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Special Issue

A Simple Near-Infrared Fluorescent Probe for the Detection of Peroxynitrite

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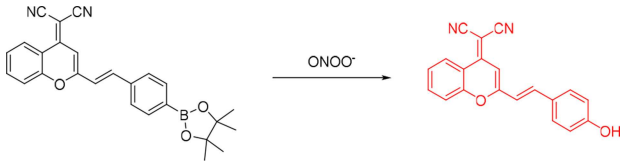
Herein, we report the evaluation and synthesis of a reaction based fluorescent probe **DCM-Bpin** for the detection of Peroxynitrite (ONOO–). **DCM-Bpin** exhibits selective fluorescence off-on response for $ONOO^-$ over other reactive oxygen species, including H_2O_2. Moreover, **DCM-Bpin** is biocompatible and has been used to visualize exogenous $ONOO^-$ in HeLa cells.

Peroxynitrite ($ONOO^-$) is a key intracellular signaling molecule in both physiological and pathological processes, formed *in vivo* by the very fast radical coupling reaction between nitric oxide ($NO^•$) and superoxide ($O_2^{•-}$).[1] The high reactivity of $ONOO^-$ leads to the reaction with almost all types of biomolecules, such as proteins, lipids and DNA, causing oxidative stress and deleterious effects on cellular function.[2,3] As such, $ONOO^-$ has been implicated as a key pathogenic factor for a number of diseases, including inflammatory, ischemia-reperfusion and neurodegenerative diseases,[4,5] therefore new and effective technologies for $ONOO^-$ detection are of prime importance. Of particular interest is the development of novel small molecule fluorescent probes for the detection of such species, as fluorescence probes are often more selective, less invasive and more convenient than many other methods for the detection of biologically relevant analytes in cells.[6–8] Although a wide array of sensors have been developed with absorption and emission peaks in the visible range (400–650 nm), near-infrared (NIR) fluorescence sensors (emission in 650–900 nm NIR region) are still rare, despite distinct advantages for *in vitro* and *in vivo* tracing of molecular processes.[9] Not only does NIR fluorescence avoid interference with the auto-fluorescence of indigenous molecules, near-infrared light results in less scattering and deeper tissue penetration, limiting the damage to living cells.[10,11] As such, the development of NIR fluorescent sensors is of growing interest to the sensing community.[12–16]

Herein we report the evaluation of a D-π-A-based[17] fluorescent probe **DCM-Bpin**, in which a 2-(2-methyl)-4*H*-chromen-4-ylidene)malononitrile (**DCM**) serves as the NIR fluorescence acceptor, and the boronate ester Bpin moiety acts as an $ONOO^-$ reporter. The fluorescence of the **DCM** system is quenched by Bpin, and so its rapid oxidation to the corresponding phenol (donor) by ROS species can be used to trigger a turn-on response, resulting in a peroxynitrite-activated "turn-on" probe (Scheme 1). **DCM-Bpin** has previously been reported as part of upconversion nanoparticles (UCNPs) for the detection of hydrogen peroxide, however, the **DCM-Bpin** probe was not evaluated for the detection of $ONOO^-$,[18] and given that peroxynitrite reacts with boronates much more rapidly,[19] we reasoned this system would be suitable for the detection of peroxynitrite.

The synthesis of **DCM-Bpin** was successfully achieved in one step by the condensation of 4-formylphenylboronic acid, pinacol ester and **DCM** (using the previously described procedure).[20] Heating to reflux in ethanol and piperidine for 4.5 hours, followed by filtration and washing with cold ethanol produced **DCM-Bpin** as a yellow solid in 62% yield (Scheme 2).[18]

The UV-Vis (Figure S1) and fluorescence (Figure 1) behavior of **DCM-Bpin** was evaluated in pH 7.40 buffer solution (5%



Scheme 1. Fluorescence "turn on" mechanism of **DCM-Bpin** with the addition of $ONOO^-$.

