ChemComm

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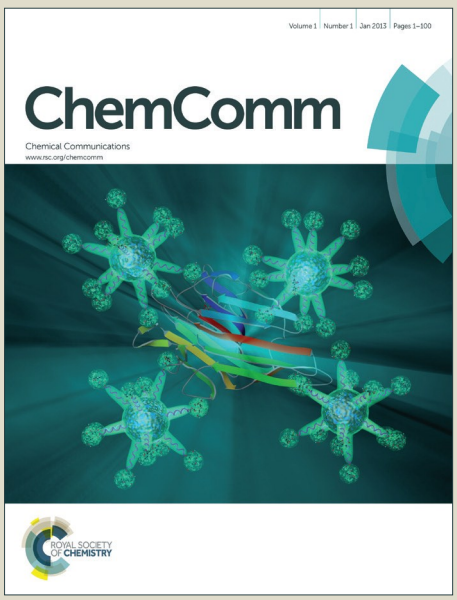

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DOI: 10.1039/C6CC06829D

Journal Name

ARTICLE

Boronate based fluorescence (ESIPT) probe for peroxynitrite

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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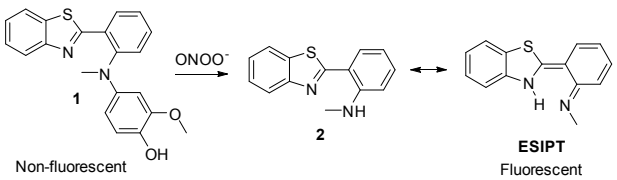
A simple probe for the detection of peroxynitrite was developed incorporating a benzyl boronic ester "protecting" unit. The "protecting" unit of the probe is removed by peroxynitrite to "turn-on" ESIPT fluorescence (4.5 fold enhancement). Furthermore, the probe was cell permeable and was used in cell imaging experiments showing an off-on response towards peroxynitrite, in HeLa and RAW 264.7 cells.

Peroxynitrite ($ONOO^-$) is a short lived reactive nitrogen species (RNS)[1,2] with a half-life of ~10-20 ms known to be a key pathological intermediate in a wide range of diseases. Including inflammatory, ischemia-reperfusion and neurodegenerative diseases.[3-5] Peroxynitrite behaves as a strong oxidant and nitrating agent towards a wide range of biological targets such as lipids, proteins, and DNA.[6] Therefore the development of peroxynitrite selective fluorescent molecular probes is highly desirable.[7-15] However, current commercial peroxynitrite probes including aminophenyl fluorescein (APF) and hydroxyphenyl fluorescein (HPF) lack selectivity and react with other ROS.[16]

Recently Li *et al.* developed a "turn-on" fluorescent probe **1** for tracking the *in situ* generation of peroxynitrite in cells and mice using ischemia-induced neurovascular damage.[6] Benzothiazole was shown to be a good fluorescent probe with high "turn-on" (600 fold), high selectivity towards peroxynitrite and rapid transport across the blood brain barrier. As shown in Scheme 1 in its normal form **2** is weakly fluorescent but when excited it can isomerise *via* excited state intramolecular proton transfer (**ESIPT**), resulting in a large increase in fluorescence.

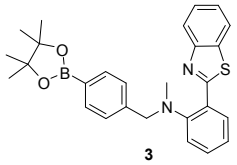
Probe **1** was designed to contain a saturated C-N bond thus blocking the ESIPT process, however, on reaction with peroxynitrite the *p*-hydroxyaniline group undergoes oxidative cleavage to form benzoquinone when exposed to peroxynitrite to generate the highly fluorescent *N*-methyl-benzothiazole proton donor (Scheme 1).



Scheme 1.

We realised that the fluorescent *N*-methyl-benzothiazole fragment **2** provides a new core on which to develop a range of selective probes. Our group is particularly interested in incorporating benzyl boronic ester units, which have been elegantly employed by Chang and Shabat,[17-19] this "protecting group" is oxidatively cleavage by hydrogen peroxide to generate highly fluorescent products.

