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Traditionally, fluorescence probes have focused on the detection of a single biomarker for a specific process. In this work, we set out to develop a number of fluorescence probes that enable the detection of a chosen analyte in the presence of reactive oxygen/nitrogen species (ROS/RNS). These fluorescence probes when activated result in the formation of the highly fluorescent pink dye, resorufin. Therefore, we have labelled these fluorescent probes as 'Pinkments'. Our first 'Pinkment' was shown to detect biologically relevant concentrations of ONOO^- and have an excellent selectivity against other ROS/RNS. Pinkment-OH was developed to provide a core unit which could be easily functionalised to produce a range of 'AND' based fluorescence probes for the detection of ROS/RNS and a second analyte. For proof of concept, we synthesised Pinkment-OTBS and Pinkment-OAc. These 'AND'-based probes were successfully shown to detect ROS/RNS and F^- or esterase, respectively.

Since the discovery of the first fluorescence-based probe in 1867, fluorescence probes have revolutionised the understanding of biological systems.^{1–4} Historically, these probes have focused on the detection of a single analyte or biomarker. However, biological systems are complex with more than one chemical species being released/present during any biological processes. For example, glutathione (GSH) accumulates at the

'AND'-based fluorescence scaffold for the detection of ROS/RNS and a second analyte[†]

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nucleus during the cell cycle to aid transcription factors binding to DNA⁵ and the pathological role of Zn^{2+} is believed to be associated with the glutamate system.⁶ Furthermore, the sensitivity of a cell towards peroxynitrite (ONOO^-) largely depends on the concentration of intracellular GSH.^{7–10} Therefore, in order to further understand cellular functions and the root causes of disease it is important to be able to study biologically important species simultaneously.

Alongside the development of the field of fluorescence probes, the field of molecular logic gates has developed.¹¹ Molecular logic gates are molecules that have the ability to bind or react with multiple analytes (input) and turn it into a measurable optical output. Consequently, these attractive molecules are now emerging in the literature demonstrating the ability to simultaneously detect multiple analytes in biological systems.^{12–21} Within our research group, we are interested in developing reaction-based fluorescence probes including 'AND'-based fluorescence probes for the detection of biologically important analytes.^{22–24} Dual responsive ('AND') fluorescence probes require both analytes being present to produce a fluorescence response. In this work, we identified a previously reported boronate-based fluorescence probe developed by Chang *et al.* **PR1**, with a free amino group attached (Fig. 1). Boronates have well-known reactivity towards ONOO^- and hydrogen peroxide (H_2O_2),²⁵ therefore we believed **PR1** could provide a suitable

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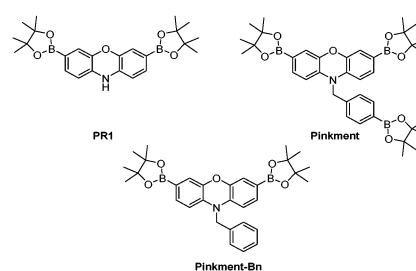


Fig. 1 **PR1** and **Pinkment** fluorescence probes for the detection of reactive oxygen and reactive nitrogen species (ROS/RNS). **Pinkment-Bn** – demonstrates the requirement elimination to produce a free $\text{N}-\text{H}$.



scaffold for the development of 'AND'-based systems for the detection of ROS/RNS and a second analyte.²⁶ In this work, we initially functionalised **PR1** with an additional ROS/RNS trigger, benzyl boronic acid ester, which afforded a selective ONOO^- probe known as **Pinkment** (Fig. 1).

