a and b) and presence (c and d) of NBD-SCN (5 μM). e and f represent bright-field and fluorescence images of Raw 264.7 macrophages which were pre-treated with NEM (12.5 μM) for 30 min and then incubated with NBD-SCN for 30 min, respectively. The scale bar represents 50 μm.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.01.009.

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