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A novel fluorescent probe with large Stokes shift for two-photon imaging of biothiols in living cells, liver tissues and tumor tissues

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ABSTRACT

Cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), the small molecule biological thiols, play essential roles in maintaining human biological systems. Research of quantitative determination of biothiols is always significant. We have designed and synthesized a fluorescent probe **ANBI** based on a naphthalene-benzo[g]indol-1-ium scaffold, quick respond to biothiols in water. The probe exhibits high stability, high selectivity, good water solubility and biocompatibility. Upon addition of biothiols, notably, probe **ANBI** showed an intense fluorescence within 5 min and displayed a large Stokes shift of 130 nm (Ex = 460 nm, Em = 590 nm). From the linear relationship of absorbance and intensity vs concentrations of Cys, it was determined that the limits of detection are 2.3 μM and 0.35 μM. Therefore, **ANBI** can qualitatively and quantitatively detect Cys by spectrometry in biological samples. Successfully, probe **ANBI** has been applied for biothiols in living HeLa cells by one- and two-photon excitation. Furthermore, we obtain the two-photon excited fluorescence images of **ANBI** in liver tissues and tumor tissues.

1. Introduction

Small molecule biological thiols including glutathione (GSH), cysteine (Cys) and homocysteine (Hcy) always play various crucial roles in maintaining cellular redox homeostasis [1–3]. Abnormal levels of them are implicated in a variety of diseases, such as edema, carotid therosclerosis, Alzheimer's disease and Parkinson's disease [4–6]. The significant roles of biothiols have spurred researchers to developing useful methods for detecting biothiols in biological systems.

The existing analytical techniques to detect biothiols include spectroscopy, electrochemistry, chromatography-mass spectrometry and magnetic resonance imaging method and so on [7–10]. Among them, fluorescence spectroscopy has become popular approach in analyte detection because of their simple operation, low cost, good specificity, real time monitoring and non-invasiveness in intracellular imaging [11–14]. Numerous fluorescent probes for biothiols have been reported, and the different sensing mechanisms include Michael addition, cleavage reaction, redox reaction and others [15–19]. Previous reported thiols probes always developed with some limitations, including organic medium, ultraviolet excitation wavelength and time-consuming. The most serious issue among them is interfered from hydrogen sulfide (H_2S), which has similar nucleophilic property. Consequently, more effective detection methods for these problems should be research.

Based on these analyses, the aim of our work is to develop a novel fluorescent probe which detects biothiols (GSH, Cys and Hcy) accurately. Acrylate group is an ideal functional trigger to detect biothiols, meanwhile, most of the reported fluorescent probes respond to H_2S [20,21]. On the contrary, hymicyanine group as a trigger is specific to H_2S [22,23]. Upon the different reactivity of the two reaction sites with biothiols, we designed a fluorescent probe **ANBI** to quantitatively detect Cys in water. Fortunately, this new probe **ANBI** features a fast response (5 min), large Stokes shift (130 nm), the use of a water medium, and applies in one- and two-photon living cell imaging. Furthermore, the two-photon liver and tumor tissues images demonstrate that probe **ANBI** has potential two-photon properties and application. Thus, **ANBI** possesses several desirable attributes for use in the rapid sensing of biothiols in biological samples conveniently.

2. Materials and methods

2.1. Apparatus and chemicals

Thin-layer chromatography (TLC) analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the

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Qingdao Ocean Chemicals. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using DMSO-d_6 as a solvent and tetramethylsilane (TMS) as internal reference. High-resolution mass spectra (HRMS) for the characterization of structures were collected using a Bruker apex-Ultra mass spectrometer (Bruker Daltonics Corp., USA) in electrospray ionization (ESI) mode. The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter. The fluorescence spectra and relative fluorescence intensity were measured with a Hitachi F-4600 spectrofluorimeter with a 1-cm quartz cuvette. UV/vis spectra were made with a Shimadzu UV-2700 spectrophotometer. Fluorescence imaging was performed with a Nikon A1R confocal microscope. All solvents and reagents were purchased from J&K, Meryer or Sinopharm Chemical Reagent Co., and used without further purification unless for special needs. Doubly distilled water was used in the experiments.

