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General

Dual mode monitoring probe for mitochondrial viscosity in single cell

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ABSTRACT

Viscosity strongly influences intracellular transportation of mass and signal, and although bulk viscosity can be well measured with macroscopic samples quantities, microscale measurements in live cells still remain a challenge. By introducing a CHO group into the *meso*-position of the pentamethine chain, a thiazole-Cy5 dye based two-photon fluorescence probe, **Mito-V**, was developed. It exhibited a selective response to solution viscosity by fluorescence ratio and lifetime methods. More importantly, **Mito-V** can localize in mitochondria and quantitatively image its viscosity changes by the dual fluorescence mode during the apoptosis.

1. Introduction

Viscosity strongly influences intracellular transportation of mass and signal, interactions between biomacromolecules, and diffusion of reactive metabolites such as ROS and RNS at the intracellular level [1,2]. The sudden changes of viscosity have been related to a wide range of diseases and dysfunction at the cellular level. Mitochondrion is the energy-producing compartments in the cells, and its function can be inhibited by decreasing the mitochondrial membrane fluidity, reducing electron transport chain (ETC) activating, increasing ROS production, and facilitating cytochrome *c* release [3]. It has been widely demonstrated that increase of mitochondrial viscosity can cause some diseases, like Alzheimer disease (AD) and Parkinson disease (PD) [4,5]. Therefore, it has a great significance to quantify the mitochondrial viscosity in living cells.

Although bulk viscosity can be well measured by mechanical or fluid-dynamics approaches with macroscopic samples quantities [6], microscale measurements in live cells still remain a challenge. In response to this, Theodorakis [7,8], Kuimova [9–13], Suhling [10–12], and some groups [14–16] have recently developed a group of molecular rotors which typically comprise a conjugated domain that can freely rotate in low-viscosity solutions and be restrained in viscous environments. As expected these molecules have made good progresses on the investigation of intracellular viscosity by fluorescence ratiometric or lifetime method. Nevertheless, these probes showed nonspecific intracellular distributions, and fluorescent probes which can well-suited for mitochondrial applications is barely reported [17,18].

Herein, we report a fluorescent molecular rotor **Mito-V** by attaching two benzyl groups to a Cy5 with a CHO group at the *meso*-position of the pentamethine chain (Scheme 1). As known two-photon microscopy imaging technique (TPM) can minimize background absorption to living biological samples and improve the spatial resolution, sensitivity and the ability to image rather thick specimens [19–25]. Significantly, this kind of Cy5 dyes showed good two-photon absorption properties. Referring to the previous work [14], the two benzyl groups were introduced to further regulate the lipid solubility of **Mito-V**. The hydrophilicity–lipophilicity is modeled by the logarithm of the water–octanol partition coefficient (log *P*). Probes specifying mitochondrial accumulation are assigned numerically by the following criteria: electric charge ($Z > 0$) and $0 < \log P < +5$. Since $Z_{\textbf{Mito-V}}$ and $\log P_{\textbf{Mito-V}}$ values of Cy5 dyes based on benzothiazole are 1 and 2.8 respectively, it is probably that **Mito-V** can be trapped in mitochondria [26,27].

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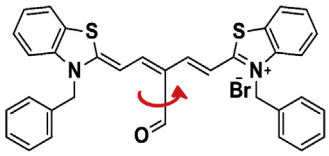

Scheme 1. Chemical structure of mitochondrial viscosity probe **Mito-V**.

2. Experimental

2.1. Sample preparation

2-Methylbenzothiazole, thiazole quaternary ammonium salts and other intermediates were prepared according to literature methods. All solvents and reagents used were reagent grade. All reactions were carried out under a nitrogen atmosphere with dry, freshly distilled solvents under anhydrous conditions. Silica gel (100–200 mesh) was used for flash column chromatography for purifications. Bovine Serum Albumin (BSA), Rabbit Ig *G* (*H*+*L*) and Human fibrin-ogen were purchased from Shanghai Sangon Biotech Co., Ltd. Etoposide (20 mg/mL in NS) was purchased from Key GEN Biotech. Water used in all experiments was doubly purified by Milli-Q systems equipment. The **Mito-V** solution was typically prepared from 1.0 mM stock solution in DMSO.

Mass spectrometric studies were carried out using LC/Q-Tof mass spectrometer. NMR spectra were obtained using a Varian INOVA 400 MHz spectrometer. Visible absorption spectra were measured on a Lamda LS35 spectrophotometer. Fluorescence spectra were obtained with a FP-6500 spectrophotometer (Jasco, Japan).

2.2. Synthesis of intermediates and probe ***Mito-V***

2.2.1. Synthesis of compound ***1*** [28]

Benzyl bromide (0.85 g, 5 mmol) was added under nitrogen to 2-methyl benzothiazole (0.90 g, 6 mmol) with stirring at 60–70 °C for 3 h. The mixture was then cooled, the precipitate filtered off, and washed with ether to give 3-benzyl-2-methylbenzothiazolium bromide, 3-benzyl-2-methylbenzothiazolium bromide salt **1** was obtained as white solid as crude product (1.9 g, yield 81%).

