DOI: 10.1002/chem.201200671

Sensitive Near-Infrared Fluorescent Probes for Thiols Based on Se–N Bond Cleavage: Imaging in Living Cells and Tissues

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Abstract: Cy-NiSe and Cy-TfSe were designed and synthesized as sensitive near-infrared (NIR) fluorescent probes for detecting thiols on the basis of Se–N bond cleavage both in cells and in tissues. Since a donor-excited photoinduced electron transfer (d-PET) process occurs between the modulator and the fluorophore, Cy-NiSe and Cy-TfSe have weak fluorescence. On titration with glutathione, the free dye exhibits significant fluorescence enhancement. The two probes are sensitive and selective for thiols over other relevant biological species. They can function rapidly at pH 7.4, and their emission lies in the NIR region. Confocal imaging confirms that Cy-NiSe and Cy-TfSe can be used for detecting thiols in living cells and tissues.

Keywords: cleavage reactions · fluorescent probes · imaging agents · Se–N bonds · thiols

Introduction

Thiol compounds in vivo, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play key roles in physiological and pathological processes. Abnormal levels of cellular thiols have been linked to a number of diseases, such as leucocyte loss, liver damage, cancer, and AIDS.[1] Specifically, cysteine is a nonessential amino acid, deficiency of which is involved in many syndromes, such as slow growth in children, liver damage, skin lesions, and loss of muscle and fat.[2,3] At elevated levels in plasma, Hcy is a risk factor for Alzheimer's disease and cobalamin (vitamin B12) deficiency.[4] More importantly, GSH is the most abundant intracellular nonprotein thiol (1–10 mM).[5] It has vital roles in maintenance of intracellular redox activity, intracellular signal transduction, and gene regulation.[6] Glutathione exists in reduced (GSH) and oxidized (GSSG) states. An increased or decreased GSSG-to-GSH ratio is considered indicative of diseases in vivo.

Accordingly, the detection of thiol levels in biological systems is very important. Several analytical techniques, including HPLC,[7] capillary electrophoresis,[8] mass spectrometry,[9] electrochemical assay,[10] UV/Vis assay,[11] and fluorescence spectroscopy[12] have been used for detection of thiols. Compared with other technologies, the fluorescence method provides greater sensitivity, less invasiveness, and the availability of bioimaging in living cells and tissues.[13] In the past few years, various mechanisms have been exploited, including Michael addition,[14] cyclization with an aldehyde,[15] cleavage by thiols,[16] and so on.[17] Most of the reported fluorescent probes emit in the ultraviolet or visible region, which can lead to cell autofluorescence.[18,19] In contrast, long-wavelength probes with emission in the near-infrared (NIR) region are optimal for biological imaging applications due to minimal photodamage to biological samples and minimum interference from background autofluorescence in living systems. Recently, fluorescent NIR imaging probes are being increasingly used for optical imaging of live animals.[20] Therefore, it is necessary to develop fluorescent probes that can be used for rapid detection of thiols under physiological conditions, preferably with emission in the NIR region.

Glutathione peroxidase (GPx) is an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage.[21] Ebselen (2-phenyl-1,2-benzoisoselen-azol-3(2*H*)one), a well-known mimetic of GPx with low toxicity, exhibits anti-inflammatory, anti-atherosclerotic, and cytoprotective properties.[22] According to previous studies,[23] the Se–N bond in ebselen is readily cleaved by thiols to produce the corresponding selenenyl sulfide. This mechanism inspired us to design fluorescent probes containing an Se–N bond to detect thiols.

As shown in Scheme 1, we designed fluorescent NIR probes Cy-NiSe and Cy-TfSe containing an Se–N bond for detecting thiols via donor-excited photoinduced electron transfer (d-PET). A cyanine dye, as one type of widely employed NIR fluorophores with high molar absorption coefficient, was selected as signal transducer, and strongly electron withdrawing 2-nitrophenylselane and 3-(trifluoro-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201200671.

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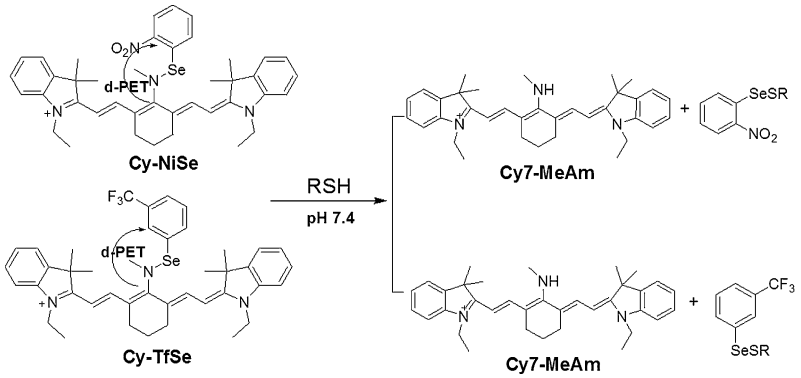


Scheme 1. Structure and design concept of Cy-NiSe and Cy-TfSe probes for thiols.

