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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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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  $\text{ONOO}^-$  over  $\text{H}_2\text{O}_2$  *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** ( $\text{ONOO}^-$  “AND” fluoride) and **Pinkment-OAc** ( $\text{H}_2\text{O}_2$  “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  $\text{ONOO}^-$  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  $\text{ONOO}^-$  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  $\text{ONOO}^-$  (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  $\text{ONOO}^-$  over other ROS species (Fig. S2–S8†). Surprisingly, the probes demonstrated a high sensitivity towards  $\text{ONOO}^-$  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  $\text{ONOO}^-$  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  $\text{ONOO}^-$  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  $\text{ONOO}^-$  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†). 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  $\text{ONOO}^-$ , using SIN-1 (500  $\mu\text{M}$ ) 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



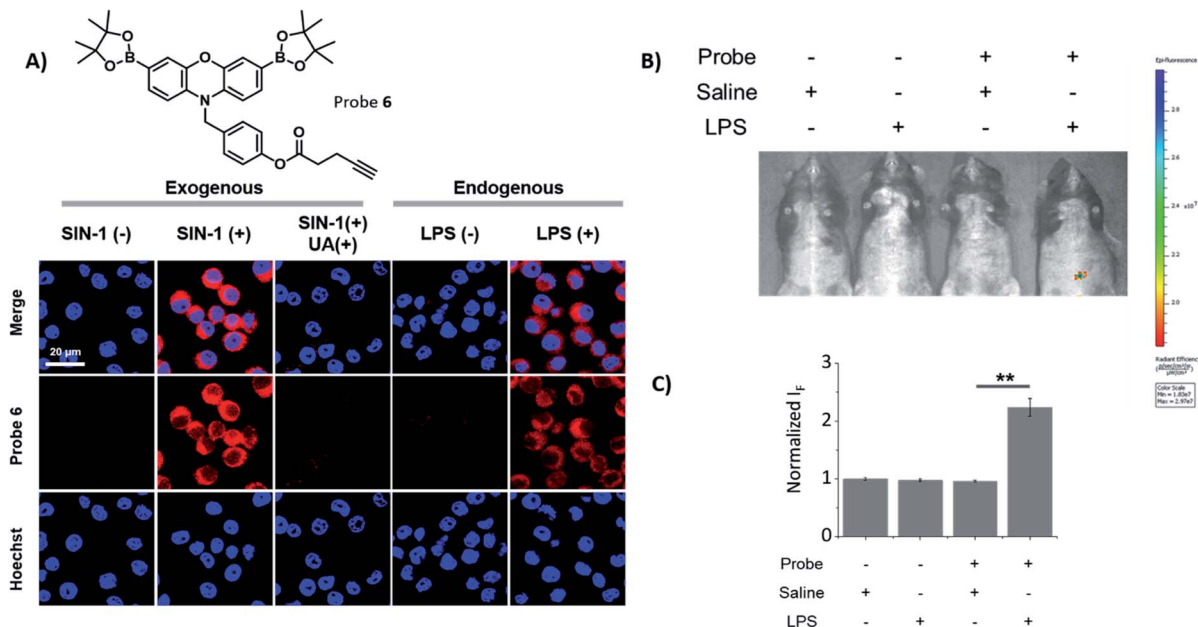


Fig. 2 (A) Confocal imaging of RAW 264.7 macrophages treated with probe 6 (20  $\mu\text{M}$ , 30 min) in the presence and absence of SIN-1 (500  $\mu\text{M}$ , 30 min) and uric acid (100  $\mu\text{M}$ , 2 h) or LPS (1  $\mu\text{g mL}^{-1}$ , 24 h) as indicated. Fluorescence data was collected using  $\lambda_{\text{ex}} = 559 \text{ nm}$  and  $\lambda_{\text{em}} = 580\text{--}650 \text{ nm}$ , respectively. The cell nuclei was 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 = 20  $\mu\text{m}$ .  $N = 3$ . (B) Intraperitoneal injection of male C57BL/6J mice with probe 6 (200  $\mu\text{M}$ ) or saline in the absence and presence of LPS (2  $\text{mg mL}^{-1}$  in saline) with  $\lambda_{\text{ex}} = 535 \text{ nm}$  and  $\lambda_{\text{em}} = 600 \text{ nm}$ .  $N = 3$ . (C) Quantification of (B) C57BL/6J male mice treated with probe 6 (200  $\mu\text{M}$ ) or saline in the absence and presence of LPS (2  $\text{mg mL}^{-1}$  in saline) with  $\lambda_{\text{ex}} = 550 \text{ nm}$  and  $\lambda_{\text{em}} = 580\text{--}620 \text{ nm}$ . Error bars represent s. d. with  $**p \leq 0.01$ .  $N = 3$ . Normalised fluorescence intensities were calculated using the saline solution fluorescence intensities.

