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Invited feature article

Development of a fast-responsive two-photon fluorescent probe for aminothiols and its application in living tissues

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ABSTRACT

Aminothiols, including cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), play important roles in various physiological processes such as cancer, neuropathy, and cardiomyopathy. Herein, a two-photon fluorescent probe based on a naphthalene-benzothiazole platform was designed and synthesized for the detection of aminothiols *in vivo*. The probe exhibited excellent properties including fast response (about 5 min), good selectivity, and low cytotoxicity. The turn-on fluorescent probe could be employed to two-photon image aminothiols in living cells and tissues.

1. Introduction

Aminothiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play very vital roles in many physiological and pathological processes. For example, abnormal level of Cys can lead to many health problem including cancer, liver damage, neuropathy, skin lesions, cardiomyopathy and slowed growth [1]. The elevated homocysteine could lead to Alzheimer's and cardiovascular diseases [2,3]. GSH are linked to numerous physiological diseases including HIV, psoriasis, liver damage, cancer and leucocyte loss [4–6]. Therefore, it is very necessary to design efficient and fast method for the detection of cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) with high sensitivity and selectivity.

Several analytical techniques for the detection of Cys, Hcy and GSH have been exploited in the previous work, such as high-performance liquid chromatography (HPLC), electrochemical assay, capillary electrophoresis, UV–vis spectroscopy, FT-IR spectroscopy, mass spectrometry, gas chromatography, immunoassay *etc.* [7–14]. Compared to the above methods, we aimed to develop an organic fluorescent probe for the detection of Cys, Hcy and GSH, which can be used in the biosystems with the advantages of real-time detection and living cell and tissue imaging. In the past few years, organic fluorescent probes, as the most powerful monitoring tools, have become an important tool, which can be used in biological studies with the advantages such as high selectivity, high sensitivity and real-time detection [15–20].

Many fluorescent probes for monitoring aminothiols in living biosystem have recently been reported [21–33], most of which are two-photon fluorescent probes. They were excited with long wavelengths avoiding photobleaching of probes and damage to living biosystem. Besides, two-photon microscopy (TPM) with the two-photon excitation has significant advantages such as three-dimensional imaging of living tissues, reduced the photodamage to biosamples, increased tissue penetration and negligible background fluorescence. Therefore, it is of great importance and challenge to construct two-photon probe which can be suitable for imaging aminothiols owing to the above advantages.

2. Experimental

2.1. Material

Unless otherwise noted, all reagents and materials were purchased from commercial company and used without further purification. Twice-distilled water was applied to all experiments. High-resolution electrospray (ESI-HRMS) mass spectra were examined from Bruker APEX IV-FTMS 7.0T mass spectrometer; NMR spectra were obtained from AVANCE III 400 MHz Digital NMR

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Spectrometer with TMS as an internal standard; Electronic absorption spectra were recorded on a LabTech UV Power spectrometer; Photoluminescent spectra were obtained with a HITACHI F4600 fluorescence spectrophotometer; The fluorescence images were collected with Nikon A1MP confocal microscopy with a CCD camera; the progress of the reaction was monitored on thin layer chromatography (TLC) and column chromatography was carried out over silica gel (mesh 200–300). Both TLC and silica gel (mesh 200–300) were purchased from the Qingdao Ocean Chemicals.

2.2. Synthesis of **NS-S** probe

6-hydroxy-2-naphthaldehyde (137.6 mg, 0.8 mmol) and 2-aminobenzenethiol (110.0 mg, 1.1 equiv) were dissolved in DMF (2.5 mL). And then sodium metabisulfite (228.1 mg, 1.5 equiv) was added to the previous solution under N_2. The reaction mixture was stirred at 120 °C for 6 h and concentrated under vacuum, and the crude product was purified by silica column chromatography to give the compound **NS** (181.3 mg, 82%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.56 (s, 1H), 8.16 (d, J = 8.0 Hz, 1H), 8.10 (dd, J = 8.8, 1.6 Hz, 1H), 8.07 (d, J = 8.0 Hz, 1H), 8.02 (d, J = 8.8 Hz, 1H), 7.86 (d, J = 8.8 Hz, 1H), 7.56 (t, J = 7.6 Hz, 1H), 7.46 (t, J = 7.6 Hz, 1H), 7.22–7.18 (m, 2H). ^{13}C NMR(100 MHz, $CDCl_3$) δ = 168.2, 157.6, 154.2, 136.6, 134.8, 131.2, 127.8, 127.8, 127.7, 127.6, 127.1, 125.8, 124.7, 123.1, 122.8, 120.3, 109.4. HRMS (ESI) m/z calcd for $C_{17}H_{11}NOS$ $[M+H]^+$: 278.0561; found 278.0644.

