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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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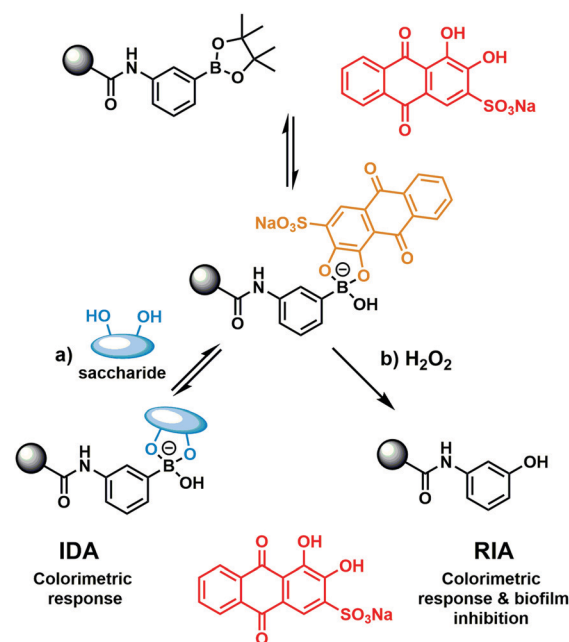
Here, a reaction-based indicator displacement hydrogel assay (RIA) was developed for the detection of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) via the oxidative release of the optical reporter Alizarin Red S (ARS). In the presence of H<sub>2</sub>O<sub>2</sub>, the RIA system displayed potent biofilm inhibition for Methicillin-resistant *Staphylococcus aureus* (MRSA), as shown through an *in vitro* assay quantifying antimicrobial efficacy. This work demonstrated the potential of H<sub>2</sub>O<sub>2</sub>-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.<sup>1</sup> Such chemistry has begun to find widespread use with marked enhancement over traditional sensing assays.<sup>1–8</sup> More complex systems containing multiple dyes also offer new paradigms for microarray development.<sup>9</sup> 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.<sup>10</sup>

Previously our group has developed boronate-based hydrogel systems as dye displacement assays (borogel) for monosaccharide detection.<sup>11,12</sup> 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<sub>2</sub>O<sub>2</sub>)-mediated oxidation to form the corresponding phenol.<sup>13</sup> This unique synthetic transformation has been exploited in organic synthesis and fluorescence sensing.<sup>13</sup> 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.<sup>11,12</sup> (b) Present work – the development of a hydrogel bound reaction-based indicator displacement (RIA) assay for the detection of H<sub>2</sub>O<sub>2</sub> and for the inhibition of MRSA biofilm formation.

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detection of  $\text{H}_2\text{O}_2$  with attendant antimicrobial activity (Scheme 1).<sup>14–16</sup> Here, we report the construction of a covalently incorporated ARS polyacrylamide hydrogel that undergoes oxidative activation in the presence of  $\text{H}_2\text{O}_2$  to release ARS and afford a reaction-based indicator displacement assay (RIA).<sup>15</sup> *In vitro* antibacterial assays with Methicillin-resistant *Staphylococcus aureus* (MRSA) indicated significant activity against biofilm formation for the combination of ARS and  $\text{H}_2\text{O}_2$ .<sup>17,18</sup>

In brief, phenylboronic acid (**PBA**) and benzoxaborole (**BOB**) acrylamide monomers were synthesised as previously reported.<sup>11,12</sup> 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  $2.5 \times 10^{-4}$  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<sup>†</sup>). For quantitative purposes, hydrogel cylinders (0.1 g) were immersed in a  $2.5 \times 10^{-4}$  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<sup>†</sup>), 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<sup>†</sup>). 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  $\text{H}_2\text{O}_2$ . Each gel (**PBA** and **BOB**) was placed into a solution of PBS (1 mL) and then exposed to various concentrations of  $\text{H}_2\text{O}_2$  (0–4 mM). As shown in Fig. 1 and 2, increasing

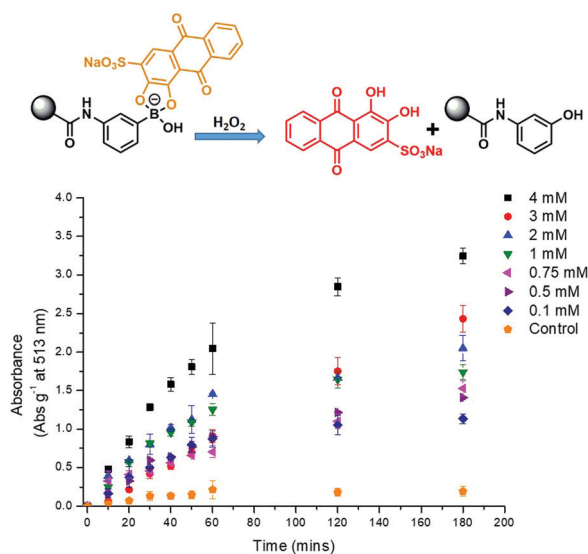


Fig. 1 UV-Vis absorption per gram of **PBA** upon addition of various concentrations of  $\text{H}_2\text{O}_2$  (0–4 mM) in PBS (pH 7.4, PBS = 0.01 M) over time (minutes). Absorbance was measured at 513 nm at 25 °C. Error bars indicate standard deviation ( $n = 3$ ).

