Probing hydrazine with a near-infrared fluorescent chemodosimeter

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Abstract: Hydrazine is highly toxic to humans and animals when inhaled or in contact with the skin. It is also a very important chemical used as propellant, metal corrosion inhibitor, pharmaceutical and intermediate. The simple detection of hydrazine in solution and biosystems becomes very important as a consequence of the foregoing applications. Herein, we report a two-photon fluorescent probing method using a NIR fluorescent chemodosimeter, displaying turn-on fluorescence and color change after cleaving an *O*-acetyl moiety by hydrazine, which exhibits good selectivity and sensitively in solution and living cells.

Keywords: two-photon; near-infrared; fluorescent chemodosimeter; hydrazine; living cells

1. Introduction

Hydrazine (N₂H₄), as an important propellant, is often applied in rocket-propulsion systems for its flammable and detonable characteristics [1]. It is an important metal corrosion inhibitor because of its strong reducing properties and scavenges oxygen in water boilers for feed and

heating systems [2]. It is also used as a common precursor in the synthesis of various polymers, direct hydrazine fuel cells in power generation sector and pharmaceutical intermediates in the materials field [3-6]. However, hydrazine and its aqueous solutions are highly toxic to humans and animals when inhaled or in contact with skin. It has been reported that hydrazine is carcinogenic and mutagenic and may cause serious damage to the liver, lungs, kidneys and the human central nervous system [7, 8]. Therefore, it is of great interest and importance to develop reliable method to detect hydrazine with satisfactory sensitivity and selectivity.

In recent years, a few analytical technologies have been developed to determination of hydrazine in the chemical system, such as chromatography [9, 10], chemiluminescence [11], spectrophotometry [12], electrochemistry [13], titrimetry [14], and surface-enhanced Raman spectroscopy [15]. However, most of these methods are effective for extracellular hydrazine and require sample preparation steps. Only a few of these methods can be used to detect intracellular hydrazine. Fluorescent probes have become powerful tools for monitoring the extracellular and intracellular levels of biologically relevant species and to understand their functions because of the rapid, nondestructive, selective, and sensitive advantages of emission signals [16-22]. Colorimetric fluorescent probes display excellent property for small molecule detection [23-26]. A ratiometric and colorimetric NIR fluorescent sensor in our previous work, which incorporated an acetyl moiety into heptamethine cyanine dye derivative, could image N_2H_4 in MCF-7 cells and nude mouse with low biological toxicity [27]. The introduced acetyl moiety in the probe could be cleaved by hydrazine with good selectivity and suitable reaction time.

Compared with the traditional one-photon microscopy technique (OPM), TPM (two-photon microscopy) acquires the excited state of a fluorophore by utilizing two-photons of lower energy. The excellent property of TPM is superior to OPM because of lower phototoxicity, better three dimensional spatial localization, deeper penetration depth and lower self-absorption. To date, there are few two-photon (TP) probes have been designed for N_2H_4 imaging. For example, Meng's group has synthesized a novel quinoline-based ratiometric two-photon fluorescent probe for the specific detection of N_2H_4 , and the TPM imaging of this probe in CHO cells [28]. However, almost all reported TP probes have short maximum emission wavelengths less than 600 nm which limit their biological applications because of low spectral interference caused by nonspecific

absorption and fluorescence of the complex matrix. Therefore, it is of great value to develop a new TP fluorescent probe with near-infrared emission for N_2H_4 determination which will definitely have more practical applications.

Herein, we report a new two-photon colorimetric fluorescent probe **BI-E** to detect the N_2H_4 with high selectivity and sensitivity *in vitro* and *in vivo*. The donor- π -acceptor structure chromophore dicyanomethylene-4*H*-pyran has been exploited as a colorimetric fluorophore in the detection of glutathione [29], hydrogen peroxide [30, 31] and fluoride [32]. We introduce an *O*-acetyl moiety into dicyanomethylene-4*H*-pyran skeleton to obtain the fluorescent probe **BI-E** which be cleaved by reaction with N_2H_4 and release a **DCPO⁻** fluorophore with a maximum emission at 680 nm (Scheme 1).

