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Specifically and wash-free labeling of SNAP-tag fused proteins with a hybrid sensor to monitor local micro-viscosity

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ABSTRACT

Viscosity, as one of the major factors of intracellular microenvironment, influences the function of proteins. To detect local micro-viscosity of a protein, it is a precondition to apply a viscosity sensor for specifically target to proteins. However, all the reported small-molecule probes are just suitable for sensing/imaging of macro-viscosity in biological fluids of entire cells or organelles. To this end, we developed a hybrid sensor **BDP-V BG** by connecting a viscosity-sensitive boron-dipyrromethene (BODIPY) molecular rotor (**BDP-V**) to O^6-benzyl-guanine (**BG**) for specific detection of local micro-viscosity of SNAP-tag fused proteins. We measured and calculated the reaction efficiency between the sensor and SNAP-tag protein *in vitro* to confirm the high labeling specificity. We also found that the labeling reaction results in a 53-fold fluorescence enhancement for the rotor, which qualifies it as a wash-free sensor with ignorable background fluorescence. The high sensitivity of protein labeled sensor (**BDP-V-SNAP**) to the changes of local viscosity was evaluated by detecting the enhancement of fluorescence lifetimes. Further, with the sensor **BDP-V BG**, we achieved high specific labeling of cells expressing two SNAP-tag fused proteins (nuclear histone H2B and mitochondrial COX8A). Two-photon excited fluorescence lifetime imaging revealed that, the micro-viscosities nearby the SNAP-tag fused two proteins are distinct. The different changes of local micro-viscosity of SNAP-tag fused histone protein in apoptosis induced by three nucleus-targeted drugs were also characterized for the first time.

1. Introduction

Recently, fluorescent probes sensitive to viscosity have attracted considerable attention, because they provide a convenient way to visualize and detect this critical factor influencing the functions of biological macromolecules, organelles and cells (Kuimova, 2012; Yang et al., 2014a). There are two general trends in the development of viscosity probes. One of them is to design various types of molecular rotors, *e.g.* 9-(dicyanovinyl)julolidine (DCVJ) (Haidekker and Theodorakis, 2016), porphyrin dimer (Kuimova et al., 2009), BODIPY (Kuimova et al., 2008), cyanine dye (Peng et al., 2011), *etc.* to enhance the sensitivity or accuracy in fluorescence imaging. The other trend is to improve the targetability *via* localizing substituents so that the probes can be accumulate in certain subcellular regions for corresponding viscosity sensing, such as plasma membranes (López-Duarte et al., 2014), lysosomes (Wang et al., 2013), mitochondria (Yang et al., 2013) and endoplasmic reticulum (Yang et al., 2014b).

The measurement of viscosity in protein locations has great important significance to clarify the physiological and pathological statuses of cells. A single protein-adjacent area is so small that local viscosity can be referred as micro-viscosity. Correspondingly, the overall viscosity of the fluids in cytoplasm or organelles should be referred to as macro-viscosity. Obviously, micro-viscosity and macro-viscosity are distinct but interrelated. The micro-viscosity of protein location is determined by the protein surrounding macro-viscosity and the restriction of protein structure (hydrophobic/hydrophilic groups, domain, *etc.*) (Gatzogiannis et al., 2012; Kao et al., 2012). So the changes of macro-viscosity may alter the micro-viscosity of protein. Micro-viscosity should be a valuable indicator for the changes of protein's structure, conformation and function (Kao et al., 2012; Gatzogiannis et al., 2012). However, to our knowledge, real time tracing such micro-viscosity has rarely been achievable, since almost all the small molecule-based fluorescent probes are, actually, probes for macro-viscosity.

