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Reaction-based indicator displacement assay (RIA) for the development of a triggered release system capable of biofilm inhibition†

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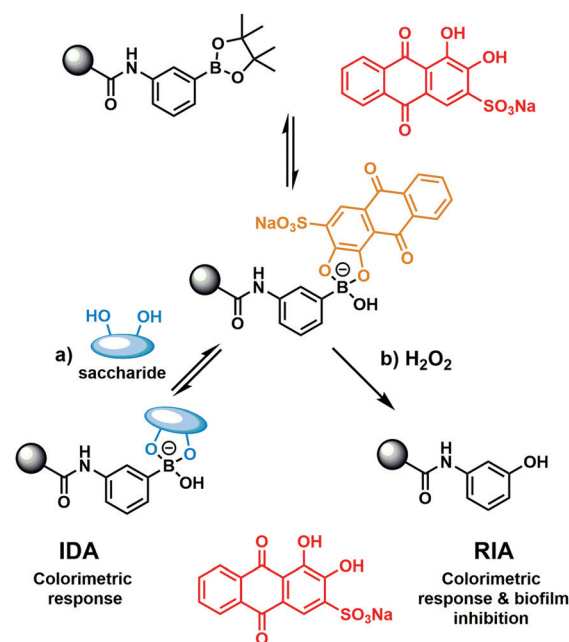
Here, a reaction-based indicator displacement hydrogel assay (RIA) was developed for the detection of hydrogen peroxide (H₂O₂) via the oxidative release of the optical reporter Alizarin Red S (ARS). In the presence of H₂O₂, the RIA system displayed potent biofilm inhibition for Methicillin-resistant *Staphylococcus aureus* (MRSA), as shown through an *in vitro* assay quantifying antimicrobial efficacy. This work demonstrated the potential of H₂O₂-responsive hydrogels containing a covalently bound diol-based drug for controlled drug release.

Dye displacement assays exploit the chemoselective reactivity of certain chemical moieties and the reversible binding of dye molecules to a specific receptor.¹ Such chemistry has begun to find widespread use with marked enhancement over traditional sensing assays.^{1–8} More complex systems containing multiple dyes also offer new paradigms for microarray development.⁹ Not surprisingly, dye displacement assays have been elegantly employed by a number of research groups. These constructs often rely on boronic acid systems as the receptor (host) subunit with a 1,2- and 1,3-diol guest.¹⁰

Previously our group has developed boronate-based hydrogel systems as dye displacement assays (borogel) for monosaccharide detection.^{11,12} As shown in Scheme 1, the commercially available 1,2-diol dye Alizarin Red S (ARS) was shown to successfully bind to the boronate hydrogel and result in a colour change from red to orange. Upon the addition of a monosaccharide, the

competitive displacement of ARS was observed with concomitant observation of an increase in absorption at 513 nm in solution (ARS wavelength).

Aryl boronic acids/boronate esters are well known to undergo hydrogen peroxide (H₂O₂)-mediated oxidation to form the corresponding phenol.¹³ This unique synthetic transformation has been exploited in organic synthesis and fluorescence sensing.¹³ We thus envisaged that modification of the previously developed ARS hydrogel bound indicator displacement assay (IDA) would yield a multimodal detection platform for the



Scheme 1 (a) Previously developed hydrogel bound dye displacement assay (IDA) for the detection of monosaccharides.^{11,12} (b) Present work – the development of a hydrogel bound reaction-based indicator displacement (RIA) assay for the detection of H₂O₂ and for the inhibition of MRSA biofilm formation.

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detection of H_2O_2 with attendant antimicrobial activity (Scheme 1).^{14–16} Here, we report the construction of a covalently incorporated ARS polyacrylamide hydrogel that undergoes oxidative activation in the presence of H_2O_2 to release ARS and afford a reaction-based indicator displacement assay (RIA).¹⁵ *In vitro* antibacterial assays with Methicillin-resistant *Staphylococcus aureus* (MRSA) indicated significant activity against biofilm formation for the combination of ARS and H_2O_2 .^{17,18}

In brief, phenylboronic acid (**PBA**) and benzoxaborole (**BOB**) acrylamide monomers were synthesised as previously reported.^{11,12} Polyacrylamide hydrogels were synthesised using water (60% w/w), acrylamide (38% w/w), methylene bisacrylamide (1% w/w), and **BOB** (1% w/w) or **PBA** (1% w/w). For qualitative purposes, hydrogel slabs containing **BOB** and **PBA** were immersed in 2.5×10^{-4} M ARS (PBS solution). Covalent incorporation was qualitatively measured *via* the observed colour change from red to orange, as measured against a blank hydrogel (Fig. S1 and S2, ESI[†]). For quantitative purposes, hydrogel cylinders (0.1 g) were immersed in a 2.5×10^{-4} M ARS solution (1 mL) and the UV-Vis absorption at 513 nm was measured over time. As shown in Fig. S3 and S4 (ESI[†]), a decrease in absorbance at 513 nm was observed, which corresponded to ARS uptake into the gel. After approximately 5 h, both **PBA** and **BOB** gels were saturated with ARS, which was indicated by no further decrease in absorption at 513 nm. Each gel was then placed into a solution of PBS (1 mL) to wash out any unbound ARS, which was shown by an increase in absorption at 513 nm (Fig. S5 and S6, ESI[†]). No further increase in absorption was observed after 3 h, which indicated the full release of any unbound ARS from each gel.