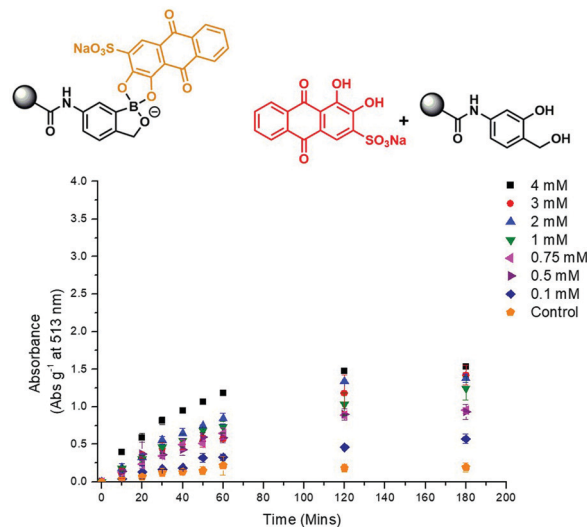


Fig. 2 UV-Vis absorption per gram of **BOB** upon addition of various concentrations of  $\text{H}_2\text{O}_2$  (0–4 mM) in PBS (pH 7.4, PBS = 0.01 M) over time (minutes). Absorbance was measured at 513 nm at 25 °C. Error bars indicate standard deviation ( $n = 3$ ).

the concentration of  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  over the **BOB**-based gels (see Fig. S7 and S8, ESI<sup>†</sup>). 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.<sup>12,19</sup> Therefore, we believe the adjacent methyl alcohol retards oxidation of ARS bound-boronic acid by  $\text{H}_2\text{O}_2$ .

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.<sup>20,21</sup> Biofilms are complex bacterial communities that can facilitate antibiotic resistance and impair wound healing.<sup>22</sup> Hence, the development of new systems that can effectively treat or inhibit biofilm formation are highly desirable.

$\text{H}_2\text{O}_2$  is a commonly used disinfectant and antiseptic in wound care. Therefore, we explored the potential of this system in the development of a  $\text{H}_2\text{O}_2$ -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  $\text{H}_2\text{O}_2$  over **BOB**-based gels (see Fig. S7 and S8, ESI<sup>†</sup>), only **PBA** gels were evaluated for biofilm inhibition. Control studies showed that the minimum inhibitory concentration (MIC) of  $\text{H}_2\text{O}_2$  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<sup>†</sup>).<sup>23–25</sup>

ARS was able to inhibit biofilm formation for *S. aureus* MRSA252 and *S. aureus* H560 at 100  $\mu\text{M}$  when added during





**Fig. 3** Biofilm inhibition of *S. aureus* MRSA252 when treated with 2 mM H<sub>2</sub>O<sub>2</sub> and solution containing released ARS from ARS-**PBA**-based gel using 2 mM H<sub>2</sub>O<sub>2</sub> (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.<sup>21</sup> However, ARS was unsuccessful in the inhibition of *P. aeruginosa* PAO1 and *E. coli* NCTC 10418 biofilms at concentrations below 100  $\mu\text{M}$  (see Fig. S14–S16, ESI<sup>†</sup>). Additionally, H<sub>2</sub>O<sub>2</sub> 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<sup>†</sup>). H<sub>2</sub>O<sub>2</sub> (2 mM) with ARS (50  $\mu\text{M}$ ) acted synergistically, effecting biofilm inhibition of *S. aureus* MRSA252 when added during the lag phase (Fig. S20, ESI<sup>†</sup>). Unfortunately, this combination was unable to inhibit biofilm formation at all other growth phases for each bacterial strain (Fig. S21 and S22, ESI<sup>†</sup>).

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<sub>2</sub>O<sub>2</sub> for ARS release and no off-target gel toxicity (Fig. S23, ESI<sup>†</sup>). Control “blank” acrylamide gels were subsequently tested to evaluate the requirement of the boronic acid units for the H<sub>2</sub>O<sub>2</sub>-mediated release of ARS. Following the usual ARS-loading protocol (see ESI<sup>†</sup>), acrylamide gels loaded with ARS (ARS uptake through passive diffusion – see Fig. S2, ESI<sup>†</sup>) were treated with H<sub>2</sub>O<sub>2</sub>. No biofilm inhibition was observed, which illustrated the requirements of the boronic acid units for H<sub>2</sub>O<sub>2</sub>-mediated release of ARS from the hydrogel (Fig. S24, ESI<sup>†</sup>). To capitalize upon the H<sub>2</sub>O<sub>2</sub>-mediated release of ARS from the boronic acid containing polyacrylamide hydrogel, **PBA**-based gels (0.1 g comprising of  $2.5 \times 10^{-4}$  M ARS) were incubated with H<sub>2</sub>O<sub>2</sub> (2 mM) for 3 h to achieve maximum 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<sub>2</sub>O<sub>2</sub> with concomitant release of ARS for antimicrobial application. The greatest reactivity towards H<sub>2</sub>O<sub>2</sub> 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.<sup>26</sup>

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## Conflicts of interest

No conflicts of interest.

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