The current literature route to obtain **PR1** involves a very low yielding first step (10% overall yield 7%), which uses harsh conditions (hydrobromic acid (HBr)).²⁶ Therefore, we alkylated commercially available phenoxazine using NaH and 4-bromobenzyl bromide to afford **1** in excellent yield (78%). **1** was then subsequently dissolved in CHCl_3 and NBS was added portion wise to afford dibrominated intermediate **2** in good yield (48%), which required only trituration (EtOAc) for purification. Lastly, Suzuki–Miyaura conditions were applied to **2** using bis(pinacolato)diboron (B_2pin_2) as a boron transfer agent to furnish **Pinkment** in 36% yield (see ESI† – Scheme S4). In the presence of ONOO^- , all three boronates were oxidised on **Pinkment**, forming the highly fluorescent pink dye, resorufin (see ESI† – Scheme S1) and a large fluorescence increase was observed at 590 nm (see ESI† – Fig. S1). **Pinkment** was able to detect biologically relevant concentrations of ONOO^- ^{27,28} and displayed excellent selectivity towards ONOO^- (see ESI† – Fig. S2–S4). To demonstrate the requirement of an 'eliminating' benzyl group on the free N–H, **Pinkment-Bn** (Fig. 1) was synthesised (see ESI† – Scheme S5) and as expected no increase in fluorescence intensity was observed with the addition of ONOO^- (10 μM) (see ESI† – Fig. S5).

From these results, we believed we could develop a range of 'AND'-based **Pinkment** fluorescence probes by altering the functionality on the benzyl group to react with a specific target analyte. To achieve this, we developed a synthesis for **Pinkment-OH** (Fig. 2), which provides a novel fluorescence core probe that can be functionalised with any chosen reactive chemical trigger for the detection of a chosen analyte in the presence of ROS/RNS (Fig. 2a).

For the synthesis of **Pinkment-OH**, the phenol of 4-hydroxybenzyl alcohol was selectively piv protected to afford

5 using pivaloyl chloride and NEt_3 in DCM . **5** was then converted to its corresponding bromide **6** using MsCl and NEt_3 followed by the addition of LiBr . Phenoxazine was then alkylated with **6** to produce **7** using NaH and DMF in satisfactory yield (51%). **7** was then dibrominated using NBS in CHCl_3 to afford **8**, which was subsequently deprotected using DIBAL-H to afford **9** in good yield (67%). The same Suzuki–Miyaura conditions were then applied to furnish **Pinkment-OH** in excellent yield (83%) (see ESI† – Scheme S6). To illustrate the stability of **Pinkment-OH**, an X-ray structure was obtained (Fig. 2b). Interestingly, **Pinkment-OH** formed dimers *via* intermolecular hydrogen bonding (see ESI† – Fig. S20).

Pinkment-OH was shown to have a high sensitivity and excellent selectivity towards ONOO^- making it suitable for cellular imaging experiments (see ESI† – Fig. S6–S9). As proof of concept for 'AND'-based pinkment sensors, we chose to synthesise **Pinkment-OTBS** and **Pinkment-OAc** (Fig. 2c and d), which can be used to detect ROS/RNS and fluoride (F^-) or esterase, respectively.

To afford **Pinkment-OTBS**, **Pinkment-OH** was silyl protected using standard silyl protection conditions, however, the reaction was found to be very slow (3 d) and **Pinkment-OTBS** was isolated in very low yield (11%) (see ESI† – Scheme S7). We then evaluated the ability of **Pinkment-OTBS** to detect F^- 'AND' ONOO^- in a buffer solution (52% w/w $\text{MeOH} : \text{H}_2\text{O}$). This $\text{MeOH}/\text{H}_2\text{O}$ buffer solution was required for the silyl-ether deprotection reaction to proceed effectively. As shown in ESI† – Fig. S10, 100% PBS produced a much smaller response when both analytes were added, which is consistent with literature reported TBS fluorescence probes.^{29,30} As shown in Fig. 3, addition of *tert*-butylammonium fluoride (TBAF – source of F^-) led to no increase in fluorescence intensity, however, subsequent additions of ONOO^- (0–20 μM) led to a significant increase in fluorescence intensity.

To ensure both analytes were required, the addition of TBAF and ONOO^- were then repeated in reverse order. Due to the high reactivity of ONOO^- ,^{8,31} a small increase in fluorescence intensity was observed on addition to **Pinkment-OTBS** (20 μM).