Scheme 1. The structure and reaction mechanism of the probe **BI-E**.

2. Materials and Methods

2.1. General

All solvents and reagents used were reagent grade and were used without further purification unless specified. The stock solution of **BI-E** was dissolved in DMSO at a concentration of 1 mM and stored in a refrigerator for use. ^1H NMR and ^{13}C NMR spectra were recorded on a VARIAN INOVA-400 (or a Bruker Avance II 400 MHz or 500 MHz) spectrometer. Chemical shifts (δ) in ppm (in CDCl_3 or Acetone-d_6 , with TMS as the internal standard). VARIAN CARY Eclipse

fluorescence spectrophotometer (Serial No. FL0812-M018) was used for fluorescence spectroscopy. A Perkin Elmer Lambda 35 UV/VIS spectrophotometer (Perkin Elmer) was used for measuring absorption spectroscopy. HRMS spectrometric data were reported on HP1100LC/MSD MS and LC/Q-TOF-MS instruments. A Model PHS-3C meter was used for all pH measurements. The HCl or NaOH stock solution was prepared at a concentration of 1 M for slight pH changes. Silica gel (100 - 200 mesh) and aluminum oxide (neutral, 100 - 200 mesh) bought from Qingdao Ocean Chemicals were used for flash column chromatography. Doubly purified water used in all experiments was from Milli-Q systems. All measurements were recorded at room temperature.

Scheme 2. The synthetic route of probe **BI-E**. Reagent and conditions: (a) Na, CH₃COOC₂H₅, 4 h, 49.6%; (b) AcOH, H₂SO₄, 30 min, 78.0%; (c) malononitrile, AcOH, H₂SO₄, 14 h, 35.5%; (d) 4-hydroxybenzaldehyde, toluene, piperidine, AcOH, 12 h, 50.3% (e) acetyl chloride, Et₃N, CH₂Cl₂, r.t., 90.0%.

2.2. Synthesis of compounds

2.2.1. Synthesis of compound **DCPO**

2-(2-(4-Hydroxystyryl)-4H-chromen-4-ylidene) malononitrile (**DCPO**) was synthesized according to the procedure published in literature [33]. 2-(2-Methyl-4H-chromen-4-ylidene) malononitrile (208 mg, 1.00 mmol) and 4-hydroxybenzaldehyde (387.5 mg, 1.10 mmol) were dissolved in anhydrous toluene (25 mL). After adding piperidine (1.21 ml) and acetic acid (0.5 ml), the solution was heat to reflux for 12h. The mixture was cooled to room temperature and the solvent removed under vacuum. The residue was purified by a flash column chromatography and

the red solid product was obtained (251.5mg, 50.3%). **Mp:** 257—259 °C. ¹H NMR (400 MHz, Acetone) δ 8.81 (dd, J = 8.4, 1.3 Hz, 1H), 7.87 (ddd, J = 8.5, 7.3, 1.4 Hz, 1H), 7.71 (dd, J = 8.6, 7.6 Hz, 2H), 7.63 (d, J = 8.6 Hz, 2H), 7.59 – 7.52 (m, 1H), 7.12 (d, J = 15.9 Hz, 1H), 6.93 (d, J = 8.6 Hz, 2H), 6.84 (s, 1H). ¹³C NMR (101 MHz, Acetone) δ 160.12, 158.78, 152.74, 152.43, 139.20, 134.89, 130.21, 126.78, 125.79, 125.08, 118.85, 117.55, 116.98, 116.06, 115.87, 115.61, 105.65, 60.70. **IR (KBr, cm⁻¹):** 3396, 2939, 2210, 1693, 1631, 1598, 1556, 1513, 1496, 1481, 1442, 1405, 1265, 1168, 973, 840, 823, 745. **HRMS (ESI) calcd for C₂₀H₁₂N₂O₂ [M-H]⁻ 311.0826, found 311.0826.**

