





Scheme 1 Synthesis of substrate 1.

Scheme 2 Structure of substrate 1 and the proposed mechanism of  $\beta$ -Gal catalysed breakdown with subsequent release of ferrocenylamine 3.

peaks being approximately 250 mV and were completely resolved which allowed for the peaks to be integrated independently (Fig. 1). The conversion of substrate 1 was calculated using eqn (1).

$$\text{Conversion (\%)} = \frac{\left( \int 3 \right)}{\left( \int 3 + \int 1 \right)} \times 100 \quad (1)$$



Fig. 1 Differential pulse voltammogram obtained for substrate 1 (0.1 mM) and ferrocenylamine 3 (0.1 mM) in 50 mM pH 7 Tris buffer.

Substrate 1 (0.1 mM) was initially subjected to varying concentrations of  $\beta$ -Gal in Tris buffer (pH 9, 50 mM) at room temperature (21 °C) and subjected to electrochemical analysis every 3 minutes for 60 minutes (see ESI, Fig. S2†). The voltammogram of each sample was then integrated and conversions calculated using eqn (1). At high  $\beta$ -Gal concentrations, 5 and 10 U mL<sup>-1</sup>,<sup>20</sup> quantitative conversion was observed within 18 minutes, and 60 minutes for 1 U mL<sup>-1</sup>. Pleasingly, no background substrate hydrolysis was observed in the absence of  $\beta$ -Gal allowing for a  $\beta$ -Gal concentration as low as 0.1 U mL<sup>-1</sup> to be detected within 60 minutes. Intriguingly, the presence of a third peak was present in the voltammogram at 390 mV (see ESI, Fig. S3†). This was confirmed to be 4-hydroxybenzyl alcohol 10, formed when quinone methide 8, produced as a by-product from the result of self-immolation, reacts with water or hydroxide (Scheme 3).<sup>21</sup> Despite being produced in an equimolar concentration as ferrocenylamine 3, the peak obtained was still significantly smaller than the ferrocene





**Scheme 3** Proposed formation of 4-hydroxybenzyl alcohol from quinone methide.

peaks showing the clear benefit of using the organometallic redox label compared with other commonly used organic ones.

Next, the effect of pH was investigated (see ESI, Fig. S4†). A  $\beta$ -Gal concentration of  $1 \text{ U mL}^{-1}$  was chosen as it allowed for both positive and negative effects due to the pH to be observed. At pH 8 there was a significant increase in the rate of conversion, with quantitative conversion observed in under 30 minutes. There was a marginal increase in rate from pH 8 to pH 7, but importantly, at the lower pH the presence of the third peak was suppressed, presumably due to protonation of the electrochemically active phenolate ion **9**. The suppression of the peak produced a cleaner voltammogram, allowing for more accurate conversion to be calculated and as a result, pH 7 was used moving forward.

According to previous literature, the optimum working temperature for  $\beta$ -Gal is  $37^\circ\text{C}$ .<sup>22</sup> A lower  $\beta$ -Gal concentration was chosen, specifically  $0.1 \text{ U mL}^{-1}$  in Tris buffer (pH 7, 50 mM) (see ESI, Fig. S5†), to allow for changes in the rate of conversion to be noticeable. Interestingly, increasing the temperature from room temperature had minimal effect on the rate of conversion, and above  $37^\circ\text{C}$  the rate was retarded. At  $57^\circ\text{C}$  negligible conversion was observed due to denaturing of the enzyme. Substrate **1**, however, remained stable to hydrolysis even at elevated temperatures, exhibiting the high stability of the substrate. With no improved rate of conversion, the assays were continued to be conducted at room temperature ( $21^\circ\text{C}$ ).

The concentration of substrate **1** in the assay was then screened, utilising a  $\beta$ -Gal concentration of  $1 \text{ U mL}^{-1}$  (Fig. 2). Increasing the probe concentration to  $0.25 \text{ mM}$  from  $0.1 \text{ mM}$  had minimal effects on the rate of conversion with quantitative conversion still observed within 24 minutes. However, increasing the concentration further showed no discernible increase in the rate of reaction. When the substrate concentration was decreased to  $0.05 \text{ mM}$ , the reduced current observed was susceptible to artefacts on the voltammogram affecting accurate conversion calculations, which were unavoidable when using disposable screen-printed carbon graphite electrode cells. At concentrations above  $0.1 \text{ mM}$ , additional sample manipulation, *via* serial dilutions, was required before analysis, due to overloading of the electrodes, and therefore, an optimal substrate concentration of  $0.1 \text{ mM}$  was chosen.

