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# Pinkment: a synthetic platform for the development of fluorescent probes for diagnostic and theranostic applications†

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Reaction-based fluorescent-probes have proven successful for the visualisation of biological species in various cellular processes. Unfortunately, in order to tailor the design of a fluorescent probe to a specific application (*i.e.* organelle targeting, material and theranostic applications) often requires extensive synthetic efforts and the synthetic screening of a range of fluorophores to match the required synthetic needs. In this work, we have identified **Pinkment-OH** as a unique "plug-and-play" synthetic platform that can be used to develop a range of ONOO<sup>−</sup> responsive fluorescent probes for a variety of applications. These include theranostic-based applications and potential material-based/bioconjugation applications. The as prepared probes displayed an excellent sensitivity and selectivity for ONOO<sup>−</sup> over other ROS. *In vitro* studies using HeLa cells and RAW 264.7 macrophages demonstrated their ability to detect exogenously and endogenously produced ONOO<sup>−</sup>. Evaluation in an LPS-induced inflammation mouse model illustrated the ability to monitor ONOO<sup>−</sup> production in acute inflammation. Lastly, theranostic-based probes enabled the simultaneous evaluation of indomethacin-based therapeutic effects combined with the visualisation of an inflammation biomarker in RAW 264.7 cells.

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## Introduction

There is a growing need for new and effective diagnostic tools that can evaluate biomarkers involved in inflammatory based diseases.<sup>1–6</sup> Inflammation is the innate defence mechanism of the body that recognises damaged cells, pathogens and

infections. The inflammatory response often results in the generation of reactive oxygen species/reactive nitrogen species (ROS/RNS), which are involved in the functional regulation of M1 and M2 macrophages.<sup>7,8</sup> The M1 pro-inflammatory phenotype is induced by lipopolysaccharide (LPS), which triggers the generation of ROS from NADPH using NADPH oxidase (NOX).<sup>9</sup> This production of ROS regulates an array of cellular events including the activation of the nuclear factor kappa-B (NF-κB), the production of cytokines and cell survival whereas, high levels of ROS are associated with programmed cell death, *i.e.* apoptosis.<sup>7,10–14</sup> The high sensitivity and high spatial and temporal resolution of fluorescent probes allow us to visualise these key cellular events. Our group and others have focused on the fluorescence-based detection of ROS/RNS such as ONOO<sup>−</sup>, H<sub>2</sub>O<sub>2</sub> and HOCl.<sup>1,15–21</sup> To achieve the selective detection of a particular ROS requires the careful consideration of both fluorophore and reactive motif. In this regard, resorufin is a particularly attractive fluorophore due to its red shifted fluorescence and easy to functionalise scaffold. Pioneering work led by Chang *et al.* developed peroxyresorufin-1 (PR1) for H<sub>2</sub>O<sub>2</sub> detection whereby resorufin is masked with boronic esters.<sup>22,23</sup> Boronic esters have been identified as a relevant sensing group for both H<sub>2</sub>O<sub>2</sub> and ONOO<sup>−</sup> detection. However, in an environment with both species present, boronic esters preferentially react with ONOO<sup>−</sup> due to the inherent faster reactivity of ONOO<sup>−</sup> in comparison to H<sub>2</sub>O<sub>2</sub>.<sup>24</sup> Previously, we have

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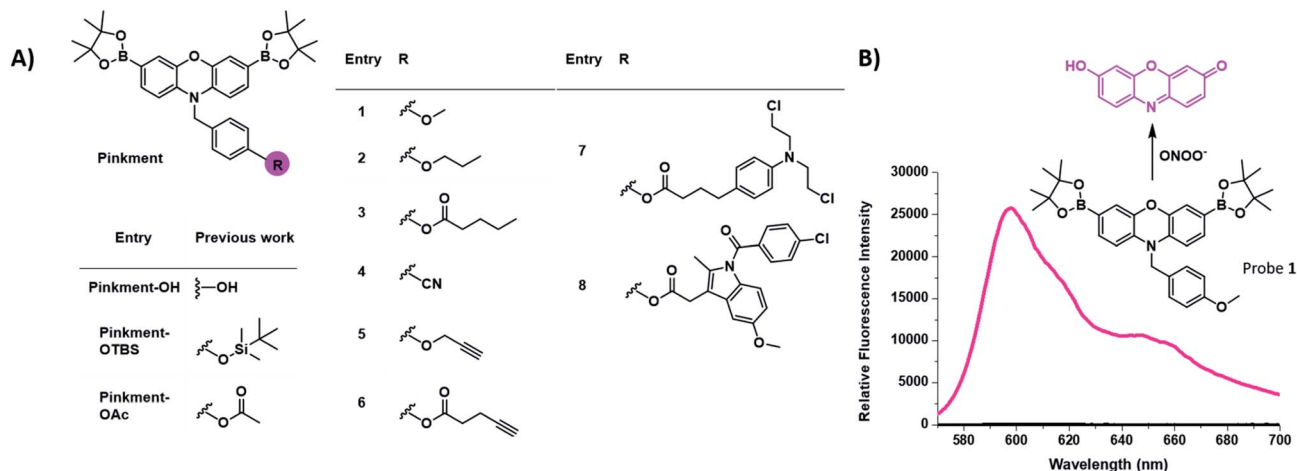
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**Fig. 1** (A) Chemical structure of the resorufin-based probes for the sensing of  $\text{ONOO}^-$ , including the previously reported **Pinkment-OH** “plug and play” scaffold and dual analyte probes **Pinkment-OTBS** and **Pinkment-OAc**.<sup>10</sup> (B) Unexpected fluorescence turn on response of **1** in the presence of  $\text{ONOO}^-$ .