2.2.2. Synthesis of probe **BI-E**

To a solution of 2-(2-(4-Hydroxystyryl)-4*H*-chromen-4-ylidene) malononitrile (**DCPO**) (300 mg, 0.96 mmol) and triethylamine (302 mg, 3.0 mmol) in anhydrous dichloromethane (20 mL) at 0 °C, acetyl chloride (73 mg, 0.93 mmol) was added under a nitrogen atmosphere. Then the mixture was stirred for another 2 h until the reaction was complete. The concentrated mixture was dissolved in dichloromethane and then washed with water, brine and dried with MgSO₄. Then the residue was purified by a flash column chromatography and the yellow solid product was obtained (305.9 mg, 90.0%). **Mp:** 235—236 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.92 (d, J = 8.3 Hz, 1H), 7.74 (d, J = 8.1 Hz, 1H), 7.68 – 7.52 (m, 4H), 7.47 (d, J = 7.5 Hz, 1H), 7.19 (d, J = 8.4 Hz, 2H), 6.88 (s, 1H), 6.78 (d, J = 16.1 Hz, 1H), 2.33 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 169.11, 157.19, 152.79, 152.32, 152.27, 137.71, 134.71, 132.35, 129.05, 126.04, 125.87, 122.45, 118.92, 118.63, 117.84, 116.68, 115.60, 107.03, 63.18, 21.16. **IR (KBr, cm⁻¹):** 3436, 2925, 2853, 2208, 1749, 1724, 1631, 1598, 1560, 1502, 1481, 1456, 1405, 1369, 1263, 1224, 1201, 1166, 1010, 983, 844, 768. **HRMS (ESI) calcd for C₂₂H₁₄N₂O₃ [M+Na]⁺ 377.0897, found 377.0902.**

2.3. Cell culture and confocal fluorescence imaging

MCF-7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO₂. For live cell imaging, cells grown in a glass bottom dish were incubated with compound for 30 min and wash with DMEM three times. Then cells were imaged using a confocal fluorescence microscope (OLYMPUS, FV1000) for one-photon

microscopy imaging (OPM) and two-photon microscopy (TPM) imaging. A high-performance mode-locked titanium-sapphire laser source (MaiTai, Spectra-Physics USA) was used for TPM imaging. The images of **DCPO** were obtained with the emission in the range of 650—750 nm for OPM and 575 nm—630 nm for TPM.

2.4. Cytotoxic assay

MCF-7 cells were used for estimating the cytotoxicity of **BI-E**. Briefly, Cells were seeded in a 96-well plate. On the following day, cells were incubated with 5 μ M **BI-E** for 12 h and 24 h at 37 $^{\circ}$ C. Subsequently, 100 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co. USA) was added into each well, followed by further incubation for 24 h at 37 $^{\circ}$ C. The DMEM was remove and DMSO (200 μ L/well) added. Optical density (OD) was measured by a microplate reader (Spectra Max M5, Molecular Devices) at 570 nm with subtraction of the absorbance of the cell-free blank volume at 630 nm. The relative cell viability (100%) was calculated using the following formula: cell viability (%) = $(OD_{\text{dye}} - OD_{K_{\text{dye}}}) / (OD_{\text{control}} - OD_{K_{\text{control}}}) \times 100$, where dye stands for the **BI-E** sample.

3. Results and discussion

3.1. Synthesis of probe **BI-E**

BI-E was synthesized according to the route shown in Scheme 2 as a yellow solid in several steps and has been characterized by IR, ^1H NMR, ^{13}C NMR and HRMS. It is slightly soluble in water, but can dissolve in normal organic solvent such as dichloromethane, chloroform and DMSO.

