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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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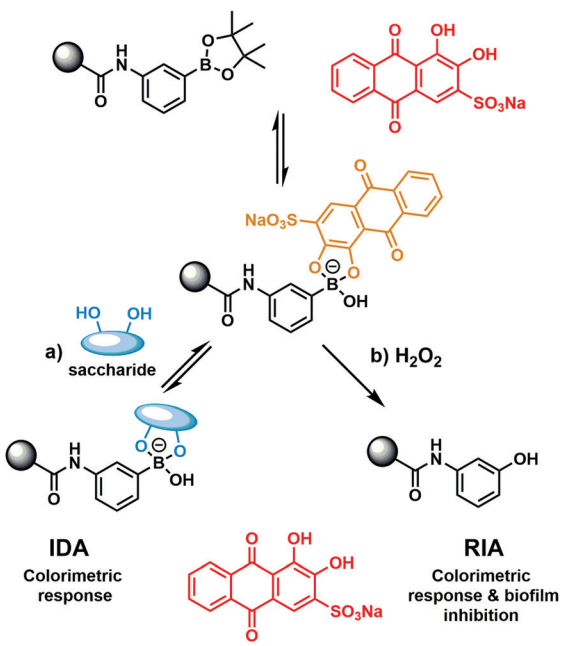
Here, a reaction-based indicator displacement hydrogel assay (RIA) was developed for the detection of hydrogen peroxide (H_2O_2) *via* the oxidative release of the optical reporter Alizarin Red S (ARS). In the presence of H_2O_2, the RIA system displayed potent biofilm inhibition for Methicillin-resistant *Staphylococcus aureus* (MRSA), as shown through an *in vitro* assay quantifying antimicrobial efficacy. This work demonstrated the potential of H_2O_2-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 (H_2O_2)-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 H_2O_2 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 absorbance 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

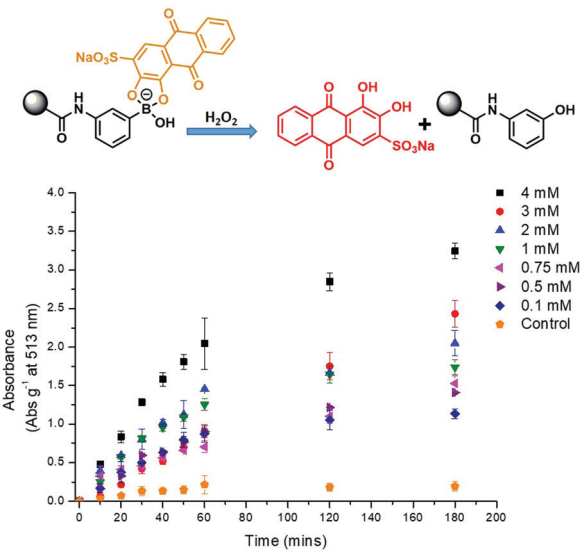

Fig. 1 UV-Vis absorption per gram of **PBA** upon addition of various concentrations of H_2O_2 (0–4 mM) in PBS (pH 7.4, PBS = 0.01 M) over time (minutes). Absorbance was measured at 513 nm at 25 °C. Error bars indicate standard deviation (n = 3).

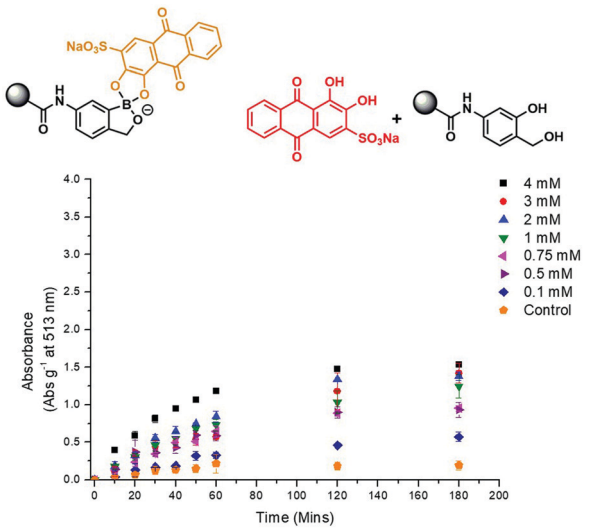

Fig. 2 UV-Vis absorption per gram of **BOB** upon addition of various concentrations of H_2O_2 (0–4 mM) in PBS (pH 7.4, PBS = 0.01 M) over time (minutes). Absorbance was measured at 513 nm at 25 °C. Error bars indicate standard deviation (n = 3).