demonstrated PR1's ability to preferentially detect ONOO<sup>-</sup> over H<sub>2</sub>O<sub>2</sub> *in vitro*.<sup>25</sup> Consequently, we decided to investigate functionalized synthetic derivatives of PR1.<sup>26</sup> This led to the development of **Pinkment-OH** for the design of dual analyte AND-logic probes, **Pinkment-OTBS** (ONOO<sup>-</sup> “AND” fluoride) and **Pinkment-OAc** (H<sub>2</sub>O<sub>2</sub> “AND” esterase) using **Pinkment-OH** (Fig. 1) as a synthetic starting point. These results revealed the potential of **Pinkment-OH** to be used as a synthetic platform for the development of ONOO<sup>-</sup> selective fluorescence probes with additional sensing, targeting or drug units. Here, we have serendipitously discovered that the benzyl unit of our ROS **Pinkment** fluorescent probe can be functionalized with a functional unit of choice without compromising ROS selectivity. As a result, **Pinkment-OH** was successfully shown as a synthetic platform to develop ONOO<sup>-</sup> selective fluorescent probes with additional functional units (Fig. 1).

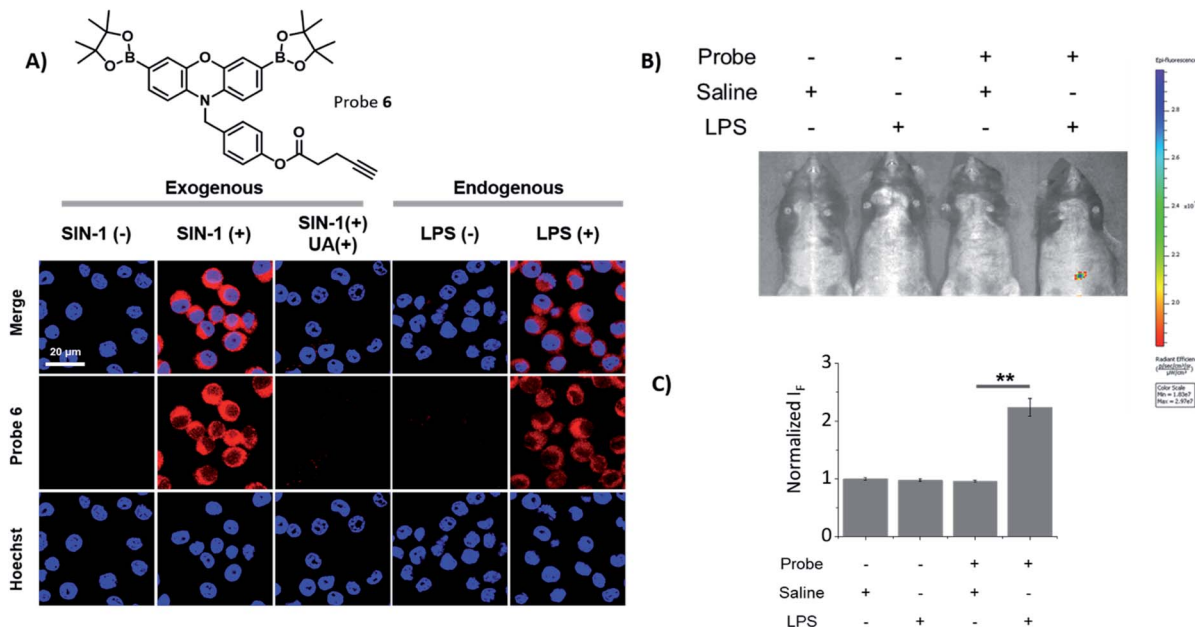
## Results and discussion

Initially, our focus was on continuing the development of “AND”-based logic-gates for biological application.<sup>1,17,26</sup> This led to the elaboration of probe **1**, which was accessed in a simple three step synthesis (Scheme S1†). Unexpectedly, we discovered that **1** “turned on” in the sole presence of ONOO<sup>−</sup> (Fig. 1B and S1†). This led to the development of **Pinkment** probes **2** and **3** to further confirm this observation. These probes were accessible from the synthetic platform **Pinkment-OH**, whose 6-step synthesis has been previously reported by our group.<sup>26</sup> Nucleophilic substitution by **Pinkment-OH** using 1-bromopropane and pentanoyl chloride respectively gave **2** and **3** in moderate yields: 50% and 51% respectively (Scheme S2 and S3†). Probes **2** and **3** showed good selectivity towards ONOO<sup>−</sup> over other ROS species (Fig. S2–S8†). Surprisingly, the probes demonstrated a high sensitivity towards ONOO<sup>−</sup> requiring concentrations in the low micromolar range. Both **2** and **3** displayed increased solubility in comparison to **1**. We decided to further explore this

