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

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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 ( $\text{ONOO}^-$ ) is a highly reactive nitrogen species that is formed *via* the diffusion controlled reaction between superoxide ( $\text{O}_2^-$ ) and nitric oxide ( $\text{NO}$ ).<sup>1,2</sup>  $\text{ONOO}^-$  acts as a signalling molecule *in vivo* for a number of pathways.<sup>1,3</sup> However,  $\text{ONOO}^-$  is more commonly known for its deleterious properties, causing irreversible damage to a range of biological targets such as lipids, proteins and DNA.<sup>4</sup> Therefore,  $\text{ONOO}^-$  has been implicated as a key pathogenic factor for a number of diseases, which include inflammation, cancer, ischemia-reperfusion and neurodegenerative diseases.<sup>5–7</sup> In biological systems,  $\text{ONOO}^-$  is difficult to measure due to it being short-lived with a half-life  $\sim 10$ – $20$  ms.<sup>1</sup> Therefore, the development of powerful tools for the detection of  $\text{ONOO}^-$  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  $\text{ONOO}^-$  fluorescent probes have been developed for imaging in live cells and mice.<sup>8–13</sup> However, despite significant progress in this area of research, there is a lack of long-wavelength  $\text{ONOO}^-$  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

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

In the literature, Sikora *et al.* reported that the reaction rates of  $\text{ONOO}^-$  with aromatic boronates are 200 times faster than hypochlorous acid ( $\text{HOCl}/\text{ClO}^-$ ) and a million times faster than hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).<sup>16</sup> Therefore, a number of boronate fluorescent probes have been recently developed for the detection of  $\text{ONOO}^-$ .<sup>8,17,18</sup>

2-Dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF)-based fluorophores have an internal charge transfer (ICT) donor- $\pi$ -acceptor (D- $\pi$ -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.<sup>19–25</sup> With this research, we developed two boronate TCF-based fluorescent probes for the detection of  $\text{ONOO}^-$  (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.<sup>26</sup> 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  $\text{K}_2\text{CO}_3$  and NaI in MeCN to afford TCFB1 in a reasonable yield (47%).

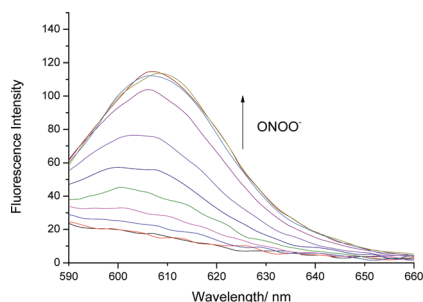


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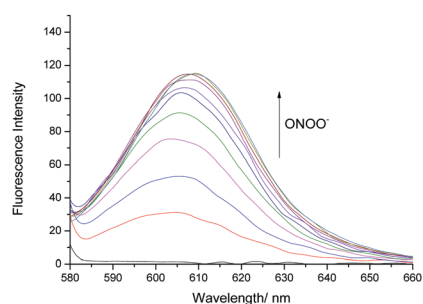


**Fig. 1** Fluorescence spectra of **TCFB1** (10  $\mu\text{M}$ ) with addition of  $\text{ONOO}^-$  (0–100  $\mu\text{M}$ ) in PBS buffer solution, 20% DMSO, pH 8.00 at 25  $^{\circ}\text{C}$ .  $\lambda_{\text{ex}}$  = 560 nm. Slit widths ex = 10 nm and em = 15 nm.

We initially evaluated the UV-Vis (Fig. S2, ESI $^{\dagger}$ ) and fluorescence behaviour (Fig. 1 and Fig. S3, ESI $^{\dagger}$ ) 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  $\text{ONOO}^-$  (0–100  $\mu\text{M}$ ). (Schemes S1, S2 and Fig. S1, ESI $^{\dagger}$ ) However, in comparison to our previously reported ESIPT probe, **TCFB1** was less sensitive towards  $\text{ONOO}^-$  despite a larger “turn on” response.<sup>8</sup>

Subsequently, we evaluated the selectivity of **TCFB1** towards other ROS (Fig. S4, S5 and S11, ESI $^{\dagger}$ ). **TCFB1** demonstrated an excellent selectivity for  $\text{ONOO}^-$ , which permitted the evaluation of its ability to detect exogenous and endogenous  $\text{ONOO}^-$  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  $\text{ClO}^-$ .<sup>20</sup> As previously reported for other aryl boronate fluorescent probes,<sup>27,28</sup> **TCFB2** was found to be initially non-fluorescent with no UV absorption beyond  $\sim 525$  nm (Fig. S6, ESI $^{\dagger}$ ). The addition of  $\text{ONOO}^-$  to **TCFB2** resulted in the appearance of a large emission peak at 606 nm (Fig. 2 and Fig. S7, ESI $^{\dagger}$ ). 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 $^{\dagger}$ ) with  $\text{ONOO}^-$  and was able to detect very low concentrations (0–10  $\mu\text{M}$ ).

