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Fluorogenic boronate-based probe-lactulose complex for full-aqueous analysis of peroxynitrite

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ABSTRACT

A selective fluorogenic boronate-based probe-lactulose complex was evaluated for the rapid analysis of peroxynitrite ($ONOO^-$) based on a reaction-based indicator displacement assay (RIA). The probe was synthesised by a simple nucleophilic substitution reaction between a boronic acid moiety and a well known laser dye, DCM. Fluorescence analyses showed that the probe had an off-on response to lactulose, forming a fluorogenic probe-lactulose complex. The subsequent addition of $ONOO^-$ selectively quenched the fluorescence of the complex over other Reactive Oxygen/ Nitrogen Species (ROS/RNS) tested. The complex can be applied for the rapid determination of $ONOO^-$ in full aqueous solution with good linear range, and has also proven suitable for monitoring $ONOO^-$ in living cells and real water samples.

1. Introduction

As a highly reactive oxidant and an efficient nitrating agent in physiological and pathological processes, peroxynitrite is formed through a combination of nitric oxide (NO) and superoxide radical anion ($O_2^{\cdot-}$) [1]. Many biomolecules in cells can be oxidized and/or nitrated by the excessive generation of $ONOO^-$, such as nucleic acids, lipids, proteins and thiols [2,3]. An abnormal peroxynitrite level can cause a number of deleterious effects including circulatory shock, cardiovascular diseases, reperfusion injury, inflammation, cancers, atherosclerosis, ischemic stroke, and diabetes [4]. As a consequence, effective and applicable detection of peroxynitrite with a simple and quick method is of great significance.

The traditional methods for measuring peroxynitrite depend on UV/Vis spectroscopy, electrochemical analysis, electron spin resonance, and immunohistochemistry [5–8]. Compared with the traditional methods, fluorescence methods [9–11] have received more attention because of their high sensitivity, selectivity, ease in manipulation and flexibility in structural modification. To date, a number of synthetic fluorescence probes for peroxynitrite have been developed [12,13]. And most of them are based on the specific oxidation reactions, such as phenol group oxidation [14–18], aryl ether group oxidation [19], heteroatom (Se, Te) oxidation [20–23], activated carboncarbon double bond oxidative cleavage [24,25] and aryl boronate oxidation [26–31]. As the precise morbigenous factor of peroxynitrite in biological systems is still not very clear because of its short life-time, high activity, low concentration, and elusive nature, a selective and sensitive fluorescent probe for peroxynitrite amongst the various of relevant reactive oxygen and nitrogen species is urgently in demand.

Here we report a novel fluorescent probe, **KB7**, based on the effective coupling between DCM (4-dicyanomethylene-2-methyl-6-[4-(dimethylamino)styryl]–4*H*pyran) dye which has long excitation and emission wavelength [32,33] and a boronic acid moiety which has been reported as a saccharides receptor [34–36]. The boronic acid moiety can rapidly interact with lactulose in aqueous media to form a fluorogenic **KB7**-Lactulose complex, which shows a fluorescence on-off response to $ONOO^-$ with high sensitivity and good selectivity over other reactive oxygen and nitrogen species tested. The **KB7**-Lactulose complex can be applied for the rapid determination of $ONOO^-$ in full aqueous solution with good linear range and has also proven amenable to monitoring $ONOO^-$ in living cells and real water samples.

2. Materials and methods

2.1. General

All purchased chemicals and reagents are of analytical grade. Solvents were purified by standard procedures. Reactions were monitored by TLC (thin-layer chromatography) using E-Merck aluminum

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precoated plates of Silica Gel. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer using tetramethylsilane (TMS) as the internal standard (chemical shifts in parts per million). High resolution mass spectra were recorded on a Waters LCT Premier XE spectrometer using standard conditions (ESI, 70 eV). All fluorescence spectra were measured on a Varian Cary Eclipse Fluorescence spectrophotometer.

2.2. Synthesis of *3*

To a sulotion of **1** (837 mg, 3.5 mmol) and **2** (500 mg, 2.9 mmol) in EtOH (20 mL) were added piperidine (0.5 mL) and acetic acid (0.8 mL). The resulting mixture was refluxed over night at 80 ℃. Then the mixture was concentrated and washed with water and brine, and extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, filtered and concentrated in vacuum. The resulting residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH=25:1, V/V) to afford compound **3** (420 mg, 42%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.35 (s, 3H), 2.43 (s, 3H), 2.72 (t, *J*=8.0 Hz, 2H), 2.99 (s, 3H), 3.51 (t, *J*=8.0 Hz, 2H), 6.61 (s, 1H), 6.73–6.77 (m, 3H), 7.01 (d, *J*=16.0 Hz, 1H), 7.44 (d, *J*=16.0 Hz, 1H), 7.53 (d, *J*=8.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.4, 35.5, 47.8, 50.6, 54.0, 99.5, 104.1, 105.0, 105.4, 111.7, 112.7, 115.8, 122.0, 129.9, 138.5, 150.6, 156.6, 161.1, 163.7. HR-ESI-MS *m/z*:$[M+H]^+$ calcd. For 347.1858, found 347.1872.

