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Two boronate fluorescent probes have been developed for the detection of peroxynitrite (TCFB1 and TCFB2). TCFB1 was shown to have a low sensitivity 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).

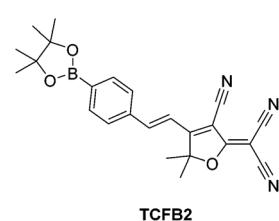
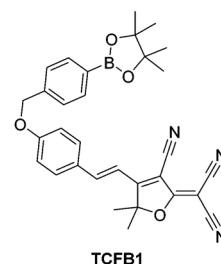
Peroxynitrite (ONOO^-) is a highly reactive nitrogen species that is formed *via* the diffusion controlled reaction between superoxide (O_2^-) and nitric oxide (NO).^{1,2} ONOO^- acts as a signalling molecule *in vivo* for a number of pathways.^{1,3} However, ONOO^- is more commonly known for its deleterious properties, causing irreversible damage to a range of biological targets such as lipids, proteins and DNA.⁴ 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 minimises

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

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 (H_2O_2).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- π -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 K_2CO_3 and NaI in MeCN to afford TCFB1 in a reasonable yield (47%).



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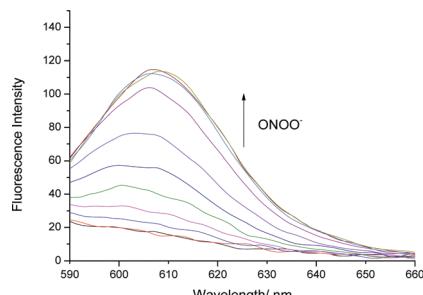


Fig. 1 Fluorescence spectra of **TCFB1** (10 μ M) with addition of ONOO[−] (0–100 μ M) in PBS buffer solution, 20% DMSO, pH 8.00 at 25 °C. $\lambda_{\text{ex}} = 560$ nm. Slit widths ex = 10 nm and em = 15 nm.

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 μ M). (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 \sim 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 \sim 590 nm. **TCFB2** demonstrated high sensitivity and rapid reaction (Fig. S8, ESI[†]) with ONOO[−] and was able to detect very low concentrations (0–10 μ M).

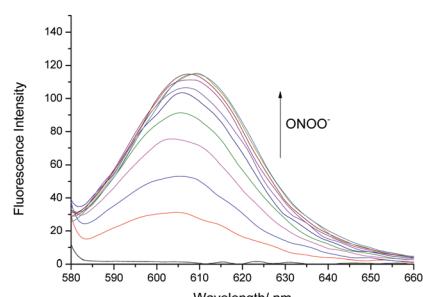


Fig. 2 Fluorescence spectra of **TCFB2** (10 μ M) with addition of ONOO[−] (0–100 μ M) in PBS buffer solution, 20% DMSO, pH 8.00 at 25 °C. $\lambda_{\text{ex}} = 560$ nm. Slit widths ex = 10 nm and em = 15 nm.

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 (HepG2: human hepatoma, HeLa: human cervical cancer, RAW 264.7: mouse macrophage and A549 cells: human lung cancer), which were incubated with **TCFB2** (10 μ M) 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

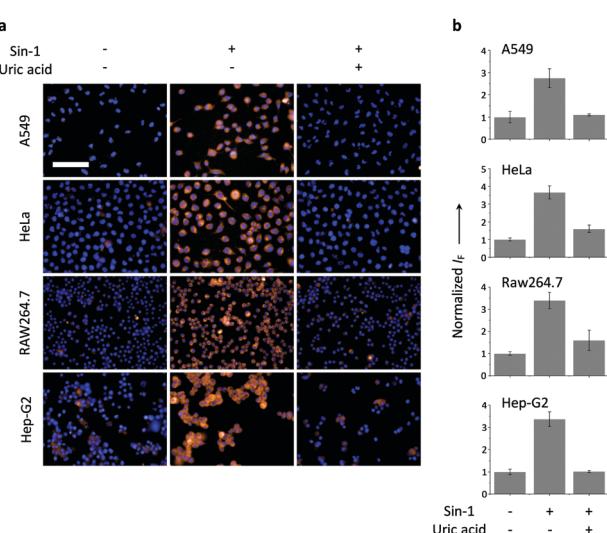


Fig. 3 (a) Fluorescence imaging (scale bar = 100 μ m) (b) quantification of different cells incubated with **TCFB2** (10 μ M) without (−/−) or with a subsequent addition of Sin-1 (500 μ M, a ONOO[−] promoter) (+/−) or a subsequent addition of uric acid (100 μ M, a ONOO[−] quencher) and then Sin-1 (+/+).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

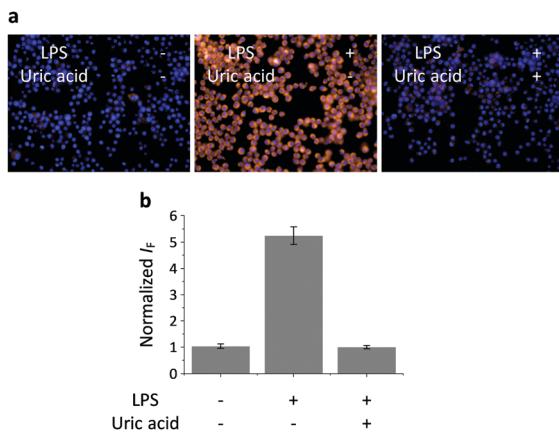


Fig. 4 (a) Fluorescence imaging (scale bar = 100 µm) (b) quantification of RAW 264.7 incubated with **TCFB2** (10 µM) without (−/−) or with a subsequent addition of lipopolysaccharide (LPS, 1 µg mL⁻¹) (+/−) or a subsequent addition of both LPS and uric acid (100 µM, a ONOO[−] quencher) (+/+). Excitation and emission wavelength for **TCFB2** are 560–580 nm and 580–650 nm, 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†). 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

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 co-localisation 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.

Notes and references

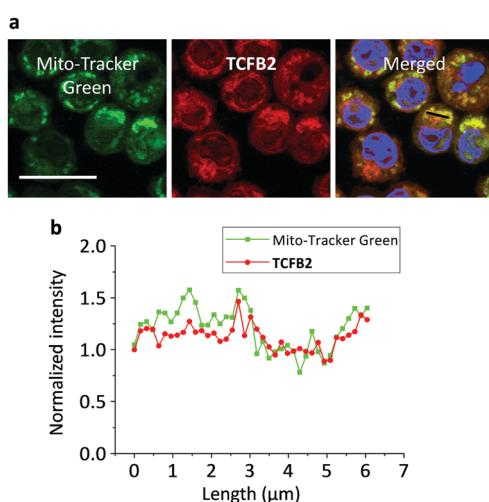


Fig. 5 (a) Fluorescence co-localisation of **TCFB2** (10 µM) with Mito-Tracker Green (1 µM) in RAW 264.7 cells (scale bar = 20 µm). (b) Fluorescence quantification of **TCFB2** and Mito-Tracker of a selected section (the black line in “Merged” panel) of a RAW 264.7 cell. Excitation wavelength for Mito-Tracker 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.

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