2.2. Absorption and fluorescence spectroscopy

Probe **ANBI** was dissolved in DMSO for a stock solution (1 mM). The amino acids (Cys, GSH, Alanine, Arginine, Aspartic acid, Glutamic acid, Histidine, Phenylalanine, Serine, Threonine, Tryptophan and Valine), cationic (K^+, Ca^{2+}, Na^+, Fe^{3+} and Zn^{2+}), H_2O_2 and Na_2S stocks were all in deionized water at 0.1 mM for absorption and fluorescence spectra analyses. Test solutions of **ANBI** (10.0 μM) in PBS buffer (20 mM, pH 7.4) with 1% DMSO was prepared, the resulting solution was shaken well and incubated with the appropriate testing species for 15 min at room temperature before recording spectra. For all measurements of fluorescence spectra, excitation wavelength was 460 nm, emission wavelength was 590 nm, slit: 10.0/10.0 nm.

2.3. Synthesis of (E)-2-(2-(6-hydroxynaphthalen-2-yl)vinyl)-1,3,3-trimethyl-3H-benzo[g]indol-1-ium iodide (*NBI*)

1,2,3,3-Tetramethyl-3*H*-benzo[g]indol-1-ium iodi (**1**) was prepared according to reported methods [24]. Compound **1** (1 mmol, 0.172 g) and compound **2** (1.2 mmol, 0.421 g) were placed in an eggplant bottle containing EtOH (30 mL), and then catalytic amount glacial acetic acid were added. The mixture was reflux 6 h until TLC indicated the end of the reaction. After cooling to room temperature, red precipitate was produced, and then filtered and washed with EtOH. The crude product was purified by the recrystallization from absolute ethanol to give **NBI** in 75% yield as a red solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.45 (s, 1H, -OH), 8.63 (d, J = 13.7 Hz, 2H), 8.46 (d, J = 8.4 Hz, 1H), 8.31 (d, J = 8.9 Hz, 2H), 8.23 (d, J = 8.1 Hz, 1H), 8.12 (d, J = 9.0 Hz, 1H), 7.94 (d, J = 8.8 Hz, 1H), 7.89 (d, J = 8.8 Hz, 1H), 7.83 (t, J = 7.2 Hz, 1H), 7.74 (dd, J = 15.6 and 7.8 Hz, 2H), 7.28–7.19 (m, 2H), 4.30 (s, 3H), 2.06 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 182.72, 159.09, 153.06, 139.97, 138.32, 137.79, 135.12, 133.59, 131.94, 131.30, 130.51, 129.83, 128.88, 127.72, 127.68, 12.53, 127.16, 124.96, 123.64, 120.37, 113.75, 111.51, 109.99, 54.09, 35.39, 25.75. HRMS: m/z $[M+H]^+$ calcd for $[C_{27}H_{24}NO]^+$ 378.1858, found 378.1858.

2.4. Synthesis of (E)-2-(2-(6-(acryloyloxy)naphthalen-2-yl)vinyl)-1,3,3-trimethyl-3H-benzo[g]indol-1-ium iodide (probe *ANBI*)