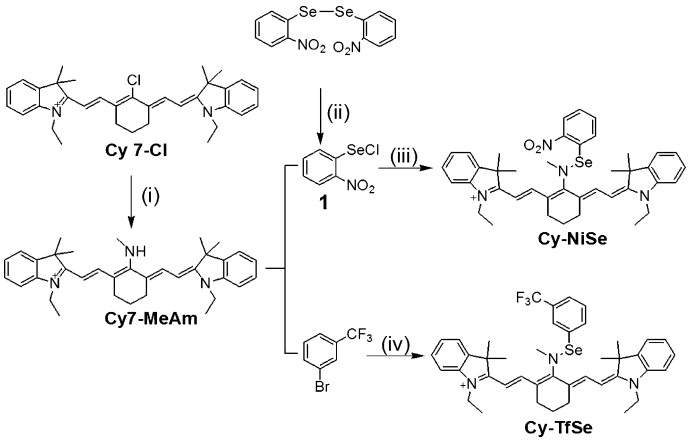
methyl)phenylselane groups as modulators. The probes feature fast response, emission in the NIR region, and stable fluorescence signal over a wide pH range in aqueous solution. Furthermore, Cy-NiSe and Cy-TfSe were used for rapid sensing of thiols in living RAW 264.7 cells and fresh rat liver tissue.

Results and Discussion

Design and synthesis of probes Cy-NiSe and Cy-TfSe: To obtain thiol-selective fluorescent probes with fluorescence emission in the NIR region under physiological conditions for potential biological applications, we selected a heptamethine cyanine dye (Cy) as fluorophore, because of its high molar absorption coefficient and NIR emission.[24] We anticipated that the fluorescence properties of cyanine can be modulated by a photoinduced electron transfer (PET) process from the excited fluorophore to a strongly electron withdrawing group (donor-excited PET, d-PET).[25] Therefore, we chose electron-withdrawing 2-nitrophenylselane and 3-(trifluoromethyl)phenylselane groups as modulators for probes Cy-NiSe and Cy-TfSe.

Our strategy was based on using the strong nucleophilicity of the sulfhydryl group to cleave the Se–N bond of probes Cy-NiSe and Cy-TfSe (Scheme 1). Owing to the presence of electron-withdrawing groups, the electron densities of the fluorophore moieties were lowered. The fluorescence of uncleaved probes Cy-NiSe and Cy-TfSe could be strongly quenched by utilizing strongly electron withdrawing 2-nitrophenylselane and 3-(trifluoromethyl)phenylselane groups as electron acceptors in a d-PET mechanism.[26] On cleavage by thiols, free dye Cy7-MeAm exhibited significantly enhanced fluorescence in its fluorescence spectrum.

The synthesis of Cy-NiSe and Cy-TfSe is depicted in Scheme 2. Intermediate Cy7-MeAm was readily prepared by reaction of Cy7-Cl with methanamine hydrochloride in 87% yield. *o*-Nitrophenyl selenochloride was obtained from *o*-nitroaniline by the classical method with some necessary modifications. A THF solution of *o*-nitrophenyl selenochloride was added dropwise to a mixture of Cy7-MeAm and triethylamine at 0°C under Ar atmosphere. After stirring at room temperature, the reaction mixture was filtered and evaporated under reduced pressure. Crude Cy-NiSe was purified by silica gel chromatography. Next, Cy-TfSe was synthesized by a simple method. KSeCN and 3-bromobenzotrifluoride were dissolved in DMF and stirred under Ar atmosphere at room temperature. Cy7-MeAm, triethylamine, and CuI were added to the mixture in turn under Ar. The solution was stirred for 24 h at room temperature, and a blue powder was obtained after workup and removal of the solvent under vacuum. NMR spectroscopy and HRMS were employed to characterize the structures of the intermediates and final products.



Scheme 2. Synthesis of probes Cy-NiSe and Cy-TfSe. i) Methylamine hydrochloride, DMF, 40°C, 24 h, 87.0%; ii) SO_2Cl_2, DMF, room temperature, 4 h, 86.0%; iii) a. Triethylamine, THF, 0°C; b. room temperature, 4 h, 82.3%; iv) KSeCN, 3-bromobenzotrifluoride, triethylamine, CuI, room temperature, 24 h, 56.9%.