2.2.2. Synthesis of ***2***

$POCl_3$ (2.8 mL, 29.4 mmol) was added dropwise to a cooled solution of DMF (C_3H_7NO, 8 mL, 103.6 mmol, at about 0 °C) over a period of 0.7 h keeping the temperature below 5 °C during the addition. The solution turned pale orange by the end of the addition. Then the ice-bath was removed and the dense mixture was stirred at room temperature for 1 h. Bromoacetic acid (1.43 g, 10.2 mmol) was added in portions, and the mixture was heated for 24 h at 65–70 °C. The brownish mixture was decomposed with ice/water (40 mL) and solid Na_2CO_3 was carefully added in large excess until pH = 8.0. Absolute ethanol was added (1 L), then the inorganic salts were filtered off. The organic filtrate was concentrated slowly under vacuum evaporation, and the pale yellowish residue was neutralized with H_2SO_4 (50%, 2.5 mL), extracted with $CHCl_3$ (3 × 80 mL) and dried over Na_2SO_4. After solvent removal, triformylmethane **1** was obtained as yellowish solid as crude product (0.4 g, yield 36% based on bromoacetic acid).

2.2.3. Synthesis of ***Mito-V***

Compound **2** (0.11 g, 1.1 mmol) and **1** (0.64 g, 2.0 mmol) were mixed in 20 mL anhydrous ethanol and several drops of pyridine were added. The solution was refluxed under nitrogen for 2 h and cooled down. Then the solvent was removed by rotary evaporation. The residue was washed by 50 mL saturated saline, and then extracted by DCM (3 × 50 mL). The extracts were combined and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum to obtain a deep blue residue which was purified by chromatography (silica gel, 80:1 DCM/methanol, v/v as eluting solvent). Eluent was concentrated by evaporating most DCM and then poured into a large amount of ethyl ether to give a powder with a metalescent solid (0.23 g, 37%).

^{1}H NMR (400 MHz, DMSO-d^6): 9.76 (s, 0.5H,CHO), 8.19 (d, *J* = 7.9 Hz, 1*H*), 7.96 (d, *J* = 14.3 Hz, 1*H*), 7.92 (d, *J* = 8.4 Hz, 1*H*), 7.71–7.58 (m, 2*H*), 7.54 (t, *J* = 7.7 Hz, 1*H*), 7.42–7.37 (m, 2*H*), 7.33 (t, *J* = 6.3 Hz, 3*H*), 5.87 (s, 2*H*,CH_2). ^{13}C NMR (101 MHz, $CDCl_3$): δ 195.60, 137.40, 123.98, 122.67, 121.29, 116.29, 93.76, 82.22, 80.91, 28.35, 26.27, 19.50. HRMS-ESI: *m*/*z* calcd M+ for $C_{34}H_{27}N_2OS_2$+, 543.1559; found, 543.1548.

2.3. Fluorescence quantum yields measurements

The relative fluorescence quantum yields were determined with Rhodamine B (Φ_F = 0.97) in ethanol as a standard and calculated using the following equation [29]:

$$\Phi_x = \Phi_s(F_x/F_s)(A_s/A_x)(\lambda_{exs}/\lambda_{exx})(n_x/n_s)^2$$

where Φ represents quantum yield; *F* stands for integrated area under the corrected emission spectrum; *A* is absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; *n* is the refractive index of the solution (because of the low concentrations of the solutions (10^{-7}–10^{-8} mol/L), the refractive indices of the solutions were replaced with those of the solvents); and the subscripts *x* and *s* refer to the unknown and the standard, respectively.

2.4. Viscosity and fluorescence lifetime detection

The solvents were obtained by mixing deionized water–glycerol and ethanol–glycerol systems in different proportions. Measurements were carried out with a NDJ-7 rotational viscometer, and each viscosity value was recorded. The solutions of **Mito-V** in different viscosity were prepared by adding the stock solution (1.0 mM, 5.0 μL) to 5.0 mL of solvent mixture (water–glycerol or ethanol–glycerol solvent systems) to obtain the final concentration of the dye (1.0 μM). These solutions were sonicated for 10 min to eliminate air bubbles. After standing for 15 min at a room temperature, the solutions were measured in a UV spectrophotometer and a fluorescence spectrophotometer. A fluorescence lifetime measuring equipment (Edinburgh Instruments) was used to obtain the fluorescence lifetimes of **Mito-V**, with the excitation wavelength at 405 nm and detection at 658 nm.

2.5. Measurement of two-photon cross section

The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described. **Mito-V** was dissolved in different solution at concentrations of 5.0×10^{-4} M and then the two-photon induced fluorescence intensity was measured at 690–900 nm by using fluorescein (8.0×10^{-5} M, pH = 11) as the reference, whose two-photon property has been well characterized in the literature. The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using equation [30]:

$$\delta = \delta_r(S_s\,\Phi_r\,\varphi_r\,c_r)/(S_r\,\Phi_s\,\varphi_s\,c_s)$$

where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*; and Φ is the fluorescence quantum yield; φ is the