A highly selective near-infrared fluorescent probe for imaging H₂Se in living cells and in vivo†

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Hydrogen selenide (H₂Se), a highly reactive Se species, is an important selenium metabolism intermediate involved in many physiological and pathological processes. This compound is of scientific interest with regard to the real-time monitoring of H₂Se in living cells and in vivo to understand the anti-cancer mechanism of selenium. However, monitoring H₂Se in living cells is still challenging due to the lack of straight forward, highly selective and rapid methods. Here, we developed a novel small-molecule fluorescent probe, NIR-H₂Se, for imaging endogenous H₂Se. NIR-H₂Se exhibited high selectivity toward H₂Se over selenocysteine (Sec), H₂S and small molecule thiols and was successfully used to image the H₂Se content in HepG2 cells during Na₂SeO₃-induced apoptosis. Increased H₂Se content and reduced ROS levels were observed under hypoxic conditions compared to normoxic conditions, which indicated that the cell apoptosis induced by Na₂SeO₃ under a hypoxic environment is via a non-oxidative stress mechanism. Thus, this probe should serve as a powerful tool for exploring the physiological function of H₂Se and Se anticancer mechanisms in a variety of physiological and pathological contexts.

Introduction

Selenium (Se) is an essential trace element for various physiological functions in the human body. Insufficient or excessive Se intake has been associated with a number of diseases.¹² Importantantly, selenium plays a role in cancer prevention and treatment.³ However, the anticancer mechanism for Se is still not fully understood. The effectiveness of Se compounds as anticancer agents is correlated to their chemical form and dose.⁴ H₂Se, a reduced form of selenium, is an important metabolite of dietary Se compounds.⁵ Endogenous H₂Se is generated by reducing selenocysteine via GSH and other reduction systems⁶ and is involved in many physiological and pathological processes.⁷ A reliable and rapid method to determine H₂Se in vivo must be developed to investigate the physiological function of H₂Se and the anticancer mechanism of Se.

Fluorescent probes with high sensitivity, good selectivity, and short response times via direct observation are powerful tools for the in vivo detection of biomolecules.⁸ Several fluorescent probes for detecting Se species were recently reported. Maeda et al. developed the first fluorescent probe, BESThio, to discriminate selenols from their Cys counterparts based on their different nucleophilicity at pH = 5.8.⁹ Using the recognition group Maeda reported, Fang and Lin exploited probes by adjusting the fluorophore to detect selenols with high selectivity under physiological conditions.¹⁰ In addition, Fang et al. developed another novel fluorescent probe (TRFS-green) using a 1,2-dithiolane reporter group to selectively image thioredoxin reductase (TrxR) at pH = 7.4.¹¹ Wu reported a cadmium sulfide (CdS) quantum dot (QD) probe for HSe⁻ ions in aqueous solution.¹² However, a small-molecule fluorescent probe for imaging the highly reactive H₂Se in living cells and in vivo has not been reported.

Previously, our group discovered that the Se–N bond can be cleaved using sulphydryl groups and we developed a series of fluorescence methods to monitor thiols and their redox state in living cells.¹³⁻¹⁵ We subsequently found that the Se–N bond in 2,1,3-benzoselenadiazole (BS) can be specifically broken by selenol via direct nucleophilic addition and we designed a new fluorescent probe (HB) to image endogenous Sec in living cells and in vivo.¹⁶ These results inspired us to develop a H₂Se fluorescent probe based on the BS group by modifying the probe molecule structure.

Here, we integrated the BS moiety into a near infrared (NIR) merocyanine dye¹⁷ to develop a novel small-molecule fluorescent probe for detecting H₂Se (NIR-H₂Se). NIR-H₂Se responds to H₂Se rapidly, with a high selectivity over H₂S, Sec and biological thiols and was used to successfully image endogenous H₂Se in living cells and in vivo. Moreover, we used a radical oxygen species (ROS) probe and found that the H₂Se contents increased during HepG2 cell apoptosis induced by Na₂SeO₃ in a time- and
dose-dependent manner, while the ROS level did not change under a hypoxic environment. This probe will provide a powerful tool for investigating the metabolism and antitumor mechanism of Se compounds.

Results and discussion

The syntheses are illustrated in Scheme 1, and the probe and intermediate structures were fully characterized by $^1$H NMR, $^{13}$C NMR, and HRMS. We first examined the spectroscopic properties of this probe and optimized the fluorescence measurement conditions (Fig. S1 and S2, ESI†). **NIR-H$_2$Se** exhibits an emission maximum at 735 nm with weak fluorescence (φ = 0.019) in aqueous solutions buffered at physiological pH (10 mM phosphate buffered saline, PBS, pH = 7.4) due to effective fluorescence quenching of the BS via the heavy atom effect of Se. As predicted, the BS moiety in **NIR-H$_2$Se** reacts selectively with H$_2$Se over Sec, H$_2$S, and small molecule thiols. The HPLC analysis confirmed that the fluorescent product was a diamino product (Scheme 2 and Fig. S3 and S4, ESI†).