3.2. UV-vis and fluorescence responses

The absorption and fluorescence properties of **BI-E** were determined in PBS (pH = 7.4) solution containing 50% of ethanol (Fig. 1 and Fig. S1). **BI-E** has two main absorption peaks at around 414 nm and 434 nm. After treatment of **BI-E** with N_2H_4 for 1min, two new absorption peaks appeared at 388 nm and 551 nm with a decrease of peak at 414 nm and disappearing at 434 nm. The maximum absorption peak shifted from 434 nm to 551 nm. Addition of **BI-E** with increasing

concentrations of N_2H_4 , the absorption titration curves decreased gradually at 414 nm and increased at 551 nm indicating the capacity of **BI-E** for colorimetric detection of N_2H_4 . There was a good linearity between the absorption intensity ratio (A_{551}/A_{414}) and concentration of N_2H_4 ranging from 0—40 μM . The photo-stability of both **BI-E** and **DCPO** is much better than **Cy7-Cl** (structure in Fig. S2b) after exposure to high density light (500W, tungsten lamp) for 100 min, which is a critical factor for bioimaging *in vivo* (Fig. S2a).

Probe **BI-E** showed almost no fluorescence emission upon excitation at 560 nm. Addition of N_2H_4 resulted in an obvious increase at 680 nm. With increasing concentrations of N_2H_4 , the fluorescence titration spectrum showed a gradual enhancement. N_2H_4 elicited a more than 110-fold fluorescence increase. Mass spectrometry data along with absorption and emission spectra showed that the N_2H_4 -mediated acetyl deprotection of **BI-E** generated **DCPO** as the fluorescence product (Fig. S3 and Fig. S4). The fluorescence intensity of **BI-E** increasing obviously at 680 nm after treatment of N_2H_4 with a Stokes shift 120 nm indicated that it was suitable for the detection of N_2H_4 *in vivo* and *in vitro*.

Fig. 1. (a) Absorption spectra of probe **BI-E** (10 μM) in the presence of different concentration of N_2H_4 (0, 5, 10, 20, 30, 40, 60, 70, 80, 90 and 100 μM , 1 min) in solution (Ethanol/PBS = 5/5, pH = 7.4), (b) a linear correlation between absorption intensity ratio (A_{551}/A_{414}) and concentration of N_2H_4 , (c) from left to right refer to color

changes of **BI-E** (10 μM) to different concentration of N_2H_4 (0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM).

To further identify the performance of **BI-E**, the fluorescence of the probe was measured by fluorescence titration experiment. In a certain range, the fluorescence intensity increased gradually following the increased concentration of N_2H_4 (Fig. 2a and Fig. 2b). There was a good linearity between the fluorescence intensity at 680 nm and the concentration of N_2H_4 ranging from 0 - 40 μM (Fig. 2c). The detection limit of N_2H_4 was calculated from the equation $\text{DL} = 3\sigma/S$, where σ is the standard deviation of a blank measurement and S is the slope between intensity versus sample concentration. The detection limit of **BI-E** was calculated to be 5.7×10^{-8} M. The time-dependent fluorescent change measurements showed the response time of **BI-E** toward N_2H_4 was 1 min (Fig. 2d). The pH experiments indicated that **BI-E** could detect N_2H_4 in physiological pH range (Fig. S5).

Fig. 2. (a) and (b) Emission spectra of probe **BI-E** (10 μM) in the presence of different concentration of N_2H_4 (0,

5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM , 1 min) ; (c) a linear correlation between emission intensity and concentration of N_2H_4 at 680 nm; (d) Time-dependent fluorescence changes of **BI-E** upon addition of N_2H_4 (100 μM) at 680 nm. Emission spectra obtained in solution (Ethanol/PBS = 5/5, pH = 7.4) upon excitation at 560 nm.