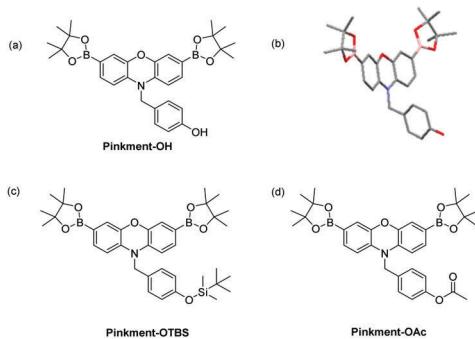


Fig. 2 (a) **Pinkment-OH** – a core fluorescence unit that enables the synthesis of 'AND' based fluorescence probes for the detection of (ROS/RNS) and a second analyte. (b) Molecular structure of **Pinkment-OH** with thermal ellipsoids shown at 50% probability. Hydrogen atoms are omitted for clarity except for the OH hydrogen atom. (c) **Pinkment-OTBS** (d) **Pinkment-OAc** for the detection of ROS/RNS 'AND' fluoride or esterase, respectively. See ESI† – Fig. S4. CCDC 1844385 contain the supplementary crystallographic data for **Pinkment-OH**.†

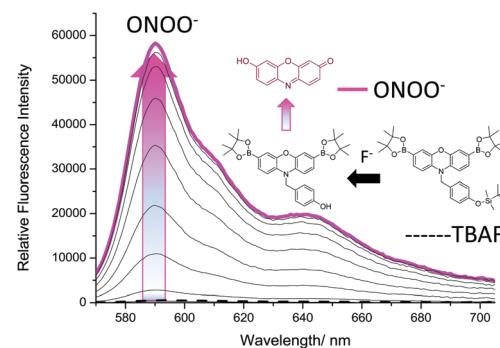


Fig. 3 Fluorescence spectra of **Pinkment-OTBS** (0.5 μM) with addition of TBAF (10 mM) and incubated for 1 h and then measured. Followed by incremental additions of ONOO^- (0–20 μM) The data was obtained in PBS (52% w/w $\text{MeOH} : \text{H}_2\text{O}$, pH = 8.2 at 25 °C. $\lambda_{\text{ex}} = 545$ –20 nm. Dashed line represents TBAF (10 mM) addition only.



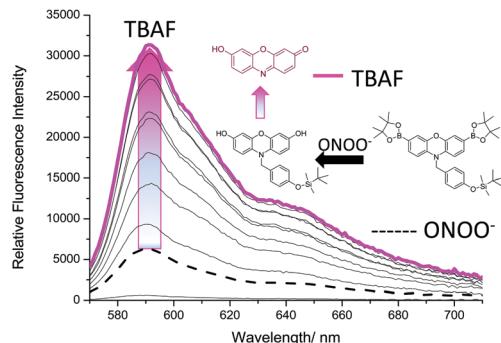


Fig. 4 Fluorescence spectra of **Pinkment-OTBS** ($0.5 \mu\text{M}$) with the addition of ONOO^- ($20 \mu\text{M}$) followed by incremental additions of TBAF (0 – 10 mM) measurements were taken after 90 min of both additions. The data was obtained in PBS (52% w/w MeOH : H_2O), $\text{pH} = 8.2$ at 25°C . $\lambda_{\text{ex}} = 545$ – 20 nm . Dashed line represents ONOO^- ($20 \mu\text{M}$) addition only.

However, like Fig. 3, a large increase in fluorescence intensity was only observed after the subsequent addition of TBAF (0 – 10 mM) (Fig. 4). As shown in ESI[†] – Fig. S12 and S13, **Pinkment-OTBS** displayed an excellent selectivity towards ONOO^- against other ROS in the presence of TBAF. **Pinkment-OTBS** was then screened against other halide sources (TBAB, TBAC and TBAI) in the presence of ONOO^- ($20 \mu\text{M}$) see ESI[†] – Fig. S14. As expected **Pinkment-OTBS** displayed excellent selectivity towards TBAF in the presence of ONOO^- .

We then turned our attention towards the synthesis of **Pinkment-OAc**. Despite the poor reactivity with TBS-Cl, **Pinkment-OH** reacted with AcCl smoothly and produced **Pinkment-OAc** in excellent yield (71%) (see ESI[†] – Scheme S8). Due to the known ability of ONOO^- to react with carbonyls, its addition to **Pinkment-OAc** was avoided (see ESI[†] – Fig. S15 and S16). Therefore, as proof of concept, we evaluated **Pinkment-OAc** with H_2O_2 ‘AND’ esterase. As shown in Fig. 5, the addition of porcine liver esterase, PLE (0.6 U) led to no increase in fluorescence intensity. However, subsequent additions of H_2O_2 (0 – 1 mM) led to a large increase in fluorescence intensity.