The yield of Cy-NiSe was much higher than that of Cy-TfSe. This could be explained by the stability of the probes. As previously proposed for other *o*-nitrophenylselenium compounds,[27] a specific interaction between the nitro group and the selenium atom possibly prevents bimolecular displacement of selenium. The Se···O nonbonding interaction, though expected to be weaker than the Se–N bond, were shown to increase the stability of probe Cy-NiSe. The stability of Cy-NiSe is associated with intermolecular secondary Se···O interactions with a CH_2 group in the five-membered heterocyclic ring,[28] while it is impossible to integrate such a structure in Cy-TfSe. As a result, the stability of Cy-TfSe may be much lower than that of Cy-NiSe.

Investigation of spectral properties of Cy-NiSe and Cy-TfSe with GSH: We examined the spectral properties of Cy-NiSe

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and Cy-TfSe in 15 mM phosphate buffered saline (PBS, pH 7.4) under physiologically mimetic conditions. Cy-NiSe and Cy-TfSe exhibited almost identical absorption peaks with maxima at 609 nm, as shown for Cy-NiSe as representative of the two probes in Figure 1.

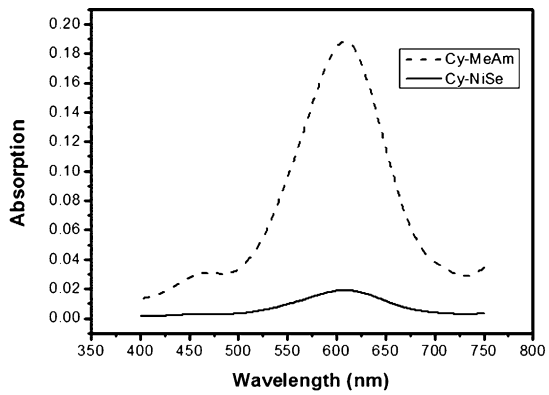

Figure 1. UV/Vis absorption of probe Cy-NiSe (5.0 μM) before (solid line) and after (dashed line) addition of GSH (5.0 μM) at 25 °C for 3 min in 15 mM PBS (pH 7.4). The maximum absorption wavelength was at 609 nm.

On addition of 1 equivalent of GSH to a buffer solution containing Cy-NiSe or Cy-TfSe, the absorbance was greatly enhanced without wavelength shift. The enhancement of absorption intensity suggests formation of Cy-MeAm ($\varepsilon = 3.75\times10^4\,M^{-1}cm^{-1}$) when the strongly electron withdrawing 2-nitrophenylselane and 3-(trifluoromethyl)phenylselane groups leave the probes.

As a typical biological thiol, GSH was used to further investigate the fluorescence response of Cy-NiSe and Cy-TfSe. The changes in the fluorescence emission spectra of the two probes in the presence of GSH in 15 mM PBS (pH 7.4) are displayed in Figure 2. The final concentrations of the probes were maintained at 5 μM, while the concentrations of GSH varied from 0 to 5.0 μM. Cy-NiSe and Cy-TfSe were essentially nonfluorescent owing to d-PET between fluorophore and modulator. The initial quantum yields of the probes were determined to be 0.002 for Cy-NiSe, and 0.003 for Cy-TfSe. However, the fluorescence intensity ($\lambda_{em} = 750$ nm) increases significantly on addition of GSH. On addition of 5.0 μM GSH, the quantum yield of Cy-MeAm was calculated to be 0.13. When the concentration of GSH is increased to 5.0 μM, impressive fluorescence enhancement of the probes provides evidence for a fluorescence turn-on response. The aforementioned d-PET is turned off in the product because of cleavage of the Se–N bonds in the probes, which could explain the fluorescence intensity enhancement. For convenient and accurate determination of intracellular GSH levels, a linear relationship is necessary. The fluorescence intensities at 750 nm were plotted as a function of GSH concentration to obtain a calibration graph (Figure 2, insets). The fluorescence signal is linearly related to the concentration of GSH in the given concentration range. The regression equations are $F_{750\,nm} = 16236.17[GSH/\mu M] - 17657.72$ with a coefficient of $r = 0.9814$ for Cy-NiSe, and $F_{750\,nm} = 6599.80[GSH/\mu M] - 3179.94$ with a coefficient of $r = 0.9867$ for Cy-TfSe. These results imply that Cy-NiSe and Cy-TfSe are potentially useful for quantitative determination of thiol concentration.

