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

Adam C. Sedgwick,<sup>a</sup> Hai-Hao Han,<sup>b</sup> Jordan E. Gardiner,<sup>a</sup> Steven D. Bull,<sup>a</sup> Xiao-Peng He<sup>b</sup> and Tony D. James<sup>a</sup>

**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<sup>−</sup>) is a highly reactive nitrogen species that is formed *via* the diffusion controlled reaction between superoxide (O<sub>2</sub><sup>−</sup>) and nitric oxide (NO).<sup>1,2</sup> ONOO<sup>−</sup> acts as a signalling molecule *in vivo* for a number of pathways.<sup>1,3</sup> However, ONOO<sup>−</sup> 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, ONOO<sup>−</sup> 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, ONOO<sup>−</sup> is difficult to measure due to it being short-lived with a half-life ~10–20 ms.<sup>1</sup> Therefore, the development of powerful tools for the detection of ONOO<sup>−</sup> 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<sup>−</sup> 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 ONOO<sup>−</sup> 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 ONOO<sup>−</sup> with aromatic boronates are 200 times faster than hypochlorous acid (HOCl/ClO<sup>−</sup>) and a million times faster than hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).<sup>16</sup> Therefore, a number of boronate fluorescent probes have been recently developed for the detection of ONOO<sup>−</sup>.<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 ONOO<sup>−</sup> (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 K<sub>2</sub>CO<sub>3</sub> and NaI in MeCN to afford TCFB1 in a reasonable yield (47%).



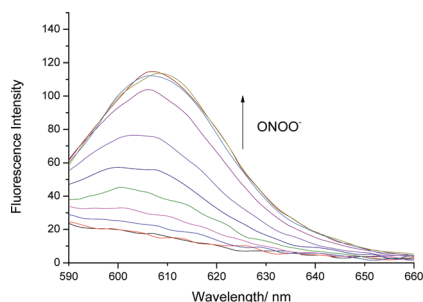
<sup>a</sup> Department of Chemistry, University of Bath, Bath, BA2 7AY, UK.

E-mail: t.d.james@bath.ac.uk, s.d.bull@bath.ac.uk

<sup>b</sup> 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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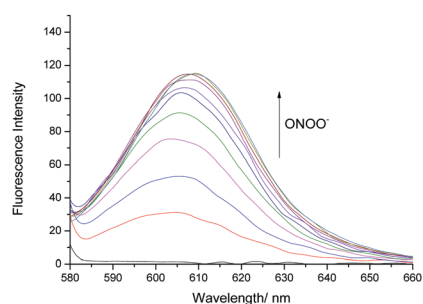


**Fig. 1** Fluorescence spectra of **TCFB1** (10  $\mu\text{M}$ ) with addition of  $\text{ONOO}^-$  (0–100  $\mu\text{M}$ ) in PBS buffer solution, 20% DMSO, pH 8.00 at 25  $^{\circ}\text{C}$ .  $\lambda_{\text{ex}}$  = 560 nm. Slit widths ex = 10 nm and em = 15 nm.

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  $\mu\text{M}$ ).

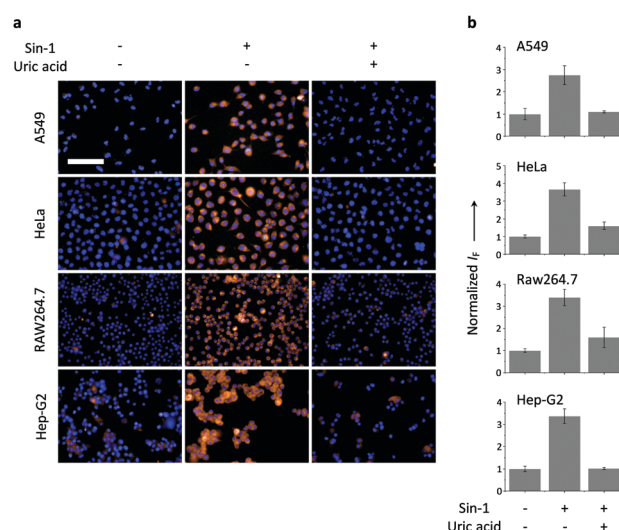


**Fig. 2** Fluorescence spectra of **TCFB2** (10  $\mu\text{M}$ ) with addition of  $\text{ONOO}^-$  (0–10  $\mu\text{M}$ ) in PBS buffer solution, 20% DMSO, pH 8.00 at 25  $^{\circ}\text{C}$ .  $\lambda_{\text{ex}}$  = 560 nm. Slit widths ex = 10 nm and em = 15 nm.

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.