3.3. N_2H_4 selective and competitive experiment

The selectivity of **BI-E** was examined towards N_2H_4 and other amino acids and various cations. As a result, only N_2H_4 induced an obvious fluorescence intensity increase at 680 nm. There was little fluorescence intensity increase when **BI-E** treated with other chemicals (Fig. 3a and Fig. S6). Besides, **BI-E** had a good selectivity for N_2H_4 when mixing with others in competitive experiment (Fig. 3b and Fig. S7). These results indicated that **BI-E** has reasonable activity and selectivity to identify N_2H_4 in a complex environment.

Fig. 3. Selective (a) and competitive (b) experiment in solution (Ethanol/PBS = 5/5, pH = 7.4) with the emission intensity at 680 nm. (a) Fluorescence response of **BI-E** (10 μM) to various analytes (100 μM). Each spectrum was recorded at 1 min after addition of the analytes (from 1 to 28 represent Ala, Arg, Asn, Asp, Gln, Glu, Gly, GSH, Hcy, Cys, His, Leu, Phe, Pro, Trp, Thr, Val, NaHS, K^+ , Na^+ , Fe^{2+} , Fe^{3+} , Ba^{2+} , Mg^{2+} , Cu^{2+} , Ca^{2+} , Zn^{2+} and **BI-E**); (b) Fluorescence intensity of **BI-E** (10 μM) in the presence of various analytes (100 μM) (from 1 to 27 represent Ala, Arg, Asn, Asp, Gln, Glu, Gly, GSH, Hcy, Cys, His, Leu, Phe, Pro, Trp, Thr, Val, NaHS, K^+ , Na^+ , Fe^{2+} , Fe^{3+} , Ba^{2+} , Mg^{2+} , Cu^{2+} , Ca^{2+} , Zn^{2+}). Black bars represent the addition of 10 equiv of the appropriate analytes to a 10 μM

solution of **BI-E**. Red bars represent the addition of **BI-E** (10 μM) to the mixture solution of 100 μM the appropriate analytes and 100 μM N_2H_4 .

3.4. Measurement of Two-Photon Cross-Section.

The two-photon cross-section of **BI-E** and **DCPO** (100 μM) were measured in 1 mL PBS buffer (pH = 7.4) containing 50% of ethanol as described in literature [34]. The quantum yield of **BI-E** and **DCPO** were measured by Absolute PL Quantum Yield Spectrometer (HAMAMATSU C11347). The quantum yield is 0.002 for **BI-E** and 0.011 for **DCPO** in solution (Ethanol/PBS = 5/5, pH = 7.4). The cross-sections of **BI-E** and **DCPO** were calculated by using the following formula: $\delta = \delta_r \times (F_s \times \phi_r \times n_r) / (F_r \times \phi_s \times n_s)$, where δ , F , ϕ and n stand for TP cross-section, spectral integral area, quantum yield and concentration; s and r stand for sample and reference. Fluorescein (aqueous, 1 μM , pH = 11) was used as a reference probe. From 710 nm to 900 nm, **DCPO** was calculated to have a two-photon absorption cross-section of $\delta_{\text{max}} = 30 \text{ GM}$ at 830 nm (Fig. 4a). The results demonstrate that **BI-E** could be used for N_2H_4 imaging by two-photon microscopy in cells.

Fig. 4. (a) Two-photon cross-section of **BI-E** and **DCPO** (10 μM) in solution (Ethanol/PBS = 5/5, pH = 7.4). (b)

Cytotoxicity of **BI-E** probe. Cell viability of MCF-7 cells incubated with **BI-E** (5 μM) for 12 h and 24 h.