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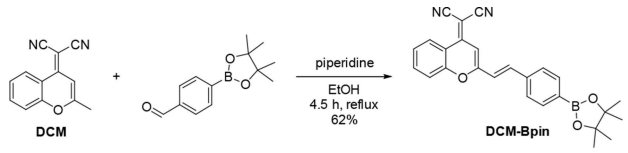

Scheme 2. Synthesis of target probe **DCM-Bpin**.

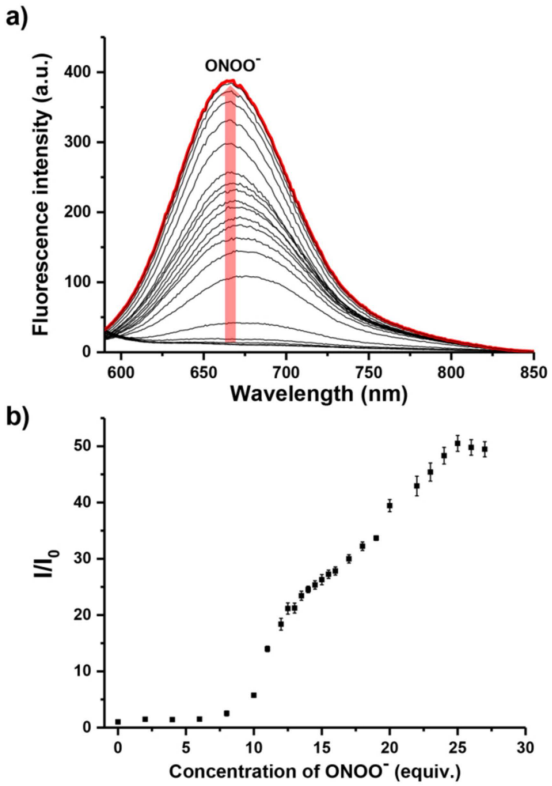

Figure 1. (a) Fluorescence spectra of **DCM-Bpin** (10 μM) with addition of $ONOO^-$ (from 0 to 27 equiv.) in PBS buffer solution (10 mM, containing 5% DMSO, pH = 7.40). The red line shows the highest intensity after addition of $ONOO^-$ (25 equiv.); (b) Fluorescence intensity changes (I/I_0) of probe **DCM-Bpin** (10 μM) with addition of $ONOO^-$ (from 0 to 27 equiv.) in PBS buffer solution (10 mM containing 5% DMSO, pH = 7.40) after 5 min. λ_{ex} = 560 nm/λ_{em} = 667 nm. Slit widths: ex = 10 nm, em = 20 nm.

DMSO was required to improve solubility). As shown in Figure S1, the maximum absorption of **DCM-Bpin** at 434 nm shifted to 530 nm with the addition of $ONOO^-$. As expected, **DCM-Bpin** was initially non-fluorescent, and upon addition of $ONOO^-$ (0-27 equiv.) a "turn-on" fluorescence response of up to 50-fold was observed at 667 nm using an excitation wavelength of 560 nm (Figure 1).

Subsequently, we compared the response of **DCM-Bpin** to $ONOO^-$ over other reactive oxygen species (ROS), including ClO^-, $^{\bullet}OH$, $O_2^{\bullet-}$, $^1O_2$, $ROO^{\bullet}$, H_2O_2 in PBS buffer (10 mM, pH 7.40). As show in Figures 2 and S2, only the addition of $ONOO^-$ to the probe lead to any distinct optical spectral changes, with no "turn-on" response observed for any of the other ROS species. This indicated that **DCM-Bpin** has highly selective optical detection for $ONOO^-$.

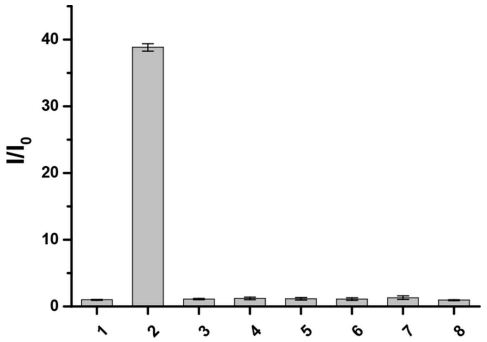

Figure 2. Selectivity bar chart of probe **DCM-Bpin** (10 μM) with addition of various ROS in PBS buffer solution.1. Blank, 2. $ONOO^-$ (200 μM), 3. $^{\bullet}OH$ (500 μM), 4. $O_2^{\bullet-}$ (500 μM), 5. $^1O_2$ (500 μM), 6. H_2O_2 (500 μM), 7. $ROO^{\bullet}$ (500 μM), 8. ClO^- (500 μM). $ONOO^-$ were measured after 5 min, other ROS were measured after 1 h. Error bar represents s.d. λ_{ex} = 560 nm/λ_{em} = 667 nm. Slit widths: ex = 10 nm, em = 20 nm.