Similarly, it has been shown that boronates/ boronic acids react more rapidly (milliseconds) with peroxynitrite when compared to hydrogen peroxide where the oxidation process can take several hours.[20] Therefore, decided to incorporate the Shabat "protecting group" with the fluorescent *N*-methyl-benzothiazole core in order to develop peroxynitrite sensor **3**.



Probe **3** was synthesised over three steps through the reaction of 2-aminothiophenol and isatoic anhydride to afford 2-(benzo[d]thiazol-2-yl) aniline in 72 % yield. A one pot reductive amination was then carried out using sodium triacetoxyborohydride and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde in 78 % yield. The intermediate formed was found to contain a particularly unreactive aniline towards a number of reagents

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x
All data created during this research are openly available from the University of Bath data archive at http://doi.org/10.15125/BATH-00252

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including NaH, consequently an excess of reagents was required, therefore, 15 equivalents of AcOH and 10 equivalents of formaldehyde were used to afford probe **3** in 14 % yield. (**Scheme S1**)

We subsequently evaluated the fluorescence behaviour of probe **3**, in in pH 8.2 buffer solution [52 wt% methanol].[21] Probe **3** produced an up to 4.5 fold fluorescence "turn on" in the presence of low concentrations of peroxynitrite (10 μM).

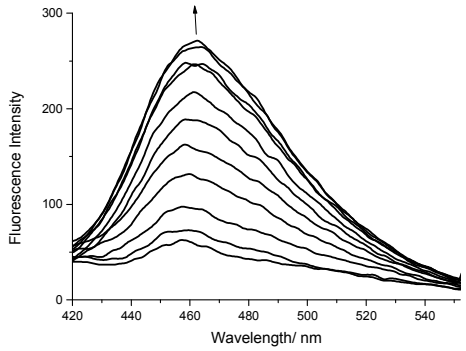

Fig 1. Fluorescence spectra of probe **3** (0.25 μM) with the addition of peroxynitrite (0-10 μM), λ_{ex} 400 nm in pH 8.2 buffer solution [52 wt% methanol].

Subsequently, we evaluated the selectivity of probe **3** towards other ROS/RNS. As expected, ClO^- (100 μM) led to a decrease in fluorescence intensity (0.71) due to its strong oxidising ability slowly destroying probe **3**. While, as reported by Sikora *et al.*, H_2O_2 led to a small fluorescence enhancement at 100 μM. Addition of 10 mM H_2O_2 is required to produce a significant fluorescence response (Fig S3 and S4), clearly demonstrating the greater reactivity of boronic acids/ esters towards peroxynitrite.

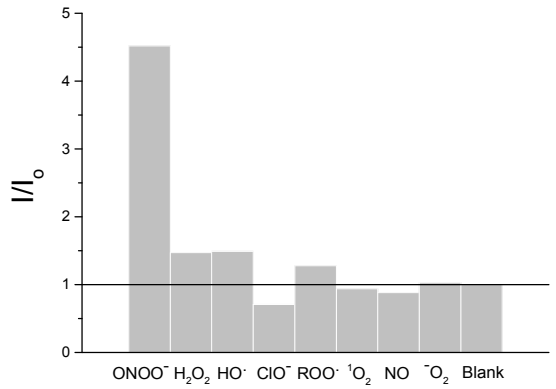

Fig 2. Selectivity of Probe **3** (0.25 μM), 10 μM peroxynitrite, All other ROS 100 μM. H_2O_2, ClO^- and ROO; incubated for 30 mins, λ_{ex} 400 nm/ λ_{em} 461 nm in pH 8.2 buffer solution [52 wt% methanol].