$\text{ONOO}^-$  and their suitability for use as fluorescence-based probes. The SIN-1 generated fluorescence signal was then evaluated with the  $\text{ONOO}^-$  scavenger, uric acid.<sup>30</sup> As expected, uric acid attenuated the fluorescent increase that was induced by SIN-1 for all probes, thus confirming the  $\text{ONOO}^-$  mediated increase in fluorescence intensity. Next, we evaluated the capability of 2, 5 and 6 to detect endogenous  $\text{ONOO}^-$  in LPS primed RAW 264.7 macrophages. All three probes were shown to detect endogenous  $\text{ONOO}^-$  in LPS primed RAW 264.7 macrophages (Fig. 2A and S11<sup>†</sup>), confirming their promise for the imaging of LPS-induced inflammatory responses. In addition, HeLa and A549 cell lines treated with or without SIN-1 were used to illustrate the versatility of the **Pinkment** probes (Fig. S12 and S13<sup>†</sup>).

Encouraged by these cell imaging results, we used a known LPS-induced inflammation mouse model<sup>31</sup> for the *in vivo* detection of  $\text{ONOO}^-$  (Fig. 2B). The injection of LPS (2  $\text{mg mL}^{-1}$  in saline) to the abdominal region of mice followed by the injection of 6 (200  $\mu\text{M}$ ) led to its fluorescence activation. The quantified fluorescence intensity in the probe(+)/LPS(+) group was significantly larger than that in the probe(+)/LPS(-) group (Fig. 1C), demonstrating the potential of using 6 for the monitoring of  $\text{ONOO}^-$  *in situ* during acute inflammation.

In order to follow our current interest in theranostics,<sup>32</sup> we then turned our attention towards the potential of **Pinkment-OH** for the design of fluorescence-based drug releasing probes. Therefore, we used the drugs chlorambucil and indomethacin to afford two distinct theranostic probes 7 and 8, respectively

(Fig. 1A). Chlorambucil is used to treat chronic lymphatic leukemia<sup>33</sup> and indomethacin is used as a non-steroidal anti-inflammatory drug (NSAID).<sup>34,35</sup> Both 7 and 8 were easily accessible from **Pinkment-OH** (Scheme S7 and S8<sup>†</sup>).

Mass spectrometry confirmed and validated the simultaneous release of each drug and fluorescent resorufin dye (Fig. S14 and S15<sup>†</sup>). Therefore, the enhancement in the fluorescence intensity over time indicates the release of each drug. As such, time-dependent fluorescence experiments with 7 and 8 in the presence of  $\text{ONOO}^-$  were performed to illustrate the time dependence of the drug release. These experiments revealed a maximum fluorescence response after  $\sim 10 \text{ min}$  (Fig. S16<sup>†</sup>).

Fluorescence studies were carried out including ROS selectivity,  $\text{H}_2\text{O}_2$  titration and  $\text{ONOO}^-$  titration studies (Fig. S17–S21<sup>†</sup>) and demonstrated high sensitivity towards these inflammation-based biomarkers. Following these initial studies, we evaluated both 7 and 8 in RAW 264.7 macrophages towards exogenous  $\text{ONOO}^-$  detection (Fig. S22<sup>†</sup>). The presence of SIN-1 significantly enhanced the intracellular fluorescence of 7 and 8, confirming the applicability of the probes *in vitro*. Despite 7 displaying significant promise, the creation of an appropriate model system to differentiate between cancerous and healthy cells would require a significant amount of development and as such was beyond the scope of this current research. Therefore, only 8 was further evaluated, since its cellular behaviour was easier to monitor. Endogenous  $\text{ONOO}^-$  was also detected by 8 in RAW 264.7 macrophages (Fig. 3A). Indomethacin, a NSAID, is an effective and non-selective





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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