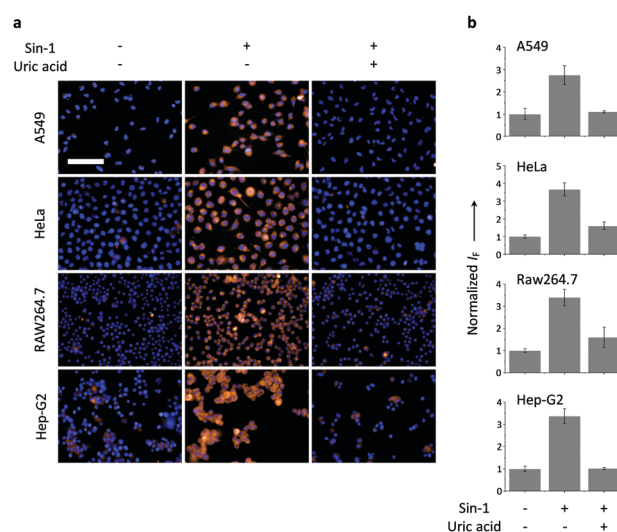


**Fig. 2** Fluorescence spectra of **TCFB2** (10  $\mu\text{M}$ ) with addition of  $\text{ONOO}^-$  (0–10  $\mu\text{M}$ ) in PBS buffer solution, 20% DMSO, pH 8.00 at 25  $^{\circ}\text{C}$ .  $\lambda_{\text{ex}}$  = 560 nm. Slit widths ex = 10 nm and em = 15 nm.

As predicted, both  $\text{ClO}^-$  and  $\text{H}_2\text{O}_2$  also resulted in a fluorescence response (Fig. S9, S10 and S12, ESI $^{\dagger}$ ), however, larger concentrations and reaction times were required. These observations clearly demonstrated the greater reactivity of the boronate towards  $\text{ONOO}^-$ .

Having determined the selectivity of **TCFB2**, we evaluated its ability to image endogenous and exogenous  $\text{ONOO}^-$  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  $\mu\text{M}$ ) 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 ( $\text{ONOO}^-$  donor). No “turn on” response was observed when the cells were pre-treated with the  $\text{ONOO}^-$  scavenger uric acid. **TCFB2** also provided a clear “turn on” response for the detection of stimulated  $\text{ONOO}^-$ . RAW 264.7 cells were used in which  $\text{ONOO}^-$  was stimulated using lipopolysaccharide (LPS).<sup>29</sup> 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  $\text{ONOO}^-$  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 $^{\dagger}$ ).

The production of superoxide occurs mainly through the mitochondrial electron transport pathway;<sup>30</sup> therefore the mitochondria are the main source of  $\text{ONOO}^-$  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  $\text{ONOO}^-$ . The results indicated that the fluorescence of the probe co-localised with that



**Fig. 3** (a) Fluorescence imaging (scale bar = 100  $\mu\text{m}$ ) (b) quantification of different cells incubated with **TCFB2** (10  $\mu\text{M}$ ) without (–/–) or with a subsequent addition of Sin-1 (500  $\mu\text{M}$ , a  $\text{ONOO}^-$  promoter) (+/–) or a subsequent addition of uric acid (100  $\mu\text{M}$ , a  $\text{ONOO}^-$  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  $\mu\text{m}$ ) (b) quantification of RAW 264.7 incubated with **TCFB2** (10  $\mu\text{M}$ ) without (–/–) or with a subsequent addition of lipopolysaccharide (LPS, 1  $\mu\text{g mL}^{-1}$ ) (+/–) or a subsequent addition of both LPS and uric acid (100  $\mu\text{M}$ , a  $\text{ONOO}^-$  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  $\text{ONOO}^-$  was produced at the mitochondria.

In conclusion, we have developed two long-wavelength reaction based fluorescent probes for the detection of  $\text{ONOO}^-$ . Unfortunately, **TCFB1** had a low solubility in aqueous solution, which led to the observation of precipitates in cell imaging experiments. A glycosylation strategy<sup>31,32</sup> 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  $\text{ONOO}^-$ . The large fluorescence response observed for **TCFB2** facilitated its use in cell imaging experiments. Therefore, **TCFB2** was able to detect exogenous and endogenous  $\text{ONOO}^-$  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  $\text{ONOO}^-$  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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Fig. 5 (a) Fluorescence co-localisation of **TCFB2** (10  $\mu\text{M}$ ) with Mito-Tracker Green (1  $\mu\text{M}$ ) in RAW 264.7 cells (scale bar = 20  $\mu\text{m}$ ). (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.



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