In the field of chemical biology, protein-tag techniques have been established as powerful tools to specifically label proteins in live cells and organisms (Giepmans et al., 2006). Through the genetically encoded methods, proteins of interest can be fused with another protein/polypeptide tag, *e.g.* Tetracysteine tag (Griffin et al., 1998), SNAP-tag (Keppler et al., 2003), and Halotag (Los et al., 2008), *etc.* If

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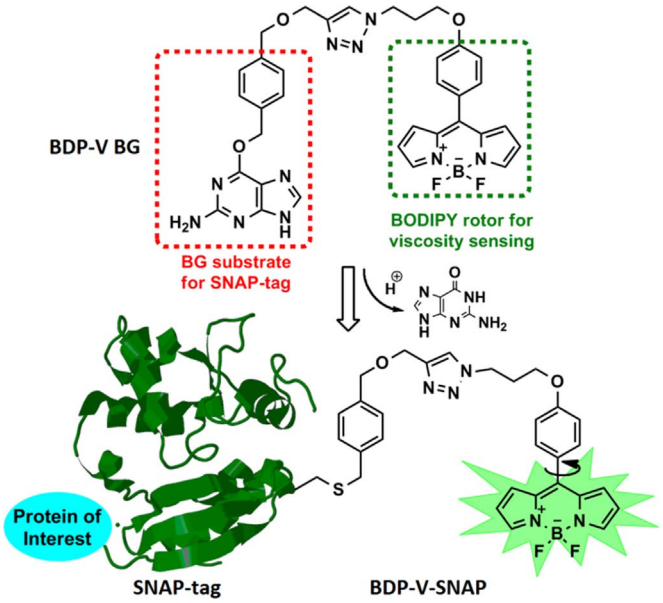

Fig. 1. Structure of **BDP-V BG** and principles of its labeling of protein fused SNAP-tag.

fluorescent probes possess special groups that can be selectively and facilely attached to the tag, they can label the target protein specifically. Such a labeled protein becomes a hybrid sensor really sense in proximity to itself. The versatility of protein tags has been justified by many applications, so they should be feasible and meaningful for the development of micro-viscosity probes (Wang et al., 2013; Gatzogiannis et al., 2012).

Among these protein tags, SNAP-tag evolved from human O^6-alkylguanine-DNA alkyltransferase (hAGT) has been widely used for stable, specific labeling and imaging of proteins with diverse small molecule fluorescent dyes and probes (Keppler et al., 2003, 2004; Lukinavicius et al., 2013). So far, SNAP-tag has not yet been adopted to develop micro-viscosity probes.

Herein, we design a hybrid sensor **BDP-V BG** consisting of a BODIPY-based rotor (**BDP-V**) and an O^6-benzylguanine (BG) moiety. **BDP-V** is adopted for its advantageous fluorescence properties of highly sensitive to viscosity but independent on other environmental factors (*e.g.* pH, polarity). BG moiety is the specific substrate of SNAP-tag. Principle of **BDP-V BG** used for sensing of viscosity in protein locations is illustrated in Fig. 1. Firstly, SNAP-tag is fused to a protein of interest. Then, the cysteine at the active site of SNAP-tag reacts with **BDP-V BG** to form covalent conjugation **BDP-V-SNAP** specifically and efficiently. Lastly, **BDP-V** senses the viscosity of the area nearby SNAP-tag fused protein of interest.

2. Materials and methods

2.1. Materials and instruments

All chemicals were obtained from commercial suppliers. The 500 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra were registered on a 500 MHz spectrometer. Mass spectra were measured with an Agilent LC/Q-TOF MS. UV–vis spectra were performed on an Agilent 8453 UV–visible spectroscopy system. Fluorescence spectra were collected on a Hitachi F-4500 spectrometer. Fluorescent images were acquired with confocal microscopy Olympus FV1000. The two-photon excited fluorescence lifetime images (FLIM) were measured with B & H DCS 120 system and tunable ultrafast lasers Mai Tai DeepSee.