unexpected result by introducing a terminal nitrile group. Probe 4 was accessible in a facile three-step synthesis (Scheme S4†) in the same manner as **1**. Again, good selectivity and sensitivity for ONOO<sup>−</sup> was observed (Fig. S2–S8†). From these results, we realized that the **Pinkment** benzyl unit can be functionalized with any unit of choice without compromising the ROS selectivity. Thus, we rationalized that **Pinkment-OH** offers a unique platform for the design of ONOO<sup>−</sup> selective fluorescence based probes that can be tailored towards a range of applications.<sup>27–29</sup> This led to the development of alkyne-based **Pinkment** probes **5** and **6** that have potential to be used in “click” chemistry.<sup>29</sup> These probes were accessed from **Pinkment-OH** and prepared in moderate yields: 48% and 47% for **5**, and **6** respectively (Scheme S5 and S6†). Fluorescence studies of **5** and **6** established good sensitivity and selectivity towards ONOO<sup>−</sup> over other ROS (Fig. S2–S8†).

We then turned our attention to assessing the imaging capacity of probes 2 and 3 and the potential “click” based probes 5 and 6 in cells and live animals. To demonstrate their suitability as imaging tools, all four probes were evaluated for cellular toxicity in murine RAW 264.7 macrophages using a MTS cell proliferation assay. Probes 2, 3, 5 and 6 were incubated at different concentrations ranging from 5 to 40  $\mu\text{M}$  for 24 h (Fig. S9<sup>†</sup>). Probes 2, 5 and 6 were found to be non-toxic. In contrast, probe 3 decreased the cell viability of RAW 264.7 macrophages by 40% at a concentration of 40  $\mu\text{M}$  compared to control conditions. As a result, 3 was not taken forward for further cell studies since high concentrations of the probe are required for *in vivo* studies.

Probes **2**, **5** and **6** were shown to be non-toxic, and were evaluated with exogenous ONOO<sup>-</sup>, using SIN-1 (500 μM) in RAW 264.7 macrophages (Fig. 2A and S10†). Each probe alone demonstrated minimal fluorescence in cells, the addition of SIN-1 led to a significant enhancement in intracellular fluorescence at a wavelength corresponding to the dye, resorufin, therefore, suggesting the intracellular reaction of the probe with