Again, to ensure that both analytes were required, the concentration of H_2O_2 was kept constant (1 mM) and the

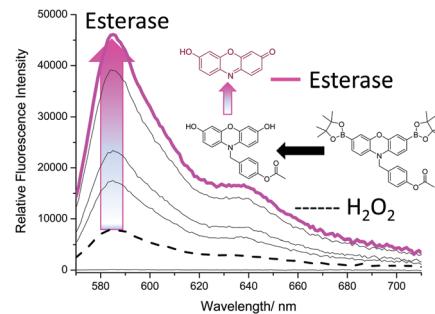


Fig. 6 Fluorescence spectra of **Pinkment-OAc** ($0.5 \mu\text{M}$) following incubation (15 min) with PLE (0.1 – 0.6 U) and subsequent addition of H_2O_2 (1 mM). The data was collected in PBS buffer (100% w/w H_2O) at $\text{pH} = 7.4$, 25°C . $\lambda_{\text{ex}} = 545$ – 20 nm . Dashed line represents H_2O_2 (1 mM) addition only.

amount of PLE was varied. Due to the known ability of H_2O_2 to hydrolyse carbonyl containing compounds, it was no surprise that there was a small increase in fluorescence intensity.^{32,33} However, with increasing additions of PLE much larger increases in fluorescence intensity were observed (Fig. 6). Interestingly, concentrations $>0.6 \text{ U}$ led to a smaller increase in fluorescence. This is believed to be the result of the excess esterase reacting with the added H_2O_2 (see ESI[†] – Fig. S17).

In summary, several fluorescence-based probes have been synthesised that when activated result in the formation of the highly fluorescent dye, resorufin. As the dye is pink in colour, we have labelled these fluorescent probes as ‘pinkments’. The original **Pinkment** was shown to detect biologically relevant concentrations of ONOO^- and have an excellent selectivity against other ROS/RNS making it suitable for cellular imaging experiments. **Pinkment** provided a suitable platform for the development of ‘AND’-based fluorescent probes for the detection of ROS/RNS and another analyte. **Pinkment-OH** is a core building block that enables easy functionalisation in order to produce a range of ‘AND’ based fluorescence probes for the detection of (ROS/RNS) and a second analyte. As proof of concept, we developed **Pinkment-OTBS** and **Pinkment-OAc** for the detection of ROS/RNS and F^- and esterase, respectively. Both **Pinkment-OTBS** and **Pinkment-OAc** were demonstrated to be successful ‘AND’-based fluorescence probes. However, both probes demonstrated slight reactivity to ROS/RNS alone, due to both silyl ether and acetate functional groups being unstable towards nucleophiles. We are now turning our attention to the attachment of palladium ion (Pd^{2+}) and mercury ion (Hg^{2+}) reactive triggers, previously developed by Ahn *et al.* and Koide *et al.*^{34,35} It is believed that the alkyl ethers will provide the appropriate stability towards ROS/RNS and Hg and Pd nanoparticles produce ROS in biological systems promoting apoptosis.^{36–39}

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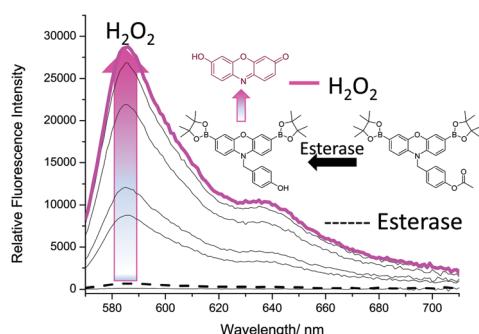


Fig. 5 Fluorescence spectra of **Pinkment-OAc** ($0.5 \mu\text{M}$) for the incubation (15 min) of porcine liver esterase, PLE (0.6 U) followed by incremental additions of H_2O_2 (0 – 1 mM) measurements were taken after 1 h . The data was collected in PBS (100% w/w H_2O) at $\text{pH} = 7.4$, 25°C . $\lambda_{\text{ex}} = 545$ – 20 nm . Dashed line represents esterase (0.6 U) addition only.

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Conflicts of interest

No conflicts of interest.

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