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Design, synthesis, cell imaging, kinetics and thermodynamics of reaction-based turn-on fluorescent probes for the detection of biothiols

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ABSTRACT

Two highly selective red-emitting fluorescent "OFF-ON" probes (**3a** and **3b**) with a BODIPY core were designed and synthesized, based on the recovery of fluorescence upon the cleavage of the fluorescence quenching unit of 2, 4-dinitrobenzenesulfonyl (DNBS) by biothiols. The probes showed 43-fold (**3a**, $\lambda_{em} = 610$ nm) and 33-fold (**3b**, $\lambda_{em} = 582$ nm) fluorescence enhancement respectively in the presence of biothiols. Compared with **3b**, the introduction of five strong electron-withdrawing fluorine atoms in **3a**, not only caused a red shift of the emission maximum for 28 nm, but also increased the fluorescence enhancement. Since they had stable fluorescence emission within physiological pH range, the two probes can be applied in imaging of living cells. In contrast, no such fluorescence response was observed in the cells pre-treated with N-ethylmaleimide (NEM), a well-known thiol scavenger. A non-linear curve-fitting of high quality was used to fit the spectroscopic data to determine the pseudo-first-order rate constants and the chemical equilibrium constants at different given temperatures (*e.g.* 310 K, 298 K and 288 K). Activation energy (E_a) and thermodynamic parameters, including Gibbs free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS), could be calculated. To the best of our knowledge, this was the first report for the chemical kinetics and thermodynamics of the reaction between the fluorescent probes and thiols. This work would greatly inspire the future design of even better fluorescent probes.

1. Introduction

Biological thiols (biothiols), including cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), play pivotal roles in human physiology [1]. Design and synthesis of fluorescent molecular probes with high selectivity and sensitivity for the detection of thiols have attracted much attention [2–4]. Specifically, Cys is a precursor of GSH, acetyl Co-A and taurine, as well as a source of sulfide in iron-sulfur clusters. Many human diseases such as slow growth, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions, and weakness, are closely related to abnormal levels of Cys [5]. Hcy is a risk factor for disorders including cardiovascular and Alzheimer's diseases, and abnormal level of plasma Hcy is related to birth defects and cognitive impairment in the elderly [6]. Glutathione (GSH), essential to human physiology, is the most abundant cellular non-protein thiol, and has broad functions including detoxification of free radicals and peroxides, regulation of cell growth and protein function, and maintenance of immune function [7].

Fluorescent molecular probes, due to their high spatial, temporal resolution, high sensitivity and the easy-to-detect signal [8,9], are promising agents to detect thiols, especially for *in vivo* fluorescent analysis such as bioimaging [10,11]. Many fluorescent probes for the detection of thiols have been successfully designed by attaching electrophilic groups, *e.g.* maleimide, to fluorophores [12]. Sippel et al. reported N-(4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl) maleimide as a thiol probe that utilized the addition reaction of thiols to the maleimide moiety [13]. In recent years, more and more reactive types have been found, *e.g.* (i) addition reaction with −CHO group [14] and nitroolefin moiety [15], (ii) intramolecular hydrogen bonding [16], (iii) disulfide

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exchange [17,18], (iv) cleavage of 2,4-dinitrobenzenesulfonyl (DNBS) moiety [19] and Se-N bond [20,21], (v) conjugation addition–cyclization reactions [22]. Fluorophores with a DNBS moiety acting as a sensing motif, demonstrated excellent "OFF-ON" emission switch effect and good selectivity [23,24]. These studies greatly promoted the research in the fluorescent detection of biothiols [25,26]. However, many of these probes suffer from relatively low sensitivity due to the inefficiency of quenching or the inadequate selectivity. Thus, new fluorescent probes for the detection of biothiols with improved performances still need to be developed.

Boron-dipyrromethene (BODIPY) is an ideal fluorophore for the development of molecular probes owing to its attractive photophysical properties [27–33], including (i) the intensive absorption above 500 nm, (ii) high fluorescence quantum yield, and (iii) pH-independent emission and excellent photostability. Herein, based on the mechanism of photoinduced electron transfer (PET), we developed fluorescent probes with BODIPY as the fluorophore/electron donor and DNBS as the electron acceptor. The results showed that they were effective probes and could be applied in cell imaging. Moreover, the interaction kinetic and thermodynamic parameters were obtained, including the pseudo-first-order rate constant (k), activation energy (E_a), equilibrium constants (K), enthalpy change (ΔH), entropy change (ΔS) and the Gibbs free energy change (ΔG). The kinetics and thermodynamics results have guiding significance for further development of thiol probes [34,35].

2. Experimental section

2.1. Chemicals and instruments

The NMR spectra were measured in appropriate deuterated solvents on a Varian Unity Inova 300 MHz spectrometer, using $SiMe_4$ as an internal standard. The electron ionization (EI) mass spectra were measured on a VARIAN 320–MS advantage mass spectrometer. The fluorescence spectra were measured by an Agilent Cary Eclipse fluorescence spectrometer. The absorption spectra were measured by an Agilent Cary 100 UV–vis double-beam spectrophotometer. The standard 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was performed using a BioTek microplate reader, and the cell imaging was carried out using a Nikon C1-si Laser Scanning Confocal Microscope.

All reagents and chemicals, unless stated, were purchased from commercial suppliers and used without further purification. Solvents were dried and distilled with drying agents under an inert atmosphere prior to use. Double-distilled water was used in all experiments.

The BODIPY derivatives **1a** [36,37], **1b** and **2b** [28,38] were prepared according to procedures reported previously.

