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Live cells imaging using a turn-on FRET-based BODIPY probe for biothiols

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ABSTRACT

We designed a red-emitting turn-on FRET-based molecular probe **1** for selective detection of cysteine and homocysteine. Probe **1** shows significant fluorescence enhancement after cleavage of the 2, 4-dinitrobenzensulfonyl (DNBS) unit from the fluorophore upon thiols treatment. The precursor of probe **1**, **BNM153**, is a moderate quantum yield FRET dye which contributes a minimum emission leakage from its donor part. We synthesized this assembly by connecting a low quantum yield (less than 1%) BODIPY donor to a high quantum yield BODIPY acceptor via a 1, 3-triazine bridge system. It is noteworthy that the majority of the non-radiative energy loss of donor (**BDN**) was converted to the acceptor (**BDM**)'s fluorescence output with minimum leaks of donor emission. The fluorescence sensing mechanism of probe **1** was illustrated by fluorescence spectroscopy, kinetic measurements, HPLC-MS analysis and DFT calculations. Probe **1** is pH-independent at the physiological pH range. Finally, live cells imaging demonstrated the utility of probe **1** as a biosensor for thiols.

1. Introduction

Biological thiols such as cysteine (Cys), homo-cysteine (Hcy), and glutathione (GSH) play important roles in a number of biological processes in living organisms [1,2]. Cys is precursor to the antioxidant GSH, and their abnormal levels are closely related to human ageing and various diseases [3]. While Hcy is a risk factor for Alzheimer's disease and cardiovascular diseases, total plasma Hcy concentrations have been proven to link to birth defects and cognitive impairment at the elderly stage [4]. In view of the importance of thiols, the design of analytical methodologies for the detection of biomolecular thiols in biological and environmental samples has consistently attracted a great deal of attention. Traditional detection methods such as high performance liquid chromatography (HPLC) [5], electrophoresis-based methods [6], as well as electrospray ionization-mass spectrometry [7] have been used to detect thiols. However, these methods are not convenient for use, since they often involve the expensive instrumentation and can be technically demanding in their handling.

On the contrary, fluorescent molecular probes are more attractive, due to their high sensitivity and easy visibility. Also, fluorescent molecular probes offer the possibility of quantitative optical detection of biothiols and broad bioimaging applications, such as detecting the carcinoma region of liver tissue based on the differences of glutathione level [8]. Till now, a number of diverse sensing mechanisms have been employed for thiol detection, including thiol–halogen nucleophilic substitution [9,10], Michael addition [11–14], –CHO attached fluorophores [15–18], and de-protection of 2,4-dinitrobenzenesulfonyl (DNBS)-protected fluorophores [19–24]. Most of these published probes show good selectivity and sensitivity in detecting certain biothiols by simple spectroscopic techniques, which monitor optical responses caused by different concentrations of biothiols. However, most of them were designed based on a single fluorophore as the fluorescence signalling profile, which may limit their applications. These single fluorophore probes have small Stokes shifts, which can lead to serious self-quenching and fluorescence detection error due to excitation backscattering effects. Some FRET-based probes, constructed with dyes that have high extinction coefficients and high quantum yields as donors, have also been explored for thiols detection. Unfortunately, instances of fluorescence leaks from the donor have been inevitable, as a result of either low energy transfer efficiencies or environmental changes [25,26].

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Hence, newly improved FRET probes with better photophysical properties, which can efficiently eliminate the interference from background fluorescence and scattered light, are worthwhile to design. Several criteria must be satisfied for the FRET probe: 1) absorbance of light at a high extinction coefficient but no fluorescence emission from donor part to ensure that there is no background influence; 2) large pseudo-Stokes shifts and emission shifts to avoid serious self-quenching and fluorescence detection errors caused by excitation backscattering effects [27,28]; 3) high efficient FRET energy transfer. Here, we designed and synthesized a turn-on FRET-based molecular probe **1** containing a 2,4-dinitrobenzenesulfonyl (DNBS) group on its acceptor, acting as the reaction site for biothiols. In addition, the application of probe **1** for sensing thiols in live cells and its sensing mechanism were also investigated.