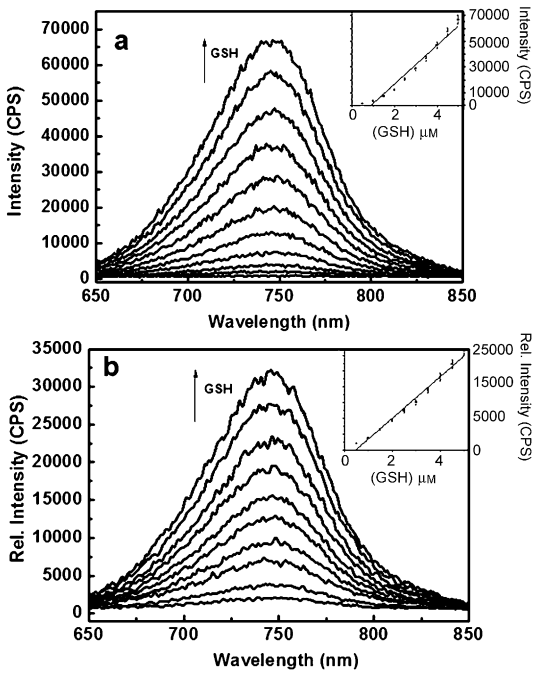

Figure 2. Fluorescence responses of a) Cy-NiSe (5.0 μM) and b) Cy-TfSe (5.0 μM) toward different concentrations of GSH (final concentration: 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 μM) after incubation at 25 °C for 3 min in 15 mM PBS (pH 7.4). Spectra were acquired with excitation at 635 nm and emission ranging from 650 to 850 nm. Inset: Relationship between the fluorescence intensity at 750 nm of probe and [GSH].

Kinetic studies: Time courses of Cy-NiSe and Cy-TfSe were tested by measuring the fluorescent response over 1 h. Figure S1 (Supporting Information) shows the time courses of fluorescence intensity of the probes (5.0 μM) in 15 mM PBS with pH 7.4 at room temperature. The fluorescence intensity was measured simultaneously at $\lambda_{ex/em} = 635/750$ nm. On introduction of GSH (1 equiv), the emission intensity essentially reached a maximum within 3 min, and leveled off thereafter for 1 h, which indicated that Cy-MeAm has good stability to light and air, and that Cy-NiSe and Cy-TfSe could rapidly detect thiols.

Effect of pH: To be useful in biological applications, it is necessary for a probe to function over a suitable pH range and particularly at physiological pH values.[29] Since our probes contain an enamine group, ionization of the methylamine moiety at different pH values may affect the fluorescence. Hence, we examined the influence of pH on the fluorescence intensity of the probes over a wide pH range of 4.0–8.6 (Supporting Information Figure S2). Standard fluorescence pH titrations were performed in 15 mM PBS at

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probe concentration of 5.0 μM. On introduction of 5.0 μM GSH, the fluorescent emission intensity showed no pronounced change. As shown in Figure S2 (Supporting Information), the pH of the medium has hardly effect on fluorescence intensity of probes within the given range. These results indicated that the fluorescence responses to GSH of our probes are pH-independent over a wide pH range. Thus, the probes can be employed to detect thiols in cells.

Selectivity studies: To test for fluorescent response to other biological analytes, Cy-NiSe and Cy-TfSe (5.0 μM) were treated with various biologically relevant analytes, such as representative amino acids, metal ions, anions, reactive oxygen species, reducing agents, and small-molecular thiols in 15 mM PBS (pH 7.4). As shown in Figure 3, no noticeable

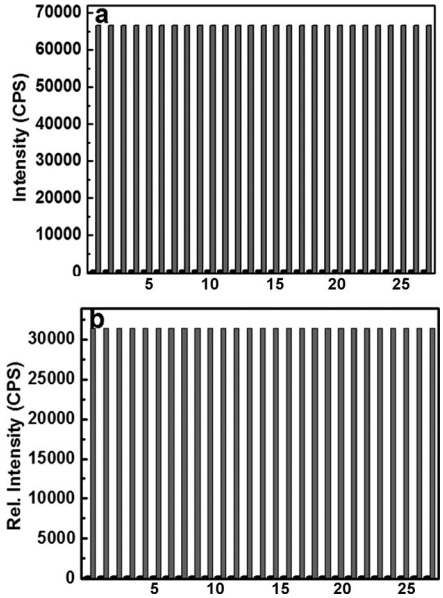

Figure 3. Fluorescence responses ($\lambda_{ex/em}$ = 635/750 nm) of a) Cy-NiSe (5.0 μM) and b) Cy-TfSe (5.0 μM) to diverse bioanalytes in 15 mM PBS (pH 7.4). 1) Na^+ (3.0 mM), 2) K^+ (3.0 mM), 3) Ca^{2+} (50 μM), 4) Mg^{2+} (50 μM), 5) Fe^{2+} (25 μM), 6) Fe^{3+} (25 μM), 7) Zn^{2+} (50 μM), 8) Cu^{2+} (0.5 mM), 9) Cl^-, 10) SO_4^{2-}, 11) CO_3^{2-}, 12) $H_2PO_4^-$, 13) NO_3^- (all 20 μM); 14) histidine, 15) glycine, 16) L-adrenaline (all 100 μM); 17) uric acid, 18) ascorbic acid, 19) dopamine, 20) mannitol (all 100 μM); 21) H_2O_2 (2 mM); 22) hydroquinone (2.5 mM); 23) HOCl, 24) HOBr, 25) O_2^-, 26) NO (all 0.4 mM). Bars represent fluorescence responses to various bioanalytes. In each group, the black bars represent the fluorescence intensity after addition of analytes, and the gray bars represent that after the subsequent addition of 5.0 μM GSH.