The prepared gels were then used to evaluate the response towards H_2O_2 . Each gel (**PBA** and **BOB**) was placed into a solution of PBS (1 mL) and then exposed to various concentrations of H_2O_2 (0–4 mM). As shown in Fig. 1 and 2, increasing

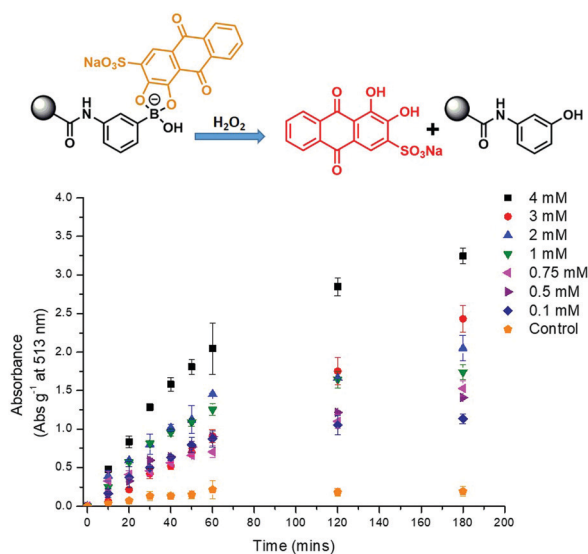


Fig. 1 UV-Vis absorption per gram of **PBA** upon addition of various concentrations of H_2O_2 (0–4 mM) in PBS (pH 7.4, PBS = 0.01 M) over time (minutes). Absorbance was measured at 513 nm at 25 °C. Error bars indicate standard deviation ($n = 3$).

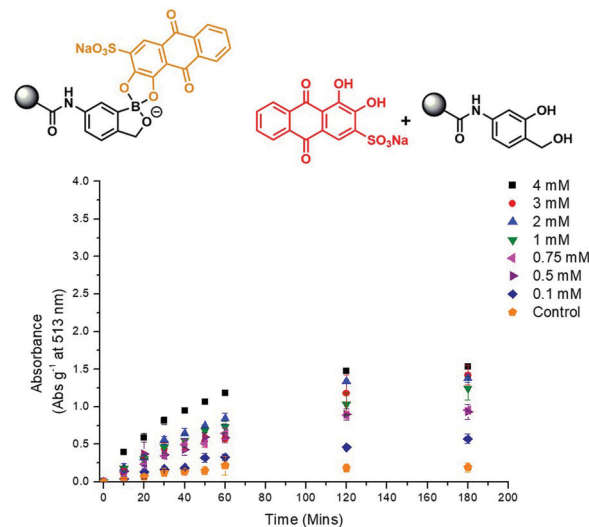


Fig. 2 UV-Vis absorption per gram of **BOB** upon addition of various concentrations of H_2O_2 (0–4 mM) in PBS (pH 7.4, PBS = 0.01 M) over time (minutes). Absorbance was measured at 513 nm at 25 °C. Error bars indicate standard deviation ($n = 3$).

the concentration of H_2O_2 led to an increased release of ARS from the borogels, as seen in the higher absorbance at 513 nm. Interestingly, the greatest sensitivity and ARS release was observed for the **PBA**-based gels, indicative of a greater reactivity towards H_2O_2 over the **BOB**-based gels (see Fig. S7 and S8, ESI[†]). This change in sensitivity is rationalised as the **BOB** moiety displays an enhanced binding affinity towards 1,2-diols due to an adjacent alkyl alcohol coordinating to the boron atom.^{12,19} Therefore, we believe the adjacent methyl alcohol retards oxidation of ARS bound-boronic acid by H_2O_2 .

Recent efforts by Lee and co-workers have demonstrated that alizarin ($10 \mu\text{g mL}^{-1}$) is an effective inhibitor of biofilm formation for three *Staphylococcus aureus* (*S. aureus*) strains and one *Staphylococcus epidermidis* strain.^{20,21} Biofilms are complex bacterial communities that can facilitate antibiotic resistance and impair wound healing.²² Hence, the development of new systems that can effectively treat or inhibit biofilm formation are highly desirable.

H_2O_2 is a commonly used disinfectant and antiseptic in wound care. Therefore, we explored the potential of this system in the development of a H_2O_2 -responsive hydrogel for the triggered release of ARS for biofilm inhibition against the three key stages of bacterial growth: lag, exponential and stationary. Due to the **PBA**-based gel displaying the greatest sensitivity towards H_2O_2 over **BOB**-based gels (see Fig. S7 and S8, ESI[†]), only **PBA** gels were evaluated for biofilm inhibition. Control studies showed that the minimum inhibitory concentration (MIC) of H_2O_2 was 3.5–7 mM for *Staphylococcus aureus* (*S. aureus*) H560 and MRSA252, 0.8–1.6 mM for *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa* PAO1) and 3–6 mM for *Escherichia coli* NCTC 10418 (*E. coli* NCTC 10418). Unfortunately, due to poor solubility, no MIC was determined for ARS against all the bacterial strains used in this study (see Fig. S9–S11, ESI[†]).^{23–25}

ARS was able to inhibit biofilm formation for *S. aureus* MRSA252 and *S. aureus* H560 at 100 μM when added during



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