NBI (1 mmol) and trimethylamine (0.5 mL) were dissolved in CH_2Cl_2 (20 mL) at ice-bath. A solution of acryloyl chloride (3 mmol) in 10 mL CH_2Cl_2 was added to the mixture in 30 min. The mixture was stirred at 0 °C for 1 h, and then stirred for 2 h at room temperature. The organic phase was washed with water (30 mL × 3), and dried over $MgSO_4$, then filtered and concentrated under reduced pressure. The crude solid was purified by column chromatography on silica gel using CH_2Cl_2:MeOH = 25:1 as eluent to obtain product (**ANBI**) in 41% yield as a dark red solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.81 (s, 1H), 8.69 (d, J = 16.5 Hz, 1H), 8.47 (t, J = 8.3 Hz, 2H), 8.33 (d, J = 8.9 Hz, 1H), 8.25 (d, J = 8.2 Hz, 1H), 8.15 (dd, J = 8.9 and 5.1 Hz, 3H), 7.82–7.90 (m, 3H), 7.76 (t, J = 7.6 Hz, 1H), 7.54 (dd, J = 8.8, 2.3 Hz, 1H), 6.63 (dd, J = 17.3 and 1.4 Hz, 1H), 6.52 (dd, J = 17.3 and 10.2 Hz, 1H), 6.25 (dd, J = 10.2 and 1.4 Hz, 1H), 4.34 (s, 3H), 2.07 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 182.93, 164.66, 151.98, 150.62, 139.94, 138.77, 135.95, 134.65, 134.04, 133.76, 132.76, 131.40, 131.26, 130.53, 129.10, 129.95, 127.99, 127.77, 127.11, 125.40, 123.76, 123.38, 119.52, 113.87, 113.59, 54.37, 35.67, 25.50. HRMS: m/z $[M+H]^+$ calcd for $[C_{30}H_{26}NO]^+$ 432.1964, found 432.1957.

2.5. Fluorescence quantum yields and two-photon absorption cross sections

Fluorescence quantum yield was determined using optically matching solutions of fluorescein (Φ_s = 0.95 in 1 M NaOH) as the standard and the quantum yield was calculated using the following Equation (1) [25]:

$$\varphi = \varphi_S(IA_S/I_SA)\left(\eta^2/\eta_S^2\right) \quad (1)$$

where, s denotes standard, A is the absorbance, I is the integrated fluorescence intensity from one-photon emission spectrum, and η is the refractive index of the solvent.

The two-photon absorption cross section (σ) was determined by using a femtosecond (fs) fluorescence measurement technique. **NBI** was dissolved in PBS buffer solution (pH 7.2, 20 mM), respectively, at a concentration of 1.0×10^{-5} M. The two-photon fluorescence was excited at 700–900 nm by using fluorescein in 1 M NaOH as the standard, whose two-photon property has been well characterized in the literature [26]. The two-photon absorption cross-section was calculated using the following Equation (2):

$$\sigma = \sigma_S(I\varphi_SC_S/I_S\varphi C)\left(\eta^2/\eta_S^2\right) \quad (2)$$

where, s denotes standard, σ is the two-photon absorption cross-section, I is the integrated fluorescence intensity form two-photon emission spectrum, Φ is the quantum yield, C is the concentration and η is the refractive index of the solvent.

2.6. Cell culture and cell imaging

HeLa cells were cultured in DMEM medium supplemented with 10% FBS and incubated at 37 °C in air atmosphere (5% CO_2). The cells were seeded into glass bottom dishes with appropriate density. After 24 h, the cells were incubated with 5 μM **ANBI** for 20 min at 37 °C. The control group of living cells was preincubated with 500 μM NEM for 20 min, and then 5 μM **ANBI** was added and incubated for another 20 min. For fluorescence imaging, the cells were washed three times with PBS (pH 7.4) and then underwent imaging measurement by a Nikon A1R confocal microscope with the 488 nm excitation filter and emission channel of 570–620 nm (red channel). Imaging analysis involved use of ImageJ.

2.7. One-photon and two-photon imaging of liver and tumor tissues

The Kunming mice for this study were purchased by the Animal Ethical Experimentation Committee of Shandong University according to the requirements of the National Act on the use of experimental animals (China). Tissues were prepared from the liver and tumor of living mice. The tissues were incubated with **ANBI** (20 μM) in an incubator at 37 °C for 1 h and then washed with