changes in fluorescence intensity were observed on addition of amino acids (His, Gly), metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}), anions (Cl^-, SO_4^{2-}, CO_3^{2-}, $H_2PO_4^-$, NO_3^-), reactive oxygen species (H_2O_2, HOCl, HOBr, O_2^-), reactive nitrogen species (NO), reducing agents (uric acid, dopamine, hydroquinone, ascorbic acid) and other analytes (histidine, L-adrenaline, mannitol) in PBS at pH 7.4. An error of ±5.0% in the relative fluorescence intensity was considered tolerable. These results indicated that Cy-NiSe and Cy-TfSe can be useful for selectively sensing thiols at physiological pH values, even in the presence of these biologically relevant analytes.

Reactivity of Cy-NiSe and Cy-TfSe with thiol-containing analytes: Cy-NiSe and Cy-TfSe were designed to sensitively detect thiols. We tested the reactivity of Cy-NiSe and Cy-TfSe towards nonprotein and protein thiols by measuring the increase of fluorescence intensity at 750 nm (Figure 4). Nonprotein thiols detected included glutathione, cysteine, *N*-acetylcysteine, and dithiothreitol with a concentration of 5 mM, as GSH has an intracellular level of 1–10 mM. Protein thiols such as thioredoxin, glutathione reductase, and metallothionein were detected at a concentration of 10.0 μM. Cy-NiSe (5.0 μM) and Cy-TfSe (5.0 μM) showed high reactivity to nonprotein and protein thiols.

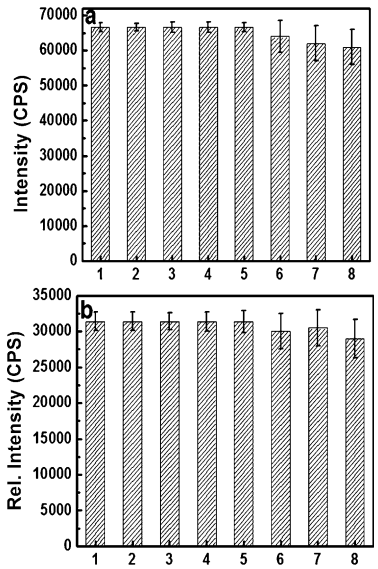

Figure 4. Fluorescence responses of a) Cy-NiSe (5.0 μM) and b) Cy-TfSe (5.0 μM) toward nonprotein (5.0 mM) and protein thiols (10.0 mM) in 15 mM PBS (pH 7.40). 1) Glutathione, 2) cysteine, 3) *N*-acetylcysteine, 4) homocysteine, 5) dithiothreitol, 6) thioredoxin, 7) glutathione reductase, 8) metallothionein. All data were obtained after incubation at 25 °C for 3 min ($\lambda_{ex/em}$ = 635/750 nm).

MTT assay: To evaluate the cytotoxicity of Cy-NiSe and Cy-TfSe, we performed an MTT assay on RAW 264.7 cells with probe concentrations from 10^{-7} to 10^{-3} M. The results showed IC_{50} values of 405 μM for Cy-NiSe and 327 μM for Cy-TfSe, that is, Cy-NiSe and Cy-TfSe are of low toxicity towards cell cultures under experimental conditions.

Cell and tissue imaging: Owing to the chemical and spectroscopic properties of the probes, Cy-NiSe and Cy-TfSe should be suited to detect thiols in living cells and tissues. We assessed the function of Cy-NiSe and Cy-TfSe within living cells by exposing RAW 264.7 cells to GSH. The RAW 264.7 cells were incubated with solution of Cy-NiSe and Cy-TfSe (5.0 μM in 1/100 DMSO/PBS v/v, pH 7.4) for 5 min at