We then tested the fluorescence response to H$_2$Se. Adding H$_2$Se increased the fluorescence intensity of **NIR-H$_2$Se** ca. 10-fold (φ = 0.13, Fig. 1a). There was good linearity between the fluorescence intensities and H$_2$Se concentrations for the range 0–12 μM (Fig. 1b). The regression equation was $F = 2096.7 + 1772.3 \times [\text{H}_2\text{Se}]$ μM with a linear coefficient of 0.9941. The limit of detection was 7.0 nM (standard deviation 3.5%, n = 11).

To evaluate the selectivity of **NIR-H$_2$Se** for H$_2$Se, we measured the fluorescence spectra for the probe with H$_2$Se, H$_2$S and thiols. High concentrations of thiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) (1 mM for each) produced low fluorescence responses, and **NIR-H$_2$Se** also gave a negative response for H$_2$S, Sec, Na$_2$SeO$_3$, N-acetyl-$\alpha$-cysteine (NAC), thioredoxin reductase (TrxR) and vitamin C (Vc). The reactivity of **NIR-H$_2$Se** towards ROS was then tested. Fig. 2a shows that biologically relevant ROS, including H$_2$O$_2$, ‘OCl$^-$, ‘OH$^-$’ and ROO$^-$, did not trigger any fluorescence changes in the probe. Moreover, considering that nitric oxide (NO) may be a potential interfering species, the fluorescence response of **NIR-H$_2$Se** to differing amounts of NO were studied both with and without H$_2$Se. The results indicated that even high NO concentrations (10 equiv.) did not induce an obvious fluorescence change in the detection system. The interference from metal ions and other amino acids were also tested as shown in Fig. S5 and S6.† Collectively, **NIR-H$_2$Se** selectively recognized H$_2$Se under physiological conditions.

Considering the variable nature and quick metabolism of endogenous H$_2$Se in biological systems, a fast-responding method for H$_2$Se detection is necessary. The response of **NIR-H$_2$Se** to H$_2$Se was evaluated via a kinetics experiment. The results indicated that the fluorescence intensity immediately increased to its maximum after adding H$_2$Se to the probe solution, which indicated that the probe instantly responds to H$_2$Se (Fig. 2b). In addition, the cytotoxicity of the probe in HepG2 cells was determined via a conventional MTT assay (Fig. S7, ESI†), which indicated that the **NIR-H$_2$Se** probe exhibited low biotoxicity and could be used as a viable probe for detecting H$_2$Se in biological samples.

After confirming the high sensitivity, selectivity and rapid response of this probe for H$_2$Se, we explored its applications to image endogenous H$_2$Se in living cells. During these cell imaging experiments, Na$_2$SeO$_3$ was used as the H$_2$Se precursor because sodium selenite can be metabolized to hydrogen selenide (H$_2$Se) via selenodiglutathione (GSsSeG) and glutathione selenenylsulfide (GSsSeH).† HepG2 cells were treated with 2–10 μM Na$_2$SeO$_3$ for 12 h or incubated with 5 μM Na$_2$SeO$_3$ for 2–12 h, as our previous study found that these action concentrations and times of Na$_2$SeO$_3$ can induce HepG2 cell apoptosis.† After treatment, the cells were loaded with 10 μM of **NIR-H$_2$Se**. The experimental results are shown in Fig. 3. Higher H$_2$Se contents were observed in hypoxic environments than under normoxic conditions during these parallel experiments.

We found that the apparent discrepancies above were related to the aerobic metabolism of H$_2$Se. Under normoxic conditions (20% O$_2$), H$_2$Se rapidly oxidized to generate a mass of superoxide anion radicals (O$_2$$^{•-}$) and an accumulation of reactive...
Fig. 2 (a) Fluorescence intensity changes for NIR-H$_2$Se (10 $\mu$M) after adding 100 equiv. of thiols, Na$_2$S, Na$_2$SeO$_3$, Sec, NAC, TrxR, Vc, various biologically related ROS and 10 equiv. of NO. The black bars show the addition of one of these interferents to a 10 $\mu$M NIR-H$_2$Se solution. The red bars represent the addition of both H$_2$Se and one interferent to the probe solution. (b) Time course for the fluorescence intensity of 10 $\mu$M NIR-H$_2$Se with 10 $\mu$M H$_2$Se in a 10 mM PBS, pH = 7.4, at room temperature.

