A novel ferrocene-based substrate for the ratiometric electrochemical detection of β-galactosidase was designed and synthesised. It was demonstrated to be an excellent electrochemical substrate for β-Gal detection with sensitivity as low as 0.1 UmL⁻¹.

β-galactosidase (β-Gal, EC 3.2.1.23) is a prominent enzyme used biologically as a reporter gene as it has been well characterised and demonstrates excellent stability.¹ The low level of background substrate hydrolysis and ready availability makes β-Gal an attractive enzyme label within enzyme-linked immunoassortment assays (ELISAs).² It can also be used in heavy-metal ion detection,³ and rapid enzyme assays have been used in the detection of coliform and E. Coli in waste water treatment.⁴ β-Gal is typically detected either chromogenically, using ortho-nitrophenyl-β-galactoside (ONPG)⁵, or 5-bromo-4-chloro-3-indoyl-β-β-o-galactopyranoside (X-Gal).⁶ or is detected fluorometrically using fluorescein-di-β-β-o-galactopyranoside (FDG).⁷ Lanthanide based coumarins have also been utilised as a luminescent probe in the detection of β-Gal.⁸ Optical substrates however, are limited by the use of expensive equipment, non-linear fluorescence, and potentially high levels of background signal.¹ The development of electrochemical enzyme substrates allows for the direct conversion of a biochemical recognition event into an electrical signal enabling facile biosensor integration within a handheld device.⁹ 4-aminophenyl-β-β-o-galactoside (PAPG) is standardly used as an electrochemical substrate for β-Gal, but high background signals prevents accurate analysis at low enzyme concentrations.⁹ A modified PAPG-style substrate, 4-methoxynaphenyl-β-β-o-galactosidase (4-MPGal) has a negligible background signal, but the unfavourable use of modified graphene oxide electrodes is currently for β-Gal.

Ferrocene-based electrochemical sensing is a continuing interest within our research group as it enables an inexpensive and convenient way to monitor enzyme activity.¹⁰ Utilising trigger-

The development of ratiometric probes has overcome the issues of reproducibility, by the ability to obtain direct conversions, minimising both sampling errors and systematic errors from instrument variation.¹¹ Ferrocene-based ratiometric chemodosimeters for enzyme detection have previously been reported for alkaline phosphatase,¹² and glucose oxidase,¹³ but none currently for β-Gal.

β-Gal catalysed breakdown with subsequent release of electrochemical probes,¹¹¹² with ferrocene derivatives widely used in biological systems due to their stability in aerobic and aqueous environments.¹³ The development of ratiometric probes has overcome the issues of reproducibility, by the ability to obtain direct conversions, minimising both sampling errors and systematic errors from instrument variation.¹¹ Ferrocene-based ratiometric chemodosimeters for enzyme detection have previously been reported for alkaline phosphatase,¹² and glucose oxidase,¹³ but none currently for β-Gal.

Due to its facile oxidation potential and excellent synthetic utility, ferrocene is implemented as the redox-active moiety in
We designed ferrocenylcarbamoylphenyl-β-D-galactosidase 1 as a ratiometric electrochemical substrate for β-Gal. Previously, ferrocenylamine 3 has been shown to be oxidised at a lower potential than carbamate derivatives. Substrate 1 would have a higher oxidation potential than 3 making them electrochemically distinguishable, allowing for the ratiometric electrochemical analysis of β-Gal activity. We propose that in the presence of β-Gal, hydrolysis at the anomeric position would afford an unstable phenolate intermediate 2. 1,6-elimination would follow releasing ferrocenylamine 3, quinone methide and CO₂ (Scheme 1).

The synthesis of substrate 1 (Scheme 2) started from the commercially available β-D-galactose pentaacetate which was converted to benzyl alcohol 5 (See ESI† for synthesis). Ferrocenoyl azide 6 was synthesised according to a literature procedure, and then coupled to the benzyl alcohol via a Curtius rearrangement. Zemplén deacetylation of 7 afforded the desired substrate 1 in a 16 % overall yield. Once synthesised, substrate 1 was found to be a bench stable orange solid with no observable degradation over several months at room temperature. Substrate 1 was also stable to hydrolysis in tris buffer (pH 7, 50 mM) solutions for several weeks at room temperature (See ESI†, Figure S1). The electrochemical behaviour of substrate 1 was tested via differential pulse voltammetry (DPV) and compared to ferrocenylamine 3.

As expected, substrate 1 had a higher oxidation potential than ferrocenylamine 3, with the difference between the two peaks being approximately 250 mV and were completely resolved which allowed for the peaks to be integrated independently (Figure 1). The conversion of substrate 1 was calculated using eqn (1).

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\text{Conversion} (\%) = \frac{\int E_3}{\int E_3 + \int E_1} \times 100
\]  

Substrate 1 (0.1 mM) was initially subjected to varying concentrations of β-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†, Figure S2). The voltammogram of each sample was then integrated and conversions calculated using eqn (1). At high β-Gal concentrations, 5 and 10 U mL⁻¹, quantitative conversion was observed within 18 minutes, and 60 minutes for 1 U mL⁻¹. Pleasingly, no background substrate hydrolysis was observed in the absence of β-Gal allowing for a β-Gal concentration as low as 0.1 U mL⁻¹ to be detected within 60 minutes. Intriguingly, the...
The concentration of substrate 1 in the assay was then screened, utilising a β-Gal concentration of 1 U mL⁻¹ (Figure 2). Increasing the probe concentration to 0.25 mM from 0.1 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 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 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 mM was chosen.

The final condition to be optimised was the buffer system used. Other common β-Gal buffer systems such as potassium phosphate buffer (50 mM, pH 7), and Z-buffer (50 mM, pH 7), 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.21 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 an β-Gal concentration of 1 U mL⁻¹ (see ESI, Figure S6). In buffered 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 voltammogramatic 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 β-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 β-Gal was tested (Figure 3). In the optimised conditions, there was no background hydrolysis observed, allowing for detection of low β-Gal concentration of 0.1 U mL⁻¹ within 60 minutes. Utilising pseudo-first order kinetics (See ESI†, Figure S7), a rate constant of 2.91 × 10⁻² s⁻¹ was calculated for a β-Gal concentration of 1 U mL⁻¹. At higher concentrations of 10 and 5 U mL⁻¹, quantitative conversions were exhibited within just 6 minutes, and at a low concentration of 0.25 U mL⁻¹, a 73±5 % conversion was achieved within 60 minutes, with an observed rate constant of 0.044 × 10⁻² s⁻¹.

Figure 2 – Conversion of the substrate 1 (0.1 mM) to the product after addition of β-Gal in tris buffer (pH 7, 50 mM) at room temperature. Error bars represent the standard deviation where n = 3.

Figure 3 – Conversion of the substrate 1 to the product after addition of β-Gal (1 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.
The calculated rate constants of 0.04 × 10⁻³ s⁻¹ and 0.06 × 10⁻³ s⁻¹ for substrate 11 and 1 respectively (see ESI, Figure 59), show only a small difference in rate between the two isomeroids. This difference in rate of hydrolysis, is more significant at 1.0 Umol⁻¹ concentrations, where the rate constant for substrate 1, 2.91 × 10⁻³ s⁻¹, is an order of magnitude higher than for substrate 11, 0.14 × 10⁻³ 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 β-galactosidase activity. The substrate with a β-galactopyranoside trigger was synthesised and was shown to be both reproducible and reliable which makes β-galactosidase sensing applicable to electrochemical point-of-care biosensors.

Notes and references
5. J. Kiernan, Biotech. Histochem., 2007, 82, 73.