2.2. Synthesis of 2a

1a (207.1 mg, 0.50 mmol), 4-hydroxybenzaldehyde (91.5 mg, 0.75 mmol), toluene (30 mL), piperidine (2 mL) and glacial acetic acid (2 mL) were added to a 50 mL round-bottomed flask equipped with a Dean–Stark trap. The reaction mixture was refluxed for 2 h. The solvent was removed under reduced pressure. The resulting residue was purified by column chromatography (CH_2Cl_2) to afford **2a** (51.9 mg, 20.1%) as a red solid powder. ^{1}H NMR (300 MHz, $CDCl_3$): 7.55–7.49 (m, 3H, 2H for Ar-H, 1H for vinylic bond), 7.21 (d, J = 6.0 Hz, vinylic bond), 6.85 (d, J = 8.4 Hz, 2H, Ar-H), 6.64 (s, 1H, pyrrole-H), 6.05 (s, 1H, pyrrole-H), 3.30 (s, 3H, pyrrole-CH_3), 2.59 (s, 3H, pyrrole-CH_3), 1.66 (s, 3H, pyrrole-CH_3). EI-MS: M^+ calcd for $C_{26}H_{19}BF_7N_2O$: 519.2; found 519.4.

2.3. Synthesis of probe 3a

2a (25.7 mg, 0.050 mmol) was dissolved in CH_2Cl_2 (10 mL). Triethylamine (100 μL) was added to the solution, and the mixture was stirred for 5 min. A solution of 2, 4-dinitrobenzenesulfonyl chloride (66.7 mg, 0.25 mmol) in CH_2Cl_2 (5 mL) was added dropwise at 0 °C. The reaction mixture was refluxed for 3 h. After removing the solvents by evaporation, the resulting crude mixture was purified by column chromatography (CH_2Cl_2) to afford **3a** (13.8 mg, 36.8%) as a purple bronzing solid. ^{1}H NMR (300 MHz, $CDCl_3$): 8.68 (s, 1H, Ar-H), 8.50 (dd, 1H, J = 1.8 Hz, J = 2.1 Hz, Ar-H), 8.15 (d, 1H, J = 8.4 Hz, Ar-H), 7.61–7.54 (t, 3H, J = 8.1 Hz, 2H for Ar-H, 1H for vinylic bond), 7.21–7.13 (t, 3H, J = 8.1 Hz, 2H for Ar-H, 1H for vinylic bond), 6.63 (s, 1H, pyrrole-H), 6.10 (s, 1H, pyrrole-H), 3.30 (s, 3H, pyrrole-CH_3), 2.59 (s, 3H, pyrrole-CH_3), 1.68 (s, 3H, pyrrole-CH_3). EI-MS: m/z calcd for $C_{32}H_{21}BF_7N_4O_7S$ M^+: 749.1; found 749.4.

2.4. Synthesis of probe 3b

3b, a purple bronzing solid, was synthesized by the same way as **3a** (14.0 mg, 42.0%). ^{1}H NMR (300 MHz, $CDCl_3$): 8.68 (s, 1H, Ar-H), 8.68 (t, 1H, J = 7.2 Hz, Ar-H), 8.15 (t, 1H, J = 8.7 Hz, Ar-H), 7.55 (t, 3H, J = 8.4 Hz, 2H for Ar-H, 1H for vinylic bond), 7.18 (d, 1H, J = 8.4 Hz, Ar-H), 7.12 (d, 1H, J = 16.5 Hz, vinylic bond), 6.96 (s, 2H, Ar-H), 6.56 (s, 1H, pyrrole-H), 6.03 (s, 1H, pyrrole-H), 2.58 (s, 3H, pyrrole-CH_3), 2.34 (s, 3H, Ar-CH_3), 2.10 (s, 6H, Ar-CH_3), 1.43 (s, 3H, Ar-CH_3), 1.41 (s, 3H, Ar-CH_3). EI-MS $[M-H]^-$ calcd for $C_{35}H_{32}BF_2N_4O_7S$: 700.1, found 700.0.

2.5. Spectroscopic methods

The stock solution of probe was prepared at 1.0 mmol L^{-1} in dimethyl sulfoxide (DMSO). The solutions of biologically relevant analytes were prepared from the 20 coding amino acids, H_2O_2, 3-mercaptopropionic acid (3-MPA), glutathione (GSH) and L-homocysteine (Hcy) in the double-distilled water. All spectroscopic measurements were performed with a mixture solution of acetonitrile and 20 mmol L^{-1} HEPES buffer (1:1, v/v, pH = 7.4). All testing solutions (except the experiments for kinetics) were set aside for 2 h for equilibrium. The fluorescence quantum yield (Φ_f) was measured relative to Fluorescein (Φ_f = 0.79 in 0.1 M NaOH) and Rhodamine 101 (Φ_f = 1.00 in Ethanol + 0.01% HCl) [39].

2.6. Cell culture, MTT assay and cell imaging

HeLa cells was used in this work and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 at 37 °C.

The cytotoxicity of the probes was assessed by the standard MTT assay. In brief, 100 μL of HeLa cells were seeded in a 96-well plate with a density of 1×10^5 cells per mL. After an incubation of 24 h at 37 °C, the culture medium was discarded and the cells were then treated with various concentrations of the probes (10–200 μM) for another 24 h. At the end of incubation, the culture medium was removed, and 20 μL of MTT (5.0 mg mL^{-1} in PBS) was added into each well. After an additional incubation for 4 h, the growth medium was removed, 150 μL of DMSO was added into each well to dissolve MTT and the plate was shaked in the dark for 15 min at 37 °C. Finally, the optical density of each sample was recorded by a microplate reader at 570 nm.

For cell imaging, the fluorescence images were photographed using a C1-si Laser Scanning Confocal Microscope. Emission signal was collected in the red channel with excitation at 543 nm by an argon laser. To prove the reaction between the fluorescent probes and biothiols, cell imaging was also conducted when cells were pre-