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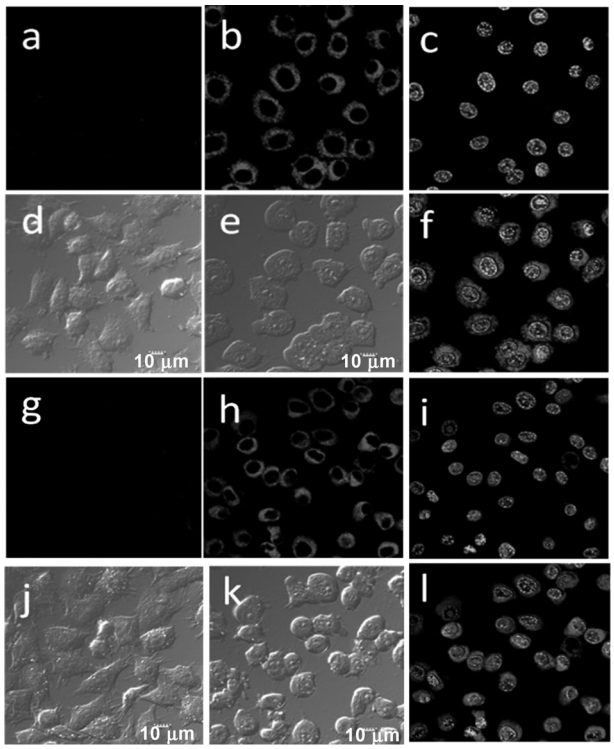

Figure 5. Confocal fluorescence images of living RAW 264.7 cells. RAW 264.7 cells incubated with a) Cy-NiSe (5.0 μM) and g) Cy-TfSe (5.0 μM) for 5 min after preincubation with NEM (5.0 mM) for 30 min. b) RAW 264.7 cells incubated with Cy-NiSe (5.0 μM) for 5 min. c) and i) RAW 264.7 cells incubated with SYTO-16 for 5 min. d) and e) Bright-field images of a) and b); (f) overlay of images of b) and c); h) RAW 264.7 cells incubated with Cy-TfSe (5.0 μM) for 5 min; j) and k) are bright-field images of g) and h). l) Overlay of images h) and i). Scale bar is 10 μm. Incubation was performed at 37 °C under a humidified atmosphere containing 5% CO_2. The fluorescence was collected at 650–800 nm on excitation at 633 nm.

37 °C and then washed three times with PBS. A strong fluorescence signal was observed from the RAW 264.7 cells (Figure 5b, h). In the control experiment, RAW 264.7 cells were pretreated with an excess of the thiol-reactive *N*-ethylmaleimide (NEM),[30] which consumed all of the free thiols within the cells, and then incubated with Cy-NiSe and Cy-TfSe. No significant fluorescence signal was observed (Figure 5a, g). This established that Cy-NiSe and Cy-TfSe are membrane-permeable, and the fluorescence changes in cells were indeed due to the changes in intracellular thiol levels. The bright-field images confirmed that the cells were viable throughout the imaging experiments (Figure 5d, e, j, k). To confirm whether Cy-NiSe and Cy-TfSe can specifically stain the cytoplasm, co-localization experiments were conducted with the RAW 264.7 cells co-stained with Cy-NiSe or Cy-TfSe and SYTO. SYTO dyes are cell-permeant nucleic acid stains that show a large fluorescence enhancement on binding to nucleic acids. Very strong green fluorescence in the nucleus of RAW 264.7 cells was observed when the cells were loaded with SYTO-16 (0.5 μM) for 5 min (Figure 5c, i). By co-staining with SYTO-16 dye, we confirmed that the probes were retained in the cytoplasm. (Figure 5f, l).

We also investigated the utility of Cy-NiSe and Cy-TfSe to image thiols in fresh rat liver tissue. After incubation with 5.0 μM Cy-NiSe or Cy-TfSe for 10 min, a fresh rat liver slice was washed with 15 mM PBS (pH 7.4). The dye-stained liver slice showed strong fluorescence due to intracellular thiols (Figure 6a, c). These results reveal that our probes have good permeability in tissues. Next, another sample was treated with 5 mM of NEM in PBS bubbled with 95% O_2 and 5% CO_2 at 37 °C for 30 min before probe was added,[31] and we observed faint fluorescence intensity (Figure 6b, d). These findings demonstrated that Cy-NiSe and Cy-TfSe are capable of sensing thiols in tissues.

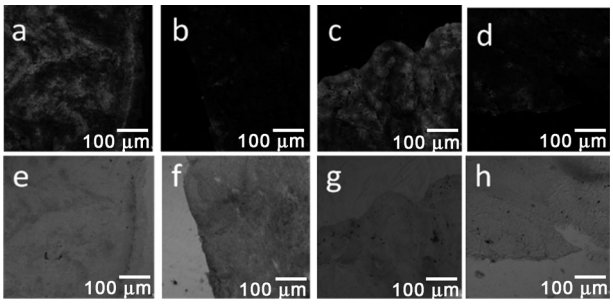

Figure 6. Detection of GSH in fresh rat liver tissue. Confocal fluorescence images of a fresh rat liver slice stained with a) 5.0 μM Cy-NiSe and c) 5.0 μM Cy-TfSe for 10 min at a depth of about 120 μm with magnification at ×20. Scale bar is 100 μm. b) Confocal image of a) pretreated with NEM (5.0 mM) before labeling with 5.0 μM Cy-NiSe. d) Confocal image of c) pretreated with NEM (5.0 mM) before labeling with 5.0 μM Cy-TfSe. e)–h) Bright-field images. Fluorescence was collected at 650–800 nm on excitation at 633 nm.