2.2. Synthesis of *BDP-V BG*

N_3-BDP-V (18 mg, 0.05 mmol), **PYBG** (15 mg, 0.05 mmol) and N,N-diisopropylethylamine (0.05 mL, 0.28 mmol) were dissolved in 3 mL DMF under a nitrogen atmosphere. $CuSO_4{\cdot}5H_2O$ (2 mg, 0.008 mmol) dissolved in 0.5 mL water was added and then sodium ascorbate dissolved in 0.5 mL water was added. The mixture was stirred for 7 h at 25 °C. Then the mixture was purified by column chromatography (gradient: CH_2Cl_2/CH_3OH, v-v 40:1-15:1) to afford **BDP-V BG** (29 mg, yields 85%). ^{1}H NMR (500 MHz, DMSO-d6) δ 8.22 (s, 1H), 8.09 (s, 2H), 7.63 (d, *J* =8.6 Hz, 2H), 7.49 (d, *J* =7.7 Hz, 2H), 7.36 (d, *J* =7.8 Hz, 2H), 7.15 (d, *J* =8.6 Hz, 2H), 7.05 (d, *J* =3.9 Hz, 2H), 6.68 (d, *J* =2.5 Hz, 2H), 6.31 (s, 1H), 5.47 (s, 2H), 4.62 - 4.50 (m, 6H), 4.11 (t, *J* =5.8 Hz, 2H), 3.33 (s, 2H), 2.35 (dd, *J* =11.7, 5.4 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 161.01 (s), 146.93 (s), 143.89 (s), 138.00 (s), 135.88 (s), 133.96 (s), 132.64 (s), 131.52 (s), 128.41 (s), 127.63 (s), 125.52 (s), 124.12 (s), 118.98 (s), 114.77 (s), 70.87 (s), 66.56 (s), 64.92 (s), 62.87 (s), 46.47 (s), 29.33 (s). m/z (FTMS+p ESI): Calcd. [M+H] for $C_{34}H_{32}BF_2N_{10}O_3$ 677.2720, found 677.2715.

2.3. Cells and SNAP-tag protein labeled with *BDP-V BG*

COS-7 cells and bacteria were cultured as we have described (Song et al., 2015). The cells were stained with **BDP-V BG** labeling solution at 37 °C for 30 min to 1h, and washed with PBS (3×1 mL). After incubation time, the culture medium was refreshed before fluorescent imaging.

SNAP-tag protein was achieved by purifying from transformed bacteria described above as we previously reported (Song et al., 2015). The labeling and kinetics of reaction between the purified SNAP-tag protein (0.5 μM) and **BDP-V BG** were also following the previous methods (Song et al., 2015). More details were described in Supplementary Material.

2.4. Photophysical properties

Two-photon absorption (TPA) spectra of **BDP-V-SNAP** were measured through a femtosecond two-photon-excited fluorescence (TPEF) technique (Xu and Webb, 1996).

The chloro-substituted BODIPY rotor (**Cl-BDP-V**) (shown in Fig. S1) was used for the calculation of lifetime–viscosity calibration curve. Fluorescence delays and fluorescence lifetimes of **Cl-BDP-V** (10 μM) were determined at different viscosity (different ratio of glycerol to methanol, v-v). Fluorescence lifetimes of **BDP-V BG** (5 μM) and **BDP-V-SNAP** (0.5 μM) were also determined at different viscosity (different ratio of glycerol to PBS with 5% methanol, v-v).

2.5. SDS-PAGE analysis of labeled SNAP-tag protein and cell lysate

The labeled SNAP-tag protein (10 μM, 8 μL) was analyzed by 15% SDS-PAGE. **BDP-V BG** labeled COS-7 cells were lysed with RIPA lysis buffer. The soluble cell extracts were also analyzed by 15% SDS-PAGE. After electrophoresis, the fluorescence of gel was imaged by using a UV transilluminator (GBOX-HR, SYNGENE) with an ethidium bromide filter.

3. Results and discussion

3.1. Design and synthesis

The synthesis of **BDP-V BG** is a facile “Click Chemistry” (Fig. S1). As is known, “Click Chemistry” needs two key modules, *i.e.* azide and terminal alkyne. For this work, we firstly synthesize an azide-substituted BODIPY rotor, **N_3-BDP-V**. As the other intermediate, **PYBG** containing a terminal alkyne had been developed by our group as a versatile building block for SNAP-tag substrates (Song et al., 2015). “Click Chemistry” between **BDP-V** and **PYBG** produced **BDP-V BG** with high yield up to 85%. The structures of **N_3-BDP-V** and **BDP-V**