Fig. 3 Confocal fluorescence imaging of endogenous H$_2$Se in HepG2 cells treated with sodium selenite under normoxic (20% pO$_2$) and hypoxic (1% pO$_2$) conditions. (a) The fluorescence changes for the HepG2 cells exposed to different sodium selenite concentrations (2–10 $\mu$M) for 12 h and incubated with NIR-H$_2$Se (10 $\mu$M). (b) The fluorescence changes of the HepG2 cells exposed to 5 $\mu$M of sodium selenite for different times (0–12 h) and incubated with NIR-H$_2$Se (10 $\mu$M). The fluorescence was imaged using a confocal microscope with 633 nm excitation and 650–750 nm collection. (c) The fluorescence intensity for (a). (d) The fluorescence intensity for (b). The data were normalized to the control, and statistical analyses were performed using a two-tailed Student’s t-test ($n \geq 4$ fields of cells) relative to the control. *p < 0.01 and the error bars are ± the standard error measurement.

Fig. 4 Confocal fluorescence imaging of H$_2$O$_2$ in HepG2 cells treated with sodium selenite under normoxic (20% pO$_2$) and hypoxic (1% pO$_2$) environments. (a) The fluorescence changes for the HepG2 cells exposed to different sodium selenite concentrations (2–10 $\mu$M) for 12 h and incubated with a H$_2$O$_2$ probe (10 $\mu$M). (b) The fluorescence changes for the HepG2 cells exposed to a 5 $\mu$M sodium selenite solution for different times (0–12 h) and incubated with a H$_2$O$_2$ probe (10 $\mu$M). The fluorescence was imaged using a confocal microscope with 532 nm excitation and 600–700 nm collection. (c) The fluorescence intensity for (a). (d) The fluorescence intensity for (b). The data were normalized to the control, and statistical analyses were performed using a two-tailed Student’s t-test ($n \geq 4$ fields of cells) relative to the control. *p < 0.01 and the error bars are ± the standard error measurement.

oxygen species during the tumor apoptosis process induced by Na$_2$SeO$_3$.$^{20}$ However, under a hypoxic environment (1% pO$_2$), H$_2$Se cannot quickly metabolize due to the lack of oxygen, which allows H$_2$Se to effectively accumulate. To further confirm our viewpoint, the H$_2$O$_2$ content was evaluated using a H$_2$O$_2$ probe$^{21}$ for parallel experiments.

HepG2 cells were treated with different Na$_2$SeO$_3$ concentrations for 12 h or incubated with 5 $\mu$M Na$_2$SeO$_3$ for differing times before loading with 10 $\mu$M of the H$_2$O$_2$ probe. Fig. 4 shows that the H$_2$O$_2$ content gradually increased under normoxic conditions in the HepG2 cells with Na$_2$SeO$_3$ in a time- and dose-dependent manner, while low H$_2$O$_2$ levels were maintained under hypoxic conditions during the parallel experiments. These results indicate that the Na$_2$SeO$_3$ anticancer mechanism is not a ROS-induced apoptosis process under hypoxic conditions. Because hypoxia is a characteristic feature of solid tumors due to the imbalance between new blood vessel formation and rapid cancer cell proliferation,$^{22}$ these results also imply that the anticancer effect of Na$_2$SeO$_3$ in solid tumors is not owed to oxidative stress.$^{23}$

The above results indicate that NIR-H$_2$Se has potential for detecting endogenous H$_2$Se in vivo. To evaluate this proposal, mice bearing subcutaneously implanted tumors grown from murine hepatoma cell line H22 were subcutaneously injected with buffer solutions containing NIR-H$_2$Se (10 $\mu$M) and sodium selenite (10 $\mu$M), and fluorescence images were then obtained at
different times using an in vivo imaging system (IVIS). Fig. 5 shows that the fluorescence signal of the probe was exclusively observed in the tumor region without a background signal, and that the fluorescence intensity increased from 3 h to 12 h post injection. The results indicated that the hypoxic solid tumor generated H₂Se from sodium selenite and that the H₂Se gradually accumulated.

Conclusions

In summary, we developed a novel small-molecule fluorescent probe, NIR-H₂Se, to detect H₂Se. The probe rapidly responded to H₂Se, exhibited high sensitivity and good selectivity for H₂Se over Sec, H₂S, ROS and other sulfur-containing species and was successfully used to image endogenous H₂Se in living cells and in vivo. Furthermore, we found that normoxic conditions increased the H₂O₂ content, while the H₂Se level rose only slightly during HepG2 cell apoptosis induced by Na₂SeO₃. However, H₂S accumulated gradually and the ROS remained low in a hypoxic environment. These findings indicate that the anticancer mechanism of Se for hypoxic solid tumors is via non-oxidative stress. We anticipate that the current probe will provide an ideal tool for further studies into the biological functions of H₂Se and Se anticancer mechanisms.