Conclusion

We have developed two fluorescent probes for thiols, Cy-NiSe and Cy-TfSe, on the basis of Se–N bond cleavage in cells and tissues. In addition, the probes are not only highly sensitive and selective for sulfhydryl-containing molecules in a wide pH range, but also emit in the NIR region (650–900 nm). Furthermore, the probes also show fast signal response and good linearity. Confocal fluorescence images indicate that the probes can be used for detecting different levels of thiols within living cells and tissues. We anticipate that the fluorescent probes will be of great benefit for biomedical researchers investigating the effects of thiols in biological systems.

Experimental Section

Instruments: Fluorescence spectra were obtained on a FluoroMax-4 Spectrofluorometer with a xenon lamp and 1.0 cm quartz cells. Absorption spectra were measured on NANO Drop 2000c UV/Vis spectrophotometer (Thermo Fisher Scientific). All pH measurements were performed with a pH-3c digital pH meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass–calomel electrode. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). Elemental analyses were obtained with a Vario MACRO cube element ana-

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lyzer. ^{1}H and ^{13}C NMR spectra were taken on a Bruker 500 MHz spectrometer. The fluorescence images of cells were taken by using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with ×20 and ×40 objective lenses.

Materials: 2-[4-Chloro-7-(1-ethyl-3,3-dimethyl(indolin-2-ylidene)]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl)-1-ethyl-3,3-dimethyl-3*H*-indolium (Cy7-Cl) was synthesized by us. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), KSeCN, and 3-bromobenzotrifluoride were purchased from Sigma-Aldrich. Mouse leukaemic monocyte macrophage cell line (RAW 264.7) was obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All other reagents and chemicals were from commercial sources, of analytical-reagent grade, and dried by standard procedures before use. Solvents used for spectroscopic studies were purified and dried by standard procedures before use. Ultrapure water (Millipore, Bedford, MA, USA) was used throughout.

Absorption analysis: Absorption spectra were obtained with 1.0 cm glass cells. The probe (DMSO, 0.1 mL, 1.0 mM) was added to a 10.0 mL color-comparison tube. After dilution to 5.0 μM with 15 mM PBS, GSH was added. The mixture was equilibrated for 5 min before measurement.

Fluorescence analysis: Fluorescence spectra were obtained with a xenon lamp and 1.0 cm quartz cells. The probe (DMSO, 0.1 mL, 1.0 mM) was added to a 10.0 mL color-comparison tube. After dilution to 5.0 μM with 15 mM PBS, GSH was added. The mixture was equilibrated for 5 min before measurement.

Cell culture: RAW 264.7 cells were cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay: Cytotoxicity in vitro was measured by MTT assay in RAW 264.7 cells. Cells were seeded into 96-well cell culture plates at 4000/well, cultured at 37°C and 5% CO_2 for 48 h, and then different concentrations of chemosensor Cy-NiSe or Cy-TfSe (0, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M) were added to the wells. The cells were then incubated for 48 h at 37°C under 5% CO_2. Subsequently, MTT (20 μL, 5 mg mL^{-1}) was added to each well and incubated for an additional 4 h at 37°C under 5% CO_2. Cells were lysed in triple liquid (10% SDS, 0.012 M HCl, 5% 2-propanol), and the amount of MTT formazan was qualified by determining the absorbance at 570 nm with a microplate reader (Tecan, Austria). IC_{50} values were calculated according to Huber and Koella.[32] The following formula was used to calculate the inhibition of cell growth: Cell viability (%) = (mean of absolute value of treatment group/mean absolute value of control) × 100%.

Confocal imaging: Fluorescence images were acquired on an Olympus Fluo View FV1000 confocal laser-scanning microscope (Japan) with ×20 and ×40 objective lenses. The excitation wavelength was 635 nm. Cell imaging was carried out after washing three times with PBS.

Preparation and staining of fresh rat liver slices: Slices were prepared from the liver of two-day-old rat. Coronal slices were cut to 300 μm thickness by using a vibrating-blade microtome in 15 mM PBS (pH 7.4). Slices were incubated with 5 μM of probe Cy-NiSe or Cy-TfSe in PBS buffer bubbled with 95% O_2 and 5% CO_2 for 5 min at 37°C[33]. Slices were then washed three times with PBS, transferred to glass-bottomed dishes (MatTek), and observed under a Olympus FV1000 confocal laser-scanning microscope with ×20 objective lens. The excitation wavelength was 635 nm. To assess the effect of NEM, the slices were treated with 5 mM of NEM for 30 min before the probe was added. Following this incubation, the slices were washed three times and imaged.