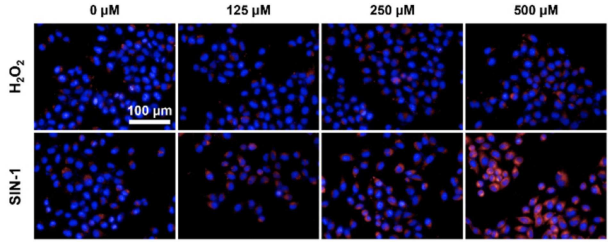

Figure 3. Fluorescence imaging of HeLa cells with **DCM-Bpin** (20 μM) in the absence or presence of H_2O_2 or SIN-1. Excitation channel = 560–580 nm, emission channel = 650–760 nm. Cell nuclei were stained with Hoechst 33342.

Having determined the selectivity of **DCM-Bpin**, we evaluated its ability to image exogenous $ONOO^-$ at the cellular level using live HeLa cells. As shown in Figure 3, **DCM-Bpin** exhibited a good "turn on" response with the addition of peroxynitrite donor SIN-1.[21] No fluorescent response was observed in the presence of up to 25 equivalents of H_2O_2, confirming the high selectivity of the **DCM-Bpin** system to $ONOO^-$ over other ROS species. Moreover, MTS cell proliferation assays indicated that **DCM-BPin** was not toxic towards HeLa cells (Figure S5).

In conclusion, **DCM-Bpin**, a near-infrared fluorescent probe, was shown to be suitable for the detection of $ONOO^-$. This probe not only exhibits an excellent "turn-on" response when exposed to peroxynitrite, it also exhibits high selectivity towards $ONOO^-$ over other common reactive oxygen species. **DCM-Bpin** is biocompatible and displays good sensitivity and selectivity towards $ONOO^-$ in HeLa cells.

Experimental Section

General Methods

All starting materials and reagents were purchased from Sigma Aldrich, Alfa Aesar, Fluorochem, or Acros Organics, and used as received without any further purification. Unless otherwise stated, all solvents used were reagent grade and were used without distillation. All water was distilled. Thin-layer chromatography was

performed by using commercially available Fluorochem aluminum-backed plates coated with a layer of silica gel (60 Å) with fluorescent indicator UV254. These plates were visualized by using ultraviolet light with a wavelength of either 254 or 365 nm. Silica gel column chromatography was carried out by using Sigma Aldrich 60 Å silica gel (200–400 mesh).

All NMR spectra were obtained using an Agilent ProPulse 500 with all spectra recorded in chloroform-*d*. LC–MS analyses were performed using an Agilent QTOF 6545 with Jetstream ESI spray source coupled to an Agilent 1260 Infinity II Quat pump HPLC with 1260 autosampler, column oven compartment and variable wavelength detector (VWD). All pH measurements taken during fluorescence/absorption experiments were recorded on a Hanna Instruments HI 9321 microprocessor pH meter, which was routinely calibrated by using Fisher Chemicals standard buffer solutions (pH 4.0: phthalate; 7.0: phosphate; 10.0: borate). UV-Vis measurements were performed on an Agilent Cary 60 UV-Vis Spectrophotometer, utilizing a Hellma silica (quartz) cuvette with a 10 mm path length (provides photometry with two windows). Fluorescence study was performed an Agilent Cary Eclipse Fluorescence Spectrophotometer.

Full synthetic procedures, characterisation data, and fluorescence analysis protocols can be found in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: near-infrared · fluorescence · boronate · peroxynitrite · probe

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