Having determined the selectivity of probe **3**, we then evaluated its ability to visualise endogenous and exogenous peroxynitrite using cell imaging experiments. The HeLa cells were incubated with probe **3** (20 μM) for 30 minutes and washed with Dulbecco's phosphate-buffered saline (DPBS). The cells were then observed using a confocal laser microscope excitation λ 405 nm, emission λ 430 – 455 nm. From Figure 3 probe **3** can penetrate live cell membranes and provide a clear "turn on" response in the presence of various concentrations of peroxynitrite added exogenously. No "turn on" was observed when the cells were pretreated with the peroxynitrite scavenger ebselen.

To detect peroxynitrite endogenously, RAW 264.7 cells were used and the immune reaction was induced using 1μg/mL and 50 ng/mL LPS, IFN-γ. As shown above in figure, probe **3** was shown to detect peroxynitrite endogenously. Probe **3** was also evaluated at longer wavelengths in cells λ_{ex} 473nm and λ_{em} 490 – 590 nm (Fig S7).

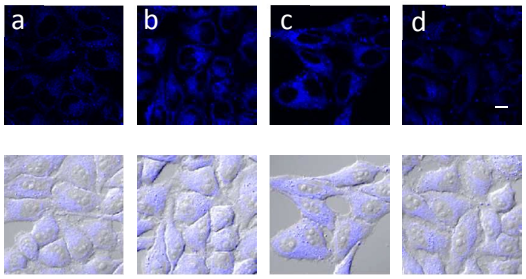

Fig 3. Fluorescence property in the live cell. HeLa cells were incubated with 20 μM probe **3** for 30 min and washed with DPBS and added (a) 0, (b) 30, (c) 100 μM ONOO- and (d) after pretreatment with 100 μM ebselen, add 100 μM ONOO- for 30 min. Fluorescence images were acquired by confocal microscopy. λ_{ex} 405 nm/ λ_{ex}. 430 – 455 nm. Scale bar: 10 μm.

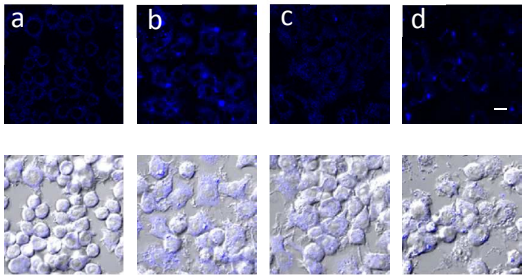

Fig 4. Detection of peroxynitrite made by immune reaction in the macrophage. RAW 264.7 cells were treated with (a) no, (b) 1 μg/ml LPS 16hr, 50 ng/ml interferon-γ 4hr, (c), LPS, IFN-γ + 100 μM ebselen and (d) LPS, IFN-γ + 100 μM uric acid. The cells were stained with 20 μM probe 3 for 30 min and washed with DPBS and imaged by confocal microscopy. λ_{ex} 405 nm/ λ_{em} 430 – 455 nm. Scale bar: 10 μm.[22-24]

Conclusions

In conclusion we have a developed a reaction based fluorescent probe that can be used to detect peroxynitrite at low concentrations allowing the detection in cells. Before exposure to ROS, probe **3** has a low fluorescence intensity becuase the ESIPT process is not possible. However, the fluorescence is enhanced once the boronic ester "protecting group" is removed selectively by

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exposure to peroxynitrite. We have demonstrated that *N*-methyl-benzothiazole core unit **2** can be used to develop probes with applications in exploring the pathological effect of peroxynitrite. Furthermore, probe **3** as a very important advantage over probe **1** since could be easily developed into a theranostic agent towards tumour cells. This approach was recently demonstrated by Kim *et al*. who have used the boronic "protecting group" to weaponize a probe by linking with the anticancer prodrug 5'-deoxy-5-fluorouridine to the boronic acid group.[25]

Acknowledgements

TDJ, XS, SDB, and TDJ would like to thank the University of Bath for support. ACS. thanks the EPSRC for a studentship. JY thanks the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (No. 2012R1A3A2048814).

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