## Notes and references

- P. Pacher, J. S. Beckman and L. Liaudet, *Physiol. Rev.*, 2007, **87**, 315.
- J. S. Beckman and W. H. Koppenol, *Am. J. Physiol.: Cell Physiol.*, 1996, **271**, C1424.
- A. Weidinger and A. V. Kozlov, *Biomolecules*, 2015, **5**, 472.
- P. Ascenzi, A. di Masi, C. Sciorati and E. Clementi, *BioFactors*, 2010, **36**, 264.
- H. Ischiropoulos and J. S. Beckman, *J. Clin. Invest.*, 2003, **111**, 163.
- P. Sarchielli, F. Galli, A. Floridi and V. Gallai, *Amino Acids*, 2003, **25**, 427.
- D. A. Wink, Y. Vodovotz, J. Laval, F. Laval, M. W. Dewhirst and J. B. Mitchell, *Carcinogenesis*, 1998, **19**, 711.
- A. C. Sedgwick, X. L. Sun, G. Kim, J. Yoon, S. D. Bull and T. D. James, *Chem. Commun.*, 2016, **52**, 12350.
- Z. N. Sun, H. L. Wang, F. Q. Liu, Y. Chen, P. K. H. Tam and D. Yang, *Org. Lett.*, 2009, **11**, 1887.
- X. Li, R.-R. Tao, L.-J. Hong, J. Cheng, Q. Jiang, Y.-M. Lu, M.-H. Liao, W.-F. Ye, N.-N. Lu, F. Han, Y.-Z. Hu and Y.-H. Hu, *J. Am. Chem. Soc.*, 2015, **137**, 12296.
- F. B. A. Yu, P. Li, G. Y. Li, G. J. Zhao, T. S. Chu and K. L. Han, *J. Am. Chem. Soc.*, 2011, **133**, 11030.
- F. B. Yu, P. Li, B. S. Wang and K. L. Han, *J. Am. Chem. Soc.*, 2013, **135**, 7674.
- D. Cheng, Y. Pan, L. Wang, Z. B. Zeng, L. Yuan, X. B. Zhang and Y. T. Chang, *J. Am. Chem. Soc.*, 2017, **139**, 285.



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.



- 14 L. Yuan, W. Y. Lin, K. B. Zheng, L. W. He and W. M. Huang, *Chem. Soc. Rev.*, 2013, **42**, 622.
- 15 R. Weissleder, *Nat. Biotechnol.*, 2001, **19**, 316.
- 16 A. Sikora, J. Zielonka, M. Lopez, J. Joseph and B. Kalyanaraman, *Free Radical Biol. Med.*, 2009, **47**, 1401.
- 17 X. Sun, Q. Xu, G. Kim, S. E. Flower, J. P. Lowe, J. Yoon, J. S. Fossey, X. Qian, S. D. Bull and T. D. James, *Chem. Sci.*, 2014, **5**, 3368.
- 18 S. Palanisamy, P. Y. Wu, S. C. Wu, Y. J. Chen, S. C. Tzou, C. H. Wang, C. Y. Chen and Y. M. Wang, *Biosens. Bioelectron.*, 2017, **91**, 849.
- 19 Y. H. Yang, J. L. Liu, H. Y. Xiao, Z. Zhen and S. H. Bo, *Dyes Pigm.*, 2017, **139**, 239.
- 20 W. Shu, L. G. Yan, Z. K. Wang, J. Liu, S. Zhang, C. Y. Liu and B. C. Zhu, *Sens. Actuators, B*, 2015, **221**, 1130.
- 21 Y. J. Wang, Y. Shi, Z. Y. Wang, Z. F. Zhu, X. Y. Zhao, H. Nie, J. Qian, A. J. Qin, J. Z. Sun and B. Z. Tang, *Chem. – Eur. J.*, 2016, **22**, 9784.
- 22 Y. R. Wang, L. Feng, L. Xu, Y. Li, D. D. Wang, J. Hou, K. Zhou, Q. Jin, G. B. Ge, J. N. Cui and L. Yang, *Chem. Commun.*, 2016, **52**, 6064.
- 23 B. C. Zhu, H. Kan, J. K. Liu, H. G. Liu, Q. Wei and B. Du, *Biosens. Bioelectron.*, 2014, **52**, 298.
- 24 C. Y. Li, M. Li, Y. Li, Z. S. Shi, Z. J. Li, X. B. Wang, J. Sun, J. W. Sun, D. M. Zhang and Z. C. Cui, *J. Mater. Chem. C*, 2016, **4**, 8392.
- 25 T. Yu, G. X. Yin, P. Yin, Y. Zeng, H. T. Li, Y. Y. Zhang and S. Z. Yao, *RSC Adv.*, 2017, **7**, 24822.
- 26 M. Ipu, C. Billon, G. Micouin, J. Samarut, C. Andraud and Y. Bretonniere, *Org. Biomol. Chem.*, 2014, **12**, 3641.
- 27 E. W. Miller, A. E. Albers, A. Pralle, E. Y. Isacoff and C. J. Chang, *J. Am. Chem. Soc.*, 2005, **127**, 16652.
- 28 B. C. Dickinson, C. Huynh and C. J. Chang, *J. Am. Chem. Soc.*, 2010, **132**, 5906.
- 29 A. Vazquez-Torres, J. Jones-Carson and E. Balish, *Infect. Immun.*, 1996, **64**, 3127.
- 30 M. D. Brand, C. Affourtit, T. C. Esteves, K. Green, A. J. Lambert, S. Miwa, J. L. Pakay and N. Parker, *Free Radical Biol. Med.*, 2004, **37**, 755.
- 31 X.-P. He, Y. Zang, T. D. James, J. Li, G.-R. Chen and J. Xie, *Chem. Commun.*, 2017, **53**, 82.
- 32 J. Zhang, Y. Fu, H.-H. Han, Y. Zang, J. Li, X.-P. He, B. L. Feringa and H. Tian, *Nat. Commun.*, 2017, **8**, 987.