2. Experimental section

2.1. Materials

The chemicals and solvents, were purchased from Sigma Aldrich, Acros and Alfa Aesar. All the chemicals were directly used without further purification. Normal phase column chromatography purification was carried out using MERCK silica Gel 60 (Particle size: 230–400 mesh, 0.040–0.063 mm).

2.2. Measurements and analysis

HPLC-MS was taken on an Agilent-1200 with a DAD detector and a single quadrupole mass spectrometer (6130 series). The analytical method, unless indicated, is A: H_2O (0.1% HCOOH), B: CH_3CN (0.1% HCOOH), gradient from 10 to 90% B in 10 min; C18 (2) Luna column (4.6 × 50 mm^2, 3.5 μm particle size).

Spectroscopic and quantum yield data were measured on a SpectraMax M2 spectrophotometer (Molecular Devices). Data analysis was performed using GraphPrism 5.0.

^1H NMR and ^{13}C NMR spectra were recorded on Bruker ACF300 (300 MHz) or AMX500 (500 MHz) spectrometers, and chemical shifts are expressed in parts per million (ppm) and coupling constants are reported as a *J* value in Hertz (Hz).

2.3. Quantum yield measurements

Quantum yields for all the fluorescent compounds were measured by dividing the integrated emission area of their fluorescent spectrum against the area of Rhodamine B in EtOH excited at 490 nm ($\Phi_{rho\text{-}B} = 0.7$) [29]. Quantum yields where then calculated using equation (1), where *F* represents the integrated emission area of fluorescent spectrum, η represents the refractive index of the solvent, and Abs represents absorbance at excitation wavelength selected for standards and samples. Emission was integrated from 530 nm to 750 nm.

$$\Phi_{flu}^{sample} = \Phi_{flu}^{sample}\left(\frac{F^{sample}}{F^{reference}}\right)\left(\frac{\eta^{sample}}{\eta^{reference}}\right)\left(\frac{Abs^{reference}}{Abs^{sample}}\right) \quad (1)$$

2.4. DFT calculations

All calculations have been performed with the Gaussian 09 code. Full geometry optimizations and electronic structure calculations were performed with density functional theory (DFT) at B3LYP/6-31G(d) level. The excited state related calculations were carried out with the time-dependent DFT (TDDFT), based on the optimized ground state geometry [30].

2.5. Cell culture and imaging experiments

HeLa cells were cultured in clear bottom, 96-well plate and maintained in an incubator at 37 °C with 5% CO_2, 24–36 h prior to imaging experiments. Cells were grown in Dulbecco's Modified Eagle Medium (Sigma) with 10% newborn calf serum, as well as 5 mM L-glutamine and 5 mg/mL gentamicin. Probe stock solution in DMSO (1 mM) was diluted to 100 μM with PBS before being added to cell culture wells to reach the final concentration of 3 μM. After 30 min incubation at 37 °C, cells were washed with PBS buffer twice prior to imaging. Cells were also treated with 1 mM N-methylmaleimide in PBS at 37 °C for 30 min and subsequently washed twice with PBS buffer, prior to incubation with probe as per described. Separately, as control experiment, cells were pre-treated with 1 mM L-buthionine sulfoximine (BSO) or 1 mM BSO in the presence of 0.1 mM hydrogen peroxide for 1 h. The latter was further treated with 1 mM L-cysteine for 1 h or left untreated before incubation with probe. Live cells imaging was done with an inverted fluorescence microscope Ti (Nikon), equipped with an Ex 480 nm/40, long-pass 510 nm filter for fluorescence image acquisition.

2.6. Synthesis

The **BDN** and **BDM153** were synthesized following our previous report [31,32].