2.3. Synthesis of *KB7*

To a sulotion of **3** (120 mg, 0.34 mmol) and **6** (75 mg, 0.35 mmol) in CH_3CN (20 mL) were added KI (50 mg, 0.30 mmol) and K_2CO_3 (100 mg, 0.73 mmol). The resulting mixture was refluxed over night at 65 ℃. Then the mixture was concentrated and washed with brine, and extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$, filtered and concentrated in vacuum. The resulting residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH=35:1, V/V) to afford compound **KB7** (132 mg, 81%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.51 (s, 3H), 3.34 (s, 6H), 3.41 (t, *J*=8.0 Hz, 2H), 3.46 (t, *J*=4.0 Hz, 2H), 4.74 (d, *J*=4.0 Hz, 2H), 5.94 (s, 2H), 6.67–6.69 (m, 1H), 6.75–6.79 (m, 2H), 6.84 (d, *J*=12.0 Hz, 1H), 7.70 (t, *J*=8.0 Hz, 1H), 7.89 (t, *J*=4.0 Hz, 1H), 8.22–8.27 (m, 2H), 8.46 (d, *J*=4.0 Hz, 1H), 8.59 (d, *J*=8.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.6, 29.5, 38.3, 38.5, 41.4, 49.1, 105.5, 105.9, 112.1, 112.2, 113.3, 116.2, 116.3, 116.3, 122.6, 127.3, 129.5, 130.3, 130.4, 135.3, 150.7, 156.9, 157.1, 161.6, 164.0, 164.2. HR-ESI-MS *m/z*:$[M+H]^+$ calcd. For 481.2411, found 481.2415.

2.4. Fluorescence spectroscopy

Stock solutions of various oligosaccharides (5 M) were prepared in deionized water. Stock solutions of ROS/RNS were prepared refer to the literature [34]. The fluorescence measurements were carried out with a path length of 10 mm and an excitation wavelength of 480 nm by scanning the spectra between 490 nm and 800 nm. The bandwidth for both excitation and emission spectra was 5 nm. Unless otherwise mentioned, all the spectra were recorded in a phosphate buffer (pH 7.4, 0.2 M) at 25 °C.

2.5. Cell imaging assay

Hep-G2 cells were cultured in DMEM supplemented with 10% FBS. Cells (1.5×10^4/well) were seeded on a black 96-well microplate with optically clear bottom overnight. After pretreatment with **KB7**-Lactulose (**KB7**, 20 μM; Lactulose, 100 mM) in PBS for 15 min (followed by three rinses using PBS), the cells were incubated with 0, 20, 40 μM peroxynitrite in PBS buffer solutions at different concentrations for another 15 min. After three rinses in PBS, the fluorescence was eventually detected and photographed with an Operetta high content imaging system.

2.6. Cell viability assay

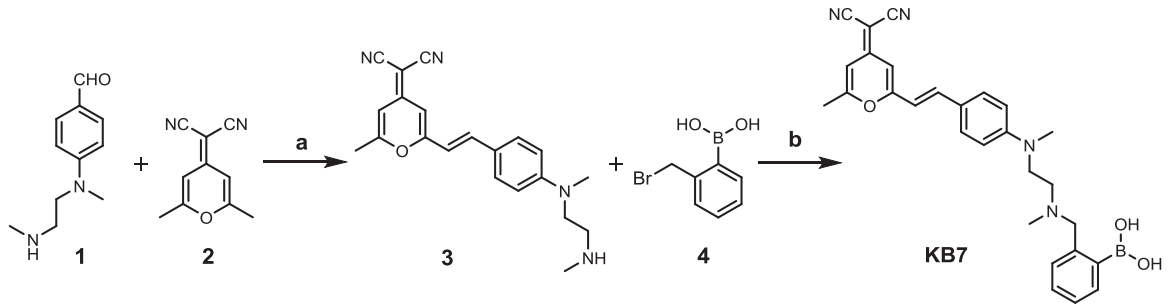
Cells were plated overnight on 96-well plates at 5000 cells per well in growth medium. After seeding, cells were maintained in growth media treated at increasing concentrations (12.5 μM, 25 μM, 50 μM, 100 μM, 200 μM) of **KB7** (dissolved in DMSO, final concentration) for 72 h. 20 μL of MTS (Promega Corp) solution (2 mg/mL) was added to each well for 2 h at 37 °C, and then the absorbance was measured on a SpectraMax 340 microplate reader (Molecular Devices, USA) at 490 nm with a reference at 690 nm. The optical density of the result in MTS assay was directly proportional to the number of viable cells. Each experiment was done in triplicate.

3. Results and discussion

The desired product **KB7** was synthesised by a simple nucleophilic substitution reaction between a boronic acid moiety and a well known laser dye, DCM. The DCM dye was obtained from the treatment of 4-(methyl(2-(methylamino)ethyl)amino)benzaldehyde (**1**) [37] and 2-(2,6-dimethyl-4H-pyran-4-ylidene)malononitrile (**2**) [38] in the presence of acetic acid, piperidine and ethyl alcohol (Scheme 1).

With the probe in hand, its sensitivity and selectivity for various saccharides were tested using fluorescence spectroscopy. As shown in Fig. 1a, upon addition of 50 mM of lactulose to the solution of **KB7** (10 μM), the off-on response of **KB7** to lactulose was tested at different incubation times. To our delight, the response could reach equilibrium within 30 s in aqueous buffer and a *ca.* 13-fold increase in fluorescence intensity (610 nm) was observed. The presence of fructose elicited the same response of the probe. In contrast, no obvious fluorescence change was observed in the presence of other saccharides including glucose (Glu), mannose (Man), galactose (Gal), lactose (Lac), sucrose (Suc), fucose (Fuc) (Fig. 1c).

Since pH may frequently impact the sensing ability of FL probes, we further tested the response of **KB7** to lactulose over a wide pH range from 4 to 10 (Fig. 1b). **KB7** showed good sensitivity with the off-on



Scheme 1. Synthetic procedures for **KB7**. Reagents and conditions: (a) piperidine, acetic acid in EtOH; (b) K_2CO_3, KI in CH_3CN.