the concentration of H_2O_2 led to an increased release of ARS from the borogels, as seen in the higher absorption 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 μg 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

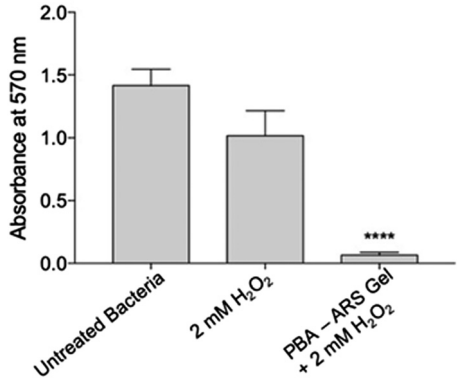

Fig. 3 Biofilm inhibition of *S. aureus* MRSA252 when treated with 2 mM H_2O_2 and solution containing released ARS from ARS-**PBA**-based gel using 2 mM H_2O_2 (3 h incubation). Experiments were repeated using three biological replicates, and error bars indicate standard deviation. Statistical significance of biofilm inhibition was assessed by performing a one-way ANOVA using GraphPad 7.0. ****$p \leq 0.001$ relative to untreated control.

the lag phase of growth (0 h), similar to other reports in the literature for Alizarin.[21] However, ARS was unsuccessful in the inhibition of *P. aeruginosa* PAO1 and *E. coli* NCTC 10418 biofilms at concentrations below 100 μM (see Fig. S14–S16, ESI†). Additionally, H_2O_2 inhibited biofilm formation, albeit at much higher concentrations, prevents growth at 5 mM for *S. aureus* MRSA252 and *S. aureus* H560, 10 mM for *E. coli* NCTC 10418, and 100 mM for *P. aeruginosa* PAO1 (see Fig. S17–S19, ESI†). H_2O_2 (2 mM) with ARS (50 μM) acted synergistically, effecting biofilm inhibition of *S. aureus* MRSA252 when added during the lag phase (Fig. S20, ESI†). Unfortunately, this combination was unable to inhibit biofilm formation at all other growth phases for each bacterial strain (Fig. S21 and S22, ESI†).

We next turned our attention towards the ability of the **PBA**-ARS hydrogel system to inhibit MRSA biofilm formation. Initial control experiments were carried out. **PBA**-ARS gel incubated in PBS solution for 3 h was shown to result in no biofilm inhibition, which indicates the requirement of H_2O_2 for ARS release and no off-target gel toxicity (Fig. S23, ESI†). Control ''blank'' acrylamide gels were subsequently tested to evaluate the requirement of the boronic acid units for the H_2O_2-mediated release of ARS. Following the usual ARS-loading protocol (see ESI†), acrylamide gels loaded with ARS (ARS uptake through passive diffusion – see Fig. S2, ESI†) were treated with H_2O_2. No biofilm inhibition was observed, which illustrated the requirements of the boronic acid units for H_2O_2-mediated release of ARS from the hydrogel (Fig. S24, ESI†). To capitalize upon the H_2O_2-mediated release of ARS from the boronic acid containing polyacrylamide hydrogel, **PBA**-based gels (0.1 g comprising of 2.5×10^{-4} M ARS) were incubated with H_2O_2 (2 mM) for 3 h to achieve maxiumum ARS release (*cf.* Fig. 1). The resultant ARS release was then applied to *S. aureus* MRSA252 at the lag phase. Remarkably, this resulted in complete biofilm inhibition (Fig. 3) thus demonstrating the potential of the **PBA**-based gels as a ''smart'' wound dressing.

In summary, we have developed a multimodal reaction-based indicator displacement (RIA) hydrogel assay for the detection of H_2O_2 with concomitant release of ARS for antimicrobial application. The greatest reactivity towards H_2O_2 was observed for the **PBA**-based gel compared to the **BOB**-based gel, attributed to attenuated reactivity of the cyclic **BOB**-boronate ester. In addition, the antimicrobial efficacy of each assay component was evaluated with the aim of developing a ''triggered release'' antimicrobial hydrogel. ARS was discovered to be a potent biofilm inhibitor in combination with hydrogen peroxide against *S. aureus* MRSA252, with the ARS loaded **PBA**-based gel successfully inhibiting biofilm formation. These results lead us to suggest that **PBA**-based gels, in combination with an early bacterial detection system for a MRSA biomarker, might find use as a ''smart'' wound dressing capable of preventing MRSA biofilm formation.[26]

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Conflicts of interest

No conflicts of interest.

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