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A novel fluorescence-based scaffold was developed enabling the production of ‘AND’ based fluorescence probes - ROS/RNS and a second analyte
Biological processes often require more than one chemical species to achieve the desired outcome. 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 nucleus during the cell cycle to aid transcription factors binding to DNA\(^5\) 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.\(^12\)\(^-\)\(^17\),\(^18\)\(^-\)\(^21\). 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 \textit{et al.} \textbf{PR1}, with a free amino group attached (Fig.1). Boronates have well-known reactivity towards ONOO\(^{-}\) and hydrogen peroxide (H\(_2\)O\(_2\)),\(^25\) therefore we believed \textbf{PR1} could provide a suitable scaffold for the development of ‘AND’-based systems for the detection of ROS/RNS and an second analyte.\(^26\) In this work, we initially functionalised \textbf{PR1} with an additional ROS/RNS trigger, benzyl boronic acid ester, which afforded a selective ONOO\(^{-}\) probe known as \textit{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 phenoazaine using NaH and 4-bromobenzyl bromide to afford 1 in excellent yield (78 %). 1 was then subsequently dissolved in CHCl₃ 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 (B₂pin₂) 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⁺ in a buffer (See ESI – Scheme S5). and displayed excellent selectivity towards ONOO⁺ (See ESI -Fig. S2-4) To demonstrate the requirement of an ‘eliminating’ benzyl group on the free N-H, Pinkment-OH –demonstrates the requirement elimination to produce a free N-H.

For the synthesis of Pinkment-OH, the phenol of 4-hydroxybenzyl alcohol was selectively piv protected to afford 5 using pivaloyl chloride and NEt₃ in DCM. 5 was then converted to its corresponding bromide 6 using MsCl and NET₃ 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 CHCl₃ 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. S6-9). As proof of concept for ‘AND’-based pinkment sensors, we chose to synthesise Pinkment-OTBS and Pinkment-OAc (Fig. 2c and 2d), 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 %). We then evaluated the ability of Pinkment-OTBS to detect F⁻ ‘AND’ ONOO⁻ in a buffer solution (52% w/ w MeOH:H₂O). This MeOH/H₂O 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-butyrammonium 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\(^{-}\), a small increase in fluorescence intensity was observed on addition to Pinkment-OTBS (20 \(\mu\)M). However, like Fig.3, a large increase in fluorescence intensity was only observed after the subsequent addition of TBAF (0 – 10 \(\mu\)M). 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 \(\mu\)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 %). Due to the known ability of ONOO\(^{-}\) to react with carbonyls, its addition to Pinkment-OAc was avoided (See ESI – S15 and S16). Therefore, as proof of concept, we evaluated Pinkment-OAc with \(\text{H}_2\text{O}_2\) \(\text{AND}\) F. 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 \(\text{H}_2\text{O}_2\) (0 – 1 mM) led to a large increase in fluorescence.

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 fluorescent 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 (Pd2+) and mercury ion (Hg2+) reactive triggers, previously developed by Ahn et al. and Koide et al. It is believed that the alkyl ethers will provide stability towards ROS/RNS in addition it is known that Hg2+ and Pd nanoparticles produce ROS in biological systems promoting apoptosis.

Acknowledgements

We would like to thank the EPSRC and the University of Bath for funding for ACS, MLO, JEG for studentships. MW would like to thank the EPSRC for funding (i) EP/L016354/1 and CDT in Sustainable Chemical Technologies. TDJ wishes to thank the National Scientific Foundation of China (21722801), the Science and Technology Commission of Shanghai Municipality (15540723800) and the Shanghai Rising-Star Program (16QA1401400) for financial support. NMR characterisation facilities were provided through the Chemical Characterisation and Analysis Facility (CCAF) at the University of Bath (www.bath.ac.uk/ccaf). The EPSRC UK National Mass Spectrometry Facility at Swansea University is thanked for analyses. All data supporting this study are provided as supplementary information accompanying this paper (ESI†).

Conflicts of interest

No conflicts of interest

Notes and references