2.6.1. Synthesis of ***BDN-Tri***

To a solution of **BDN** (115 mg, 0.37 mmol) in 70 mL of dry tetrahydrofuran (THF) was added cyanuric chloride (101 mg, 0.55 mmol). The reaction mixture was stirred for 1 h at 0 °C. After removal of the THF, the residue was purified by silica gel chromatography (hexane–EtOAc, 4:1) to give **BDN-Tri** as a yellow solid (152 mg, 90%).

^1H NMR (300 MHz, $CDCl_3$): δ 7.81 (s, 1H), 7.73 (d, *J* = 8.5 Hz, 2H), 7.70 (s, 1H), 7.40 (d, *J* = 8.5 Hz, 2H), 6.41 (s, 1H), 6.38 (s, 1H), 6.15 (s, 1H), 2.63 (s, 3H), 1.60 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$): 171.47, 170.44, 164.02, 162.37, 146.68, 142.13, 138.89, 137.22, 134.56, 133.51, 130.92, 129.95, 126.91, 123.41, 120.48, 116.20, 29.65, 15.24.

HRMS *m/z* ($C_{20}H_{15}BCl_2F_2N_6$) calculated $(M-H)^-$: 457.0718, found $(M-H)^-$: 457.0716.

2.6.2. Synthesis of ***BDM153***

Compound **2** (50 mg, 103 μmol) and 4-hydroxybenzaldehyde (25.2 mg, 206 μmol) were dissolved in acetonitrile (5 mL), with pyrrolidine (5.2 μL, 618 μmol) and 6 equiv. of AcOH (3.5 μL, 618 μmol). The mixture was heated to 85 °C and keep for 5 min. After removal of the solvent, the residue was purified by silica gel chromatography (eluents: CH_2Cl_2–MeOH, 98: 2 to 90: 10) to give **BDM153** as a purple solid (21.7 mg, 57%).

^1H NMR (500 MHz, $CDCl_3$): δ 7.38 (s, 1H), 7.30 (d, *J* = 8.6 Hz, 2H), 7.24 (dd, *J* = 34.7, 16.3 Hz, 3H), 7.09 (d, *J* = 3.8 Hz, 1H), 6.66 (s, 1H), 6.68 (d, *J* = 8.5 Hz, 2H), 6.65 (dd, *J* = 3.9, 2.1 Hz, 1H), 3.22–3.19 (m, 2H), 3.01–2.98 (m, 2H), 2.34 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$):159.32, 159.19, 144.21, 141.47, 136.46, 135.46, 135.14, 133.37, 129.74, 127.21, 122.03, 120.12, 115.71, 115.60, 114.69, 44.98, 23.80, 15.70.

HRMS *m/z* ($C_{20}H_{20}BF_2N_3O$) calculated $(M+Na)^+$: 390.1565, found $(M+Na)^+$: 390.1573.

2.6.3. Synthesis of ***BNM153***

BDN-Tri (20 mg, 54 μmol), **BDM153** (25 mg, 54 μmol) and DIEA (14 μL, 108 μmol) were dissolved in THF (10 mL) and stirred at room temperature for 2 h. After evaporation of the solvent, the crude product was purified by silica gel chromatography (eluents: CH_2Cl_2 to CH_2Cl_2–MeOH (95: 5)) to afford the corresponding **BNM153** as red solid (30.7 mg, 72%).

