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Long-wavelength fluorescent boronate probes for the detection and intracellular imaging of peroxynitrite[†]

Adam C. Sedgwick, * Hai-Hao Han, * Jordan E. Gardiner, * Steven D. Bull, * * * Xiao-Peng He * and Tony D. James *

Two boronate fluorescent probes have been developed for the detection of peroxynitrite (TCFB1 and TCFB2). TCFB1 was shown to have a low sensitvity towards peroxynitrite and have a poor solubility in aqueous solution whereas TCFB2 demonstrated high sensitivity towards peroxynitrite and mitochondria localisation with the ability to detect exogenous and endogenous peroxynitrite in live cells (Hep-G2, RAW 264.7, HeLa and A459).

Peroxynitrite (ONOO⁻) is a highly reactive nitrogen species that is formed via the diffusion controlled reaction between superoxide ($^{-}O_{2}$) and nitric oxide (NO). 1,2 ONOO $^{-}$ acts as a signalling molecule in vivo for a number of pathways. 1,3 However, ONOO is more commonly known for its deleterious properties, causing irreversible damage to a range of biological targets such as lipids, proteins and DNA. Therefore, ONOO has been implicated as a key pathogenic factor for a number of diseases, which include inflammation, cancer, ischemia-reperfusion and neurodegenerative diseases.5-7 In biological systems, ONOO is difficult to measure due to it being short-lived with a half-life $\sim 10-20$ ms.¹ Therefore, the development of powerful tools for the detection of ONOO is of significant interest.

With our research, we are particularly interested in the development of small molecule fluorescent probes for the detection of biologically relevant analytes in vivo owing to their high sensitivity, selectivity and high spatial and temporal resolution. In the past few years, a number of ONOO fluorescent probes have been developed for imaging in live cells and mice.8-13 However, despite significant progress in this area of research, there is a lack of long-wavelength ONOO fluorescent probes. The development of long wavelength/near infrared (NIR) probes is of particular interest because longer excitation/emission wavelengths allows deeper tissue penetration and minimalises

In the literature, Sikora et al. reported that the reaction rates of ONOO with aromatic boronates are 200 times faster than hypochlorous acid (HOCl/ClO⁻) and a million times faster than hydrogen peroxide (H₂O₂).¹⁶ Therefore, a number of boronate fluorescent probes have been recently developed for the detection of ONOO-.8,17,18

2-Dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF)-based fluorophores have an internal charge transfer (ICT) donor-π-acceptor (D-π-A) structure with long emission wavelengths. As a result, TCF fluorophores have been used in many applications such as non-linear optic chromophores and molecular probes. 19-25 With this research, we developed two boronate TCF-based fluorescent probes for the detection of ONOO (TCFB1 and TCFB2). The TCF fluorophore unit was synthesised in one step using the reaction of 3-hydroxy-3-methyl-2-butanone, malonitrile and NaOEt in EtOH. With the TCF unit in hand, the $(D-\pi-A)$ systems **TCFB1** and **TCFB2** were isolated in high yield using microwave reaction conditions. ²⁶ The microwave irradiation of a mixture of piperidine (Cat.), TCF and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde in EtOH followed by filtration led to the isolation of the desired TCFB2. For the synthesis of TCFB1, microwave irradiation of a mixture of piperidine (Cat.), TCF and 4-hydroxybenzaldehyde in EtOH followed by filtration led to the isolation of the intermediate TCF-OH. This was subsequently alkylated with 2-(4-(bromomethyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane using K2CO3 and NaI in MeCN to afford TCFB1 in a reasonable yield (47%).

TCFB1

TCFB2

background auto-fluorescence from proteins and photodamage to the biological samples. 14,15

^a Department of Chemistry, University of Bath, Bath, BA2 7AY, UK. E-mail: t.d.james@bath.ac.uk, s.d.bull@bath.ac.uk

^b Key Laboratory for Advanced Materials & Feringa Nobel Prize Scientist Joint Research Center, East China University of Science and Technology, 130 Meilong Rd., Shanghai 200237, P. R. China, E-mail; xphe@ecust.edu.cn

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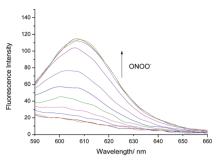


Fig. 1 Fluorescence spectra of TCFB1 (10 μM) with addition of ONOO $(0-100~\mu\text{M})$ in PBS buffer solution, 20% DMSO, pH 8.00 at 25 °C. λ_{ex} = 560 nm. Slit widths ex = 10 nm and em = 15 nm.