Compound **NS** (55.4 mg, 0.2 mmol) was dissolved in DCM (2.0 mL) at 0 °C, and 2,4-dinitrobenzenesulfonyl chloride (64.0 mg, 1.2 equiv) was added in portion. Then the reaction mixture was moved to room temperature for 4 h. Subsequently, H_2O (5.0 mL) was added to the reaction mixture, and then extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated under vacuum, and the crude product was purified by silica column chromatography to give the compound **NS-S** (85.3 mg, 84%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 9.16 (d, J = 2.0 Hz, 1H), 8.79 (s, 1H), 8.59 (dd, J = 8.4, 2.0 Hz 1H), 8.32–8.27 (m, 3H), 8.21 (d, J = 7.6 Hz, 1H), 8.16 (d, J = 8.8 Hz, 1H), 8.12 (d, J = 8.0 Hz, 1H), 7.92 (d, J = 2.4 Hz, 1H), 7.61–7.57 (m, 1H), 7.53–7.49 (m, 1H), 7.43 (dd, J = 8.8, 2.4 Hz 1H). ^{13}C NMR(100 MHz, $CDCl_3$) δ = 166.8, 153.6, 151.6, 148.2, 147.3, 134.7, 134.5, 133.7, 131.9, 131.9, 131.4, 130.1, 129.4, 127.6, 127.3, 126.9, 125.9, 125.4, 123.1, 122.5, 121.6, 121.2, 119.9. HRMS (ESI) m/z calcd for $C_{23}H_{13}N_3O_7S_2$ $[M+H]^+$: 508.0195; found 508.0260.

2.3. Determination of the fluorescence quantum yield

Fluorescence quantum yields were determined by using fluorescein (0.1 M in NaOH) according to previous report [39]. The fluorescence quantum yield of compound **NS** was

calculated according to the following equation:

$$\eta_s = \frac{A_r I_s n_s^2}{A_s I_r n_r^2}\eta_r (A \leq 0.05)$$

In the equation, s and r represent the sample and the reference (fluorescein) molecule respectively, η represents the fluorescence quantum yield, A is the absorbance of molecules that were controlled below 0.05 at the excitation wavelength for both molecules in the experiment, I means the integrated emission area and n is the refractive index of the solvent.

2.4. Two-photon absorption (TPA) cross sections

Two-photon absorption (TPA) cross sections were measured using the two-photon induced fluorescence method, and the cross section can be calculated according to the following equation:

$$\delta_s = \delta_r \frac{\Phi_r c_r n_r F_s}{\Phi_s c_s n_s F_r}$$

In the equation, s and r represent the sample and the reference (fluorescein) molecule, respectively. The terms c and n are the concentration and refractive index of the solution, respectively. F is the TP excited fluorescence integral intensity. Φ is the fluorescence quantum yield. δ_r is the TP absorbance cross-section of rhodamine 6G in methanol (d = 65 GM) at 800 nm.

2.5. Preparation of the stock and test solution

Unless otherwise noted, all the measurements were implemented with the following procedure. Stock solution of the **NS-S** probe (10 mM) was prepared in DMF (HPLC grade). The stock solutions of all the analytes were dissolved in distilled water (10 mL) to afford 10 mM aqueous solution. The slight pH variations of PBS were prepared by adding the different volumes of NaOH (0.1 M) or HCl (0.2 M). The stock solutions were used freshly and were diluted to desired concentrations by distilled water when needed.

2.6. Cells culture and fluorescence imaging

HeLa cells were grown in modified Eagle's medium (MEM) replenished with 10% fetal bovine serum (FBS) with the atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. Before imaging, the cells were washed with PBS three times and then incubated with **NS-S** (10 μM) for 30 min in an atmosphere of 5% CO_2, 95% air at 37 °C. For the control experiment 1, the cells were pretreated with 1 mM N-ethylmaleimide (NEM) for 30 min, and then incubated with **NS-S** (10 μM) in an atmosphere of 5% CO_2, 95% air at 37 °C. For the control experiment 2, the cells were pretreated with Cys (600 μM) for 30 min and then incubated with **NS-S** (10 μM) for 30 min in an atmosphere of 5% CO_2, 95% air at 37 °C. After washing with PBS three times to remove the remaining probe, the fluorescence images were acquired with a Nikon A1MP confocal microscopy with the equipment of a CCD camera.

2.7. Preparation of fresh mouse liver slices and two-photon fluorescence imaging

The Kunming mice were purchased from Shandong University Laboratory Animal Center (Jinan, China). All procedures for this study were approved by the Animal Ethical Experimentation Committee of Shandong University according to the requirements of the National Act on the use of experimental animals (China) [29]. The fresh rat liver slices were obtained from the liver of 14-day-old rat. The living liver slices were cut with 400 μm thickness using a vibrating-blade microtome in 25 mM PBS (pH 7.4). The living liver slices were pre-treated with **NS-S** (10 μM) for 0.5 h. Following this incubation for 1 h at 37 °C, the slices were washed three times by PBS buffer and imaged. The two-photon fluorescence emission was collected at between 470 and 570 nm upon excitation at 800 nm with a femtosecond laser.

2.8. Cytotoxicity assay

The cells line were incubated in DMEM (Dulbecco's Modified Eagle Medium) replenished with 10% fetal bovine serum(FBS), 100 U/mL of penicillin and 100 μg/mL streptomycin with the atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were then seeded into 96-well plates, and 0, 1, 5, 10, 15, 20 μM (final concentration) of the **NS-S** probe (99.9% DMEM and 0.1% DMSO)