The final condition to be optimised was the buffer system used. Other common  $\beta$ -Gal buffer systems such as potassium phosphate buffer (50 mM, pH 7),<sup>3</sup> and Z-buffer (50 mM, pH 7),



**Fig. 2** Conversion of the substrate **1** to the product after addition of  $\beta$ -Gal ( $1 \text{ U mL}^{-1}$ ) using different concentrations of the substrate at room temperature in Tris buffer (pH 7, 50 mM). Error bars represent the standard deviation where  $n = 3$ .

were tested. Z-buffer exhibited significant background signal, potentially due to the thiols present in Z-buffer, that obscured the peaks and prevented accurate electrochemical analysis. In phosphate buffer the stability of ferrocenylamine **3** was diminished with a second peak forming at a higher oxidation potential, assumed to be an electroactive by-product from ferrocenylamine decomposition.<sup>23</sup> Tris buffer (pH 7) was therefore chosen as this maintained a low background and ensured ferrocenylamine stability, and the effect of buffer concentration was investigated using a  $\beta$ -Gal concentration of  $1 \text{ U mL}^{-1}$  (see ESI, Fig. S6†). In unbuffered solution, the rate of conversion was significantly improved compared to 50 mM Tris buffer solution, however, the voltammogram peaks were shifted to a lower oxidation potential. At this lower potential, the presence of voltammogrammatic artefacts prevented accurate detection. At 25 mM Tris buffer concentration, a comparable rate of conversion to 50 mM Tris buffer concentration with quantitative conversion observed in 18 minutes. Further analysis showed that  $\beta$ -Gal was unstable at the lower buffer concentration, with increases in conversions stopping after 18 minutes. Increasing the concentration of the Tris buffer above 50 mM prevented accurate ratiometric analysis as we suspect the reduced stability of ferrocenylamine **3** resulted in unreliable peak integrations. Therefore, a Tris buffer (pH 7, 50 mM) was selected as the optimal buffer concentration.

With optimal conditions obtained, the sensitivity of  $\beta$ -Gal was tested (Fig. 3). In the optimised conditions, there was no background hydrolysis observed, allowing for detection of low  $\beta$ -Gal concentration of  $0.1 \text{ U mL}^{-1}$  within 60 minutes. Utilising pseudo-first order kinetics (see ESI, Fig. S7†), a rate constant of  $2.91 \times 10^{-3} \text{ s}^{-1}$  was calculated for a  $\beta$ -Gal concentration of  $1 \text{ U mL}^{-1}$ . At higher concentrations of 10 and  $5 \text{ U mL}^{-1}$ , quantitative conversions were exhibited within just 6 minutes, and at a low concentration of  $0.25 \text{ U mL}^{-1}$ , a  $73 \pm 5\%$  conversion was achieved within 60 minutes, with an observed rate constant of  $0.044 \times 10^{-3} \text{ s}^{-1}$ . The small error bars afforded, indicate the good reliability showing the benefit of using ratiometric electrochemical analysis.





Fig. 3 Conversion of the substrate **1** (0.1 mM) to the product after addition of varying concentration of  $\beta$ -Gal in Tris buffer (pH 7, 50 mM) at room temperature. Error bars represent the standard deviation where  $n = 3$ .

It has been previously shown that  $\beta$ -Gal has a large tolerance to the aglycon, as long as the D-galactose moiety remains untouched.<sup>24</sup> To further explore how the sterics of the substrate could impact  $\beta$ -Gal activity, substrate **11** was synthesised (Fig. 4), and tested utilising the optimal conditions (see ESI, Fig. S8†). Comparatively, substrate **11**, was significantly slower than substrate **1**. At low  $\beta$ -Gal concentration of 0.1 U mL<sup>-1</sup> a conversion of  $12 \pm 6\%$  within 60 minutes, compared to a conversion of  $16 \pm 1\%$  for substrate **1**. The calculated rate constants of  $0.04 \times 10^{-3} \text{ s}^{-1}$  and  $0.06 \times 10^{-3} \text{ s}^{-1}$  for substrate **11** and **1** respectively (see ESI, Fig. S9†), show only a small difference in rate between the two regioisomers. This difference in rate of hydrolysis, is more significant at 1 U mL<sup>-1</sup> concentrations, where the rate constant for substrate **1**,  $2.91 \times 10^{-3} \text{ s}^{-1}$ , is an order of magnitude higher than for substrate **11**,  $0.14 \times 10^{-3} \text{ s}^{-1}$ . The increased steric bulk around the anomeric position in substrate **11** inhibits the rate of hydrolysis, indicating the suitability of substrate **1**.

In conclusion, we have developed a new ferrocene-based electrochemical substrate for the detection of  $\beta$ -galactosidase activity. The substrate with a D-galactopyranoside trigger was synthesised and was distinguishable from the product electrochemically *via* differential pulse voltammetry. The substrate was shown to be stable to background hydrolysis, was demonstrated to be sensitive to low concentrations of  $\beta$ -galactosidase and shown to be both reproducible and reliable which makes  $\beta$ -galactosidase sensing applicable to electrochemical point-of-care biosensors.



Fig. 4 Substrate **11**.

## Conflicts of interest

There are no conflicts to declare.

## Notes and references

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- U mL<sup>-1</sup> = Units per millilitre. Where one unit will hydrolyse 1.0 micromole of O-nitrophenyl- $\beta$ -D-galactoside to O-nitrophenol and D-galactose per minute at pH 7.3 at 37 °C.



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