We initially evaluated the UV-Vis (Fig. S2, ESI†) and fluorescence behaviour (Fig. 1 and Fig. S3, ESI†) of TCFB1, in pH 8.0 buffer solution (20% DMSO). DMSO was required to improve the aqueous solubility of TCFB1. Under these conditions, TCFB1 produced an up to 6.5-fold fluorescence "turn on" in the presence of ONOO⁻ (0-100 μM). (Schemes S1, S2 and Fig. S1, ESI†) However, in comparison to our previously reported ESIPT probe, TCFB1 was less sensitive towards ONOO despite a larger "turn on" response.8

Subsequently, we evaluated the selectivity of TCFB1 towards other ROS (Fig. S4, S5 and S11, ESI†). TCFB1 demonstrated an excellent selectivity for ONOO-, which permitted the evaluation of its ability to detect exogenous and endogenous ONOO in live cells. Unfortunately, due to its poor aqueous solubility, large amounts of precipitate with TCFB1 was observed (data not shown).

Therefore, we turned our attention towards the evaluation of the UV-Vis and fluorescence properties of TCFB2, which has previously been reported for the detection of ClO⁻. ²⁰ As previously reported for other aryl boronate fluorescent probes, 27,28 TCFB2 was found to be initially non-fluorescent with no UV absorption beyond ~ 525 nm (Fig. S6, ESI†). The addition of ONOO to TCFB2 resulted in the appearance of a large emission peak at 606 nm (Fig. 2 and Fig. S7, ESI†). This was accompanied by a colorimetric response (yellow to pink) and the appearance of a large UV absorption peak at ~590 nm. TCFB2 demonstrated high sensitivity and rapid reaction (Fig. S8, ESI†) with ONOOand was able to detect very low concentrations (0-10 µM).

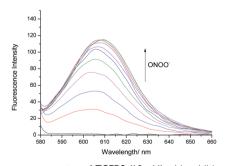


Fig. 2 Fluorescence spectra of TCFB2 (10 μ M) with addition of ONOO⁻ μ M) in PBS buffer solution, 20% DMSO, pH 8.00 at 25 °C. λ_{ex} = 560 nm. Slit widths ex = 10 nm and em = 15 nm

As predicted, both ClO and H₂O₂ also resulted in a fluorescence response (Fig. S9, S10 and S12, ESI†), however, larger concentrations and reaction times were required. These observations clearly, demonstrated the greater reactivity of the boronate towards ONOO-.

Having determined the selectivity of TCFB2, we evaluated its ability to image endogenous and exogenous ONOO in live cells. TCFB2 was evaluated in a number of different cell lines (Hep-G2: human hepatoma, HeLa: human cervical cancer, RAW 264.7: mouse macrophage and A549 cells: human lung cancer), which were incubated with TCFB2 (10 µM) for 30 minutes and washed with PBS buffer solution three times. As shown in Fig. 3, TCFB2 demonstrated a clear "turn on" response with the addition of SIN-1 (ONOO donor). No "turn on" response was observed when the cells were pre-treated with the ONOO scavenger uric acid. TCFB2 also provided a clear "turn on" response for the detection of stimulated ONOO-. RAW 264.7 cells were used in which ONOO was stimulated using lipopolysaccharide (LPS).²⁹ This led to the activation of the TCFB2 fluorescence intracellularly (Fig. 4). In contrast, no "turn on" response was observed in the presence of uric acid indicating the selectivity for ONOO in cells. A cell proliferation assay showed that the compound was not toxic towards all the cell lines used with concentrations well above that used for imaging (Fig. S13, ESI†).