Experimental

Materials and instruments

All chemicals were available commercially and the solvents were purified by conventional methods before use. Cysteine (Cys), homocysteine (Hcy), glutathione (GSH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company. Na₂SeO₃, N-acetyl-L-cysteine (NAC), thioredoxin reductase (TrxR) and selenite (10⁻⁴ M) were purchased from Sigma Chemical Company. Na₂SeO₃, N-acetyl-L-cysteine (NAC), thioredoxin reductase (TrxR) and selenite (10⁻⁴ M) in response to sodium selenite and that the H₂Se grad-

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Fig. 5  (a) In vivo fluorescence imaging of H22 tumor-bearing mice injected with the probe NIR-H₂Se (10 μM) in response to sodium selenite (10 μM) at different times. (b) The fluorescence intensity for (a). The data were normalized to the control.
The mixture was fully grinded for 30 min, and TLC showed full conversion of compound 2 to the probe. The resulting mixture was extracted by MeOH (20 mL) and the solvent was removed under reduced pressure, then the crude product was purified by silica gel chromatography with 6% MeOH in DCM to afford the desired product as a dark purple solid (87 mg, 71%). mp = 252–253 °C. 1H NMR (400 MHz, CDCl3): δ 1.61 (t, J = 6 Hz, 3H), 1.86 (s, 6H), 2.01 (t, J = 3 Hz, 2H), 2.41 (s, 2H), 2.75 (t, J = 3 Hz, 2H), 3.00 (t, J = 3 Hz, 2H), 5.00 (q, J = 6 Hz, 2H), 7.38 (d, J = 16 Hz, 1H), 7.49 (s, 1H), 7.54–7.59 (m, 4H), 7.68 (s, 1H), 7.74 (d, J = 16 Hz, 1H). 13C NMR (75 MHz, CDCl3): δ 13.7, 14.1, 20.6, 24.5, 29.3, 30.9, 42.9, 51.7, 104.7, 110.7, 114.3, 119.0, 119.6, 122.5, 124.7, 126.5, 128.0, 128.8, 129.7, 132.6, 140.7, 142.9, 146.6, 152.7, 155.5, 179.6. HR MS: [M + I]+: m/z calcd 488.1218, found 488.1237.

Fluorescence analysis

Fluorescence spectra were obtained with an FLS-920 Fluorescence Spectrometer (Edinburgh Instruments, UK). After dilution to 10 μM of the probe with 10 mM PBS, various amounts of H2Se were added. The fluorescence intensity was measured at λex/λem = 688/735 nm.

Cell culture

HepG2 cells were maintained following the protocols provided by the American Type Culture Collection. Cells were first grown in a circular Petri dish (60 mm) using high glucose Dulbecco’s Modified Eagle Medium (DMEM, 4.5 g of glucose per L) supplemented with 10% fetal bovine serum (FBS), NaHCO3 (2 g L−1) and 1% antibiotics (penicillin/streptomycin, 100 U mL−1). Cultures were maintained in a humidified atmosphere at 37 °C, in 5% CO2/95% air. One day before imaging, cells were passed and plated on 18 mm glass coverslips in a culture dish. The culture medium was refreshed every 24 h. All cells used were in the exponential growth phase.

Confocal imaging

Fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscope (Germany Leica Co., Ltd) with an objective lens (×40). Excitation of the H2Se probe-loaded cells at 633 nm was carried out with an argon laser, and emission was collected using a META detector between 650 and 750 nm. Excitation of the H2O2 probe-loaded cells at 532 nm was carried out with an argon laser, and emission was collected using a META detector between 650 and 750 nm. Prior to imaging, the medium was removed. Cell imaging was carried out after washing cells with PBS (pH = 7.4, 10 mM) three times.

Animal and tumor models

All animal experiments were carried out according to the Principles of Laboratory Animal Care (People’s Republic of China) and the Guidelines of the Animal Investigation Committee, and approved by the local Animal Care and Use Committee. Six- to eight-week-old female Kunmin male mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. During procedures, the mice were anesthetized with inhaled isoflurane. 1 × 106 H22 cells were injected into the enterocoelia of each Kunmin mouse, ascites were formed after 5 or 7 days, which were further used after three passages. Four- to six-week-old nude mice received a subcutaneous injection of 1 × 106 H22 ascite tumor cells into the axillary lateral subcutaneous of their right forelimbs. Tumors were then allowed to grow over a period of 15 to 20 days until reaching 0.5–1.5 cm in diameter.

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Notes and references

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