^1H NMR (500 MHz, CD_3CN): δ 8.21 (d, *J* = 11.6 Hz, 1H), 7.88 (d, *J* = 8.3 Hz, 1H), 7.72 (d, *J* = 8.2 Hz, 1H), 7.66 (s, 1H), 7.58 (d, *J* = 10.1 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.49 (d, *J* = 8.6 Hz, 1H), 7.44–7.40 (m, 2H), 7.32 (d, *J* = 8.2 Hz, 1H), 7.19 (s, 1H), 6.97–6.89 (m, 3H), 6.51–6.44 (m, 3H), 6.27 (d, *J* = 47.5 Hz, 1H), 3.83 (dd, *J* = 13.5, 6.8 Hz, 1H), 3.70 (dd, *J* = 14.8, 7.1 Hz, 1H), 3.32 (dd, *J* = 13.7, 6.7 Hz, 2H), 2.63–2.52 (m, 6H), 1.62 (d, *J* = 41.6 Hz, 3H); ^{13}C NMR (125 MHz, CD_3CN): 166.14, 164.40, 162.58, 159.10, 147.71, 145.81, 143.61, 143.45, 141.08, 140.72, 140.51, 140.42, 140.12, 139.81, 137.85, 137.77, 136.40, 136.17, 133.63, 129.72, 129.64, 129.59, 128.48, 127.91, 126.65, 126.54, 123.67, 123.57, 123.37, 120.26, 119.91, 119.75, 116.05, 116.02, 114.95, 42.16, 29.80, 15.96, 15.80, 14.34.

HRMS *m/z* ($C_{40}H_{34}B_2ClF_4N_9O$) calculated $(M-H)^-$: 788.2619, found $(M-H)^-$: 788.2610.

2.6.4. Synthesis of probe ***1***

BNM153 (30 mg, 38 μmol) and Et_3N (16 μL, 114 μmol) was dissolved in 10 mL of dry CH_2Cl_2. The mixture was stirred at 0 °C for 15 min under N_2 atmosphere. A solution of 2,4-dinitrobenzenesulfonyl chloride (20.3 mg, 76 μmol) in 5 mL CH_2Cl_2 was added dropwise. The reaction mixture was stirred for 2 h at rt. After evaporation of the solvent, the crude product was purified by silica gel chromatography (eluents: CH_2Cl_2 to CH_2Cl_2–MeOH (95: 5)) to afford the corresponding probe **1** as red solid (23 mg, 59%).

^1H NMR (500 MHz, $CDCl_3$): δ 8.66 (s, 1H), 8.48 (d, *J* = 8.6 Hz, 1H), 8.14 (dd, *J* = 8.6, 4.9 Hz, 1H), 7.72 (d, *J* = 8.4 Hz, 1H), 7.63–7.48 (m, 7H), 7.33 (d, *J* = 3.6 Hz, 1H), 7.27–7.15 (m, 4H), 6.48–6.34 (m, 3H), 6.10 (d, *J* = 21.9 Hz, 1H), 3.80 (s, 1H), 3.68 (s, 1H), 3.26 (dd, *J* = 15.0, 7.5 Hz, 2H), 2.58–2.43 (m, 6H), 1.55 (d, *J* = 28.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): 165.28, 162.12, 161.97, 155.68, 155.58, 150.90, 149.01, 148.82, 146.82, 139.09, 138.93, 138.75, 138.36, 138.25, 136.64, 136.56, 135.91, 135.80, 134.59, 133.91, 133.88, 133.47, 133.10, 129.52, 129.44, 129.20, 126.84, 126.50, 126.45, 124.56, 123.35, 123.23, 122.36, 122.32, 120.29, 120.11, 120.01, 119.90, 119.50, 116.64, 116.56, 115.95, 42.44, 30.72, 16.42, 14.93.

HRMS *m/z* ($C_{46}H_{36}B_2ClF_4N_{11}O_7S$) calculated $(M-H)^-$: 1018.2252, found $(M-H)^-$: 1018.2230.

3. Results and discussion

3.1. Design and synthesis of the probe 1

Here, we designed a reactive FRET probe **1** having long emission wavelength and large pseudo-Stokes shifts characteristics. **BDN** skeleton was chosen as the donor because of its high extinction coefficient and low quantum efficiency [32]. The red emitting styryl **BDM153** was chosen as acceptor part of the FRET pair of probe **1**,