The production of superoxide occurs mainly through the mitochondrial electron transport pathway;30 therefore the mitochondria are the main source of ONOO- in macrophages. Commercial Mito-tracker Green was used to localise in the mitochondrial compartments of RAW 264.7. We then used TCFB2 to investigate the subcellular distribution of ONOO. The results indicated that the fluorescence of the probe co-localised with that

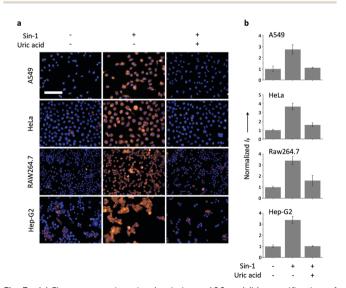


Fig. 3 (a) Fluorescence imaging (scale bar = $100 \mu m$) (b) quantification of different cells incubated with TCFB2 (10 μ M) without (-/-) or with a subsequent addition of Sin-1 (500 μ M, a ONOO promoter) (+/-) or a subsequent addition of and uric acid (100 μM , a ONOO- quencher) and then Sin-1 (+/+). Excitation and emission wavelengths for TCFB2 are 560-580 nm and 580-650 nm, respectively. The cell nuclei were stained by Hoechst 33342

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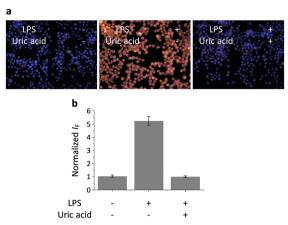


Fig. 4 (a) Fluorescence imaging (scale bar = $100 \mu m$) (b) quantification of RAW 264.7 incubated with **TCFB2** ($10 \mu M$) without (-/-) or with a subsequent addition of lipopolysaccharide (LPS, $1 \mu g m L^{-1}$) (+/-) or a subsequent addition of both LPS and uric acid ($100 \mu M$, a ONOO-quencher) (+/+). Excitation and emission wavelength for **TCFB2** are $560-580 \mu m$ and $580-650 \mu m$, respectively. The cell nuclei were stained by Hoechst 33342.

of the tracker resulting in a Pearson coefficient of 0.84 (Fig. 5). We have also carried out an additional lysosome co-localisation assay, and the result showed that the probe did not co-localise well with lysosome (Pearson's correlation = 0.38) (Fig. S14, ESI \dagger). This suggests that ONOO $^-$ was produced at the mitochondria.

In conclusion, we have developed two long-wavelength reaction based fluorescent probes for the detection of ONOO⁻. Unfortunately, **TCFB1** had a low solubility in aqueous solution, which led to the observation of precipitates in cell imaging experiments. A glycosylation strategy^{31,32} to improve the water

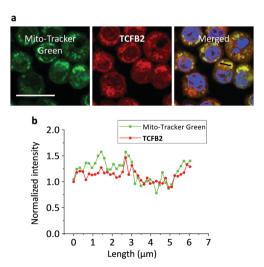


Fig. 5 (a) Fluorescence co-localisation of TCFB2 (10 μ M) with Mito-Tracker Green (1 μ M) in RAW 264.7 cells (scale bar = 20 μ m). (b) Fluorescence quantification of TCFB2 and Mito-Tracker of a selected section (the black line in "Merged" panel) of a RAW 264.7 cell. Excitation wavelength for Mito-Tracker Green and TCFB2 is 489 and 579 nm, respectively. Emission wavelength for Mito-Tracker Green and TCFB2 is 506 and 603 nm, respectively. The cell nuclei were stained by Hoechst 33342.

solubility of the insoluble TCFB1 is currently underway in our laboratories. However, TCFB2 displayed selective and sensitive "turn on" with the addition of ONOO⁻. The large fluorescence response observed for TCFB2 facilitated its use in cell imaging experiments. Therefore, TCFB2 was able to detect exogenous and endogenous ONOO⁻ with a large fluorescence "turn on" over a range of cell lines (Hep-G2, RAW 264.7, HeLa and A459). Mitochondrial localisation of TCFB2 was observed by colocalisation with Mito-Tracker Green. Overall, these results demonstrate that TCFB2 is a useful tool to understand the role of ONOO⁻ in biological systems and could lead to systems capable of disease diagnosis.

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Conflicts of interest

There are no conflicts to declare.

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