Synthesis and characterization of compounds: ^{1}H and ^{13}C chemical shifts are given in parts per million relative to internal Me_4Si. All reactions were carried out under Ar by standard vacuum-line techniques. Solvents were dried by distillation under Ar over sodium (THF), CaH_2 (CH_2Cl_2, triethylamine). Bis(*o*-nitrophenyl) diselenide was obtained from *o*-nitroaniline by the classical method with some necessary modification.[34] For synthesis of intermediates, sees Supporting Information.

Synthesis of Cy-NiSe: A solution of *o*-nitrophenyl selenochloride (0.142 g, 0.6 mmol) in THF (5 mL) was added dropwise to a solution of Cy7-MeAm (63.4 mg, 0.1 mmol) and triethylamine (421 μL, 3 mmol) in THF (10 mL) at 0°C under Ar atmosphere. After stirring at room temperature for 4 h, the reaction mixture was filtered and evaporated under reduced pressure. The crude product was purified by chromatography on silica gel with ethyl acetate/methanol (6/1, v/v) to give a deep blue solid.[35] Yield: 186 mg (82.3%). ^{1}H NMR (500 MHz, CD_3OD): $\delta = 8.91$ (d, 1H), 8.49–7.04 (m, 11H), 6.56 (t, 2H), 5.60 (d 2H), 3.28–3.36 (m, 9H), 2.48 (t, 4H), 1.62–1.65 (m, 2H), 1.51 ppm (s, 16H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 168.5$, 166.8, 144.7, 143.2, 139.3, 135.8, 134.2, 132.8, 128.5, 127.6, 122.3, 122.1, 120.1, 109.1, 92.4, 45.7, 35.9, 29.7, 28.1, 24.6, 18.7, 14.5 ppm; ^{77}Se NMR (CD_3OD-D_4, 95 MHz): $\delta = 998.5$ ppm; elemental analysis calcd (%) for $C_{41}H_{47}N_4O_2Se$: C 69.67, H 6.70, N 7.93, O 4.53, Se 11.17; found: C 69.65, H 6.71, N 7.92, O 4.54, Se 11.18; LC-MS (ESI$^+$): *m*/*z* calcd for $C_{41}H_{47}N_4O_2Se^+$: 707.2859, found: 707.2847.

Synthesis of Cy-TfSe: We synthesized probe Cy-TfSe according to the synthesis of SeN heterocycles by the classical method with some modification.[36] 3-Bromobenzotrifluoride (237 μL, 2 mmol) was added to a solution of KSeCN (0.288 g, 2 mmol) in DMF (10 mL) under vacuum at room temperature. After stirring for 1 h, Cy7-MeAm (31.7 mg, 0.05 mmol), triethylamine (Et_3N, 4.5 mL, 8.0 mmol), and copper(I) iodide (CuI, 0.38 g, 2 mmol) were added to the mixture in turn under Ar. The solution was stirred for 24 h at room temperature. Then the solution was diluted with Et_2OAc (50 mL), washed with water (25 mL) and aqueous NaOH solution (1.0 M, 25 mL), and the organic phase dried over $MgSO_4$, concentrated, and purified by chromatography on a silica gel column with ethyl acetate/methanol (9/1, v/v) to give a blue powder after solvent removal under vacuum. Yield: 20.76 mg (56.9%). ^{1}H NMR (500 MHz, CD_3OD): $\delta = 8.87$ (d, 1H), 8.50–7.01 (m, 11H), 6.52 (t, 2H), 5.62 (d 2H), 3.30–3.55 (m, 9H), 2.50 (t, 4H), 1.60–1.66 (m, 2H), 1.50 ppm (s, 16H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 169.5$, 167.8, 142.2, 139.1, 134.8, 135.2, 131.4, 128.1, 128.6, 125.6, 124.2, 122.6, 122.3, 121.1, 108.4, 92.6, 45.2, 36.1, 28.9, 28.6, 25.6, 18.9, 13.8 ppm; ^{77}Se NMR (CD_3OD, 95 MHz) $\delta = 720.07$; elemental analysis calcd (%) for $C_{42}H_{47}F_3N_3Se$: C 69.12, H 6.49, F 7.81, N 5.76, Se 10.82; found: C 69.11, H 6.48, F 7.82, N 5.76, Se 10.83; LC-MS (ESI$^+$): *m*/*z* calcd for $C_{42}H_{47}F_3N_3Se^+$: 730.2882, found: 730.2893.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (20975089, 81102415), the Chinese Academy of Sciences (KZCX2-EW-206), and the 100 Talents Program of the Chinese Academy of Sciences.

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Received: February 29, 2012
Revised: April 15, 2012
Published online: July 24, 2012

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