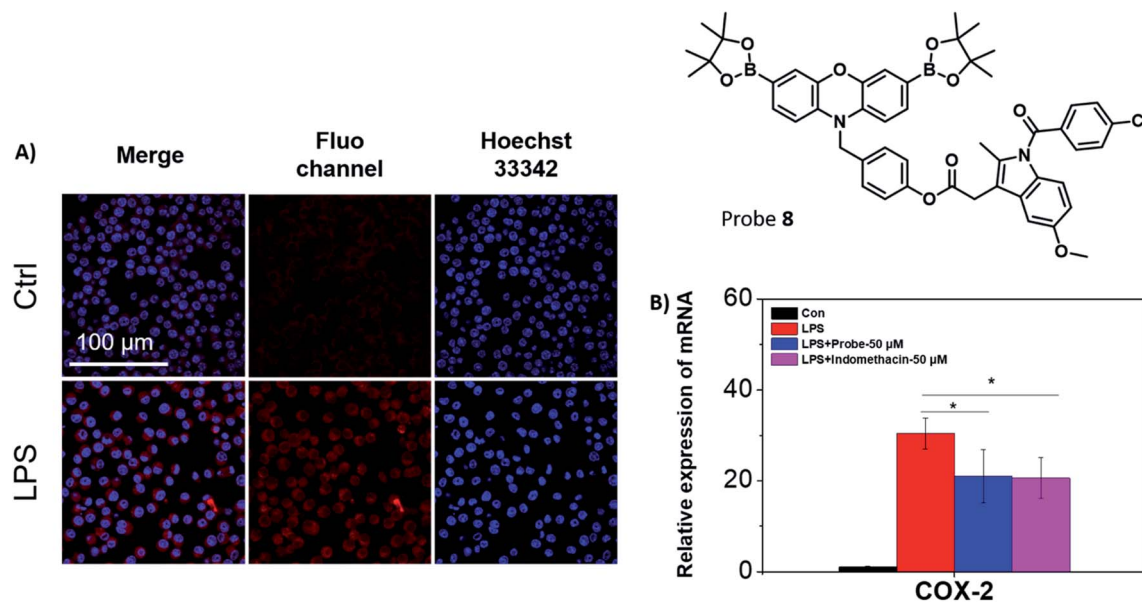


Fig. 3 (A) Confocal imaging of RAW 264.7 macrophages treated with LPS ( $1 \mu\text{g mL}^{-1}$ , 24 h) and then loaded with **8** ( $20 \mu\text{M}$ , 30 min) as indicated. Fluorescence data was collected using  $\lambda_{\text{ex}} = 559 \text{ nm}$  and  $\lambda_{\text{em}} = 580\text{--}650 \text{ nm}$ , respectively. The cell nuclei were stained using Hoechst 33342 and fluorescence collected at  $\lambda_{\text{ex}} = 405 \text{ nm}$  and  $\lambda_{\text{em}} = 450\text{--}480 \text{ nm}$ . Scale bar =  $100 \mu\text{m}$ .  $N = 3$ . (B) Effect of **8** on LPS-induced COX-2 gene expression in RAW 264.7 macrophages. Cells were treated with LPS alone ( $1 \mu\text{g mL}^{-1}$ ) or together with **8** for 24 h. Indomethacin was set as a positive control, and the relative mRNA level of COX-2 gene was normalized by GAPDH (\* $p < 0.05$ ).  $N = 4$ .

inhibitor of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), of which COX-2 is mainly responsible for the inflammatory response.<sup>36</sup> The therapeutic effects on the LPS-induced inflammatory responses in RAW 264.7 macrophages were further investigated using **8**. RAW 264.7 macrophages were treated with LPS and the expression of the pro-inflammatory gene (COX-2) was investigated using qRT-PCR in the presence or absence of **8** (Fig. 3B).<sup>37</sup> The mRNA level of COX-2 decreased in the presence of **8** ( $50 \mu\text{M}$ ) in comparison to the LPS-induced group. A similar effect to the LPS-induced group was observed with indomethacin alone. This suggests that **8** can monitor ONOO<sup>−</sup> production in acute inflammation, and in addition, reduce the inflammatory response by releasing indomethacin.

## Conclusions

The ability of the **Pinkment** scaffold to be functionalised with any unit of choice without compromising the overall ROS selectivity, opens up new possibilities for the design of highly specific ONOO<sup>−</sup> probes that can be used in a variety of applications. In this work, we have successfully illustrated the applicability of **Pinkment**-based probes for diagnostic and theranostics applications. Our probes displayed good selectivity and sensitivity towards ONOO<sup>−</sup> over a range of other ROS. Cellular studies with the **Pinkment** probes led to the identification of alkyne-functionalised **Pinkment** probe **6** as a suitable candidate for *in vivo* studies using an inflammatory mouse model. These promising results led us to design potential theranostic probes **7** and **8** with candidate **8** displaying promising properties *in vitro*. We believe this work demonstrates **Pinkment-OH** as a useful synthetic platform to enable the rapid

development of a ONOO<sup>−</sup> fluorescent probe that can be tailored to the needs of the chemical biologist. In particular, the alkyne **Pinkment** probes offer the possibility of attaching any desired unit *via* click chemistry. Therefore, we anticipate that the **Pinkment** scaffold can be further elaborated for the development of dual analyte, organelle targeting and theranostic probes for a range of diagnostic and theranostic applications.

## Conflicts of interest

There are no conflicts to declare.

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