3.5. Fluorescence bio-imaging and cytotoxicity assay

The ability of **BI-E** to detect N_2H_4 in living cells was investigated. Initially, MCF-7 cells were incubated with $100\ \mu\text{M}$ N_2H_4 for 15 min, and then washed with DMEM three times. Subsequently, the cells were treated with $5\ \mu\text{M}$ **BI-E** for 30 min at $37\ ^\circ\text{C}$ and washed with DMEM three times. Then remarkable red fluorescence was obtained in the cells by one-photon microscopy (OPM) imaging upon excitation at 559 nm. But in the control test, little fluorescence was observed when the cells were treated with $5\ \mu\text{M}$ **BI-E** for 30min at $37\ ^\circ\text{C}$. These results showed that **BI-E** has a selective response to N_2H_4 in living cells. Then imaging N_2H_4 in MCF-7 cells was examined by two-photon microscopy (TPM) imaging. Obvious red fluorescence observed in MCF-7 cells indicated that **BI-E** is a TP fluorescent probe with NIR emission for N_2H_4 in living cells (Fig. 5). 820 nm was chosen for TPM because of the difference between intracellular and extracellular environment. Moreover, the cytotoxicity of **BI-E** was investigated using a cell viability assay. MCF-7 cells were treated with $5\ \mu\text{M}$ **BI-E** for 12 h and 24 h, and then cell viability was evaluated using a MTT assay. The results showed that cell viability was over 90% indicating that **BI-E** had low cytotoxicity (Fig. 4b).

Fig. 5. Confocal microscope images of MCF-7 cells. (a), (b) and (c) cells treated with **BI-E** ($5\ \mu\text{M}$) for 30 min;

from (d) to (i) cells treated with 100 μ M N_2H_4 for 15 min and subsequent treatment of the cells with **BI-E** for 30min; (d), (e) and (e), OPM image of cells upon excitation at 560 nm, emission window 650 nm – 750 nm; (g), (h) and (i), TPM image of cells upon excitation at 820 nm, emission window 575 nm – 630 nm.

3.6. The application in the detection of hydrazine solution

The application of **BI-E** to detect hydrazine in solution was investigated. Initially, filter paper was soaked in a DCM solution of **BI-E** (5 mM) and dried. Then, the filter paper was put into the solution of hydrazine at room temperature before observation. As shown, the color of filter paper changed gradually from yellow to deep pink with the increasing of the concentration of hydrazine in PBS buffer (pH = 7.4) containing 50% of ethanol under 365 nm UV lamp (Fig. 6a). The color change of **BI-E** on the filter paper was dependent on the concentration of hydrazine in solution and was easy to distinguish by direct visualization. Besides, there was obviously fluorescence intensity increase after the filter paper exposed to 530 nm laser (Fig. 6b). The concentration of hydrazine at 0—10% results in obvious intensity increase on the paper. Compared to the probe described in literature[35], the emission of **BI-E** on the paper at 700 nm avoids low spectral interference in complex environment. These results suggested that **BI-E** offers the potential application for detecting hydrazine in solution.

Fig. 6. The UV color (a) and FL intensity (b) changes of **BI-E** (5mM) coated filter paper after immersing into different concentration of hydrazine solution for 5 second; the UV color changes were obtained using a hand-held UV lamp with an excitation at 365 nm (from left to right stand for 0, 1%, 2.5%, 5%, 10%, 20% hydrazine solution in Ethanol/PBS = 5/5, pH = 7.4), the FL intensity changes were obtained using a NightOWL II LB983 small animal in vivo imaging system with an excitation laser of 530 nm and an emission filter of 700 nm (from left to right stand for 0, 0.05%, 0.1%, 1%, 5%, 10% hydrazine solution in Ethanol/PBS = 5/5, pH = 7.4).

4. Conclusions

In summary, we have developed the two-photon probe **BI-E**, which selectively responds to N_2H_4 with an excellent NIR fluorescence turn-on effect, a good detection limit as low as 5.7×10^{-8} M and a short response time (1 min). **DCPO** provides a two-photon cross-section δ_{max} as high as 30 GM at 830 nm in solution (Ethanol/PBS = 5/5, pH = 7.4) and **BI-E** has been successfully applied to detect N_2H_4 in living cells by two-photon microscopy imaging. Moreover, the filter paper coated with probe **BI-E** could be used to detect hydrazine in solution. Therefore, this work provides a promising two-photon probing method for the rapid detection N_2H_4 in environmental and biological samples.

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