Introduction

Hydrogen peroxide (H$_2$O$_2$) is an important small molecule used in many industrial applications, such as paper-bleaching and in the manufacture of disinfectants and explosives. In nature, it has been recently established that H$_2$O$_2$ is involved within a number of crucial roles related to cell signalling. Abnormally high concentrations of H$_2$O$_2$ can be cytotoxic to cells but more significantly, however, it is a precursor to the indiscriminately reactive hydroxyl radical (OH). This, along with other reactive oxygen species (ROS), have been shown to contribute to oxidative stress, a major factor in the onset of various diseases. Additionally, H$_2$O$_2$ is produced as a byproduct in numerous enzyme-catalysed processes such as the aerobic oxidation of alcohols, urates, amino acids, and certain carbohydrates, including glucose. Moreover, H$_2$O$_2$ has become a popular signal propagator within signal amplification methodologies used to enhance the sensitivity of diagnostic assays. Since H$_2$O$_2$ can be used as a reactive biomarker for effective disease diagnosis, and disease monitoring, as well as to infer the presence of trace amounts of explosives, there is therefore a significant interest in the development of accurate and reliable hydrogen peroxide detection methods.

Results and discussion

Optimisation of probe structure

H$_2$O$_2$ is itself electrochemically active but only at a high oxidation potential. In order to decrease this overpotential and increase specificity, previous electrochemical methods for the
detection of H₂O₂ have focussed upon modifying electrodes using advanced materials. However, the use of modified electrodes is unfeasible at the point-of-need setting as they can be expensive and difficult to manufacture, and are currently unable to be easily mass produced. To obtain a ratiometric detection method at facile oxidation potentials, without the need for modified electrodes, we looked to begin our investigation by utilising ferrocene as a redox-active label. Also, by coupling a boronic acid trigger moiety into the design of the ferrocene-based probe, we hypothesised that oxidation and subsequent hydrolysis of the boronic acid trigger would only occur selectively in the presence of H₂O₂ allowing for an irreversible reaction-based detection method to be achieved (Fig. 1).²⁷

Towards this end, compounds 1–5 were designed and synthesised (see ESI†) with the aim of identifying the optimum structural criteria needed to attain a selective ratiometric electrochemical detection method for H₂O₂ (Fig. 2). Since different self-immolative linkers exhibit different elimination kinetics in response to H₂O₂,²⁸ compounds 1–3 were designed to determine the linker that delivered the quickest release of an electron-rich ferrocene reporter unit. Specifically, compound 1 utilised the commonly employed p-benzyl carbamate linker (the definitive structure of which was confirmed by X-ray crystallography (Fig. 3)),²⁹ compound 2 contained a recently-described allyl carbamate linker,⁰ and compound 3 contained no linker at all. Compounds 4–5, structural analogs of compounds 1–2 without the boronic acid trigger, were designed to determine and confirm that the specificity of the reactivity towards H₂O₂ arises from the boronic acid ester trigger unit.

With probes 1–5 in hand, 100 μM concentrations of the probes in pH 8.1 tris(hydroxymethyl)methylamine (Tris) buffer were exposed to a solution containing 1 mM (10 equivalents) of H₂O₂ and the assay analysed after 20 minutes using differential pulse voltammetry (DPV). When the reaction assays regarding probes 1 and 2 were analysed, complete disappearance of the oxidation peak corresponding to the substrate and a new peak, at a significantly lower oxidation potential, was observed (Fig. 4). This peak was found to be at an identical oxidation potential as that of aminoferrocene 6, which was synthesised separately according to a literature procedure.¹¹ As such, this observation of a ratiometric oxidation potential as that of aminoferrocene 6, which was synthesised separately according to a literature procedure.¹¹ As such, this observation of a ratiometric electrochemical detection method can be attributed to the as-designed H₂O₂-mediated oxidation of the boronic acid moiety to its corresponding alcohol, which is followed by subsequent linker elimination and carbamate decarboxylation to release aminoferrocene 6.

‡ Crystal data for compound 1. C₁₄H₂₈NO₄FeB, M = 461.13, triclinic, space group P1 (no. 2), a = 9.8839(4), b = 9.9233(4), c = 13.1134(5) Å, α = 109.219(4), β = 93.721(3), γ = 115.017(4)°, U = 1068.78(7) Å³, Z = 2, T = 150 K, μ(Cu Kα) = 5.914 mm⁻¹, D = 1.433 g cm⁻³, 10333 reflections measured [10.32° ≤ 2θ ≤ 143.94°], R = 0.1112 (all data). CCDC 1528331 contains the supplementary crystallographic data for 1.
The analysis of the peroxide assay with probe 3 also revealed the disappearance of the probe peak but no expected peak at a lower oxidation potential was seen. This could be due to H₂O₂-mediated oxidation of the ferroceneboronic acid probe 3 to hydroxyferrocene 7 but due to the known instability of 7 in aqueous conditions, rapid decomposition of the product occurred. When probes 4 and 5 were exposed to H₂O₂, no peak corresponding to aminoferrocene 6 was observed showing that the carbamate functionality is stable towards alkaline peroxide and confirms that the boronic acid ester trigger moieties are essential for achieving peroxide selectivity within reaction-based assays.

Two separate equimolar solutions (100 μM total ferrocene concentration) containing probe 1 and aminoferrocene 6 (Fig. 5), and probe 2 and 6 (see ESI†) were analysed by DPV. Differences in oxidation potentials (ΔE_{ox}) between probe and reporter were found to be 268 mV (±14 mV) and 232 mV (±4 mV) respectively. These values can be considered more than sufficient to deliver a ratiometric detection method as an ideal difference in oxidation potential between substrate and product has previously been suggested to be between 100–200 mV to ensure both compounds are not oxidised at the same oxidation potential. This allows for, through integration of the peaks on the voltammogram, reaction conversions to be calculated via:

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\text{Conv.} \, (\%) = \frac{\int 6}{\int 6 + \int 1} \times 100.
\]

In order to determine which probe showed the greatest reactivity towards H₂O₂, as well as the quickest elimination kinetics, 100 μM concentrations of the probes were exposed to differing concentrations of H₂O₂ and monitored over time through ratiometric electrochemical analysis (Fig. 6 and ESI†). Pleasantly, all concentrations of 1 eq. H₂O₂ and higher afforded positive production of compound 6. Importantly, a <2% background rate was also observed exemplifying the excellent stability of probes with carbamate linkages in aqueous buffers. This allowed a 100 μM concentration of H₂O₂ to be determined from the background. Despite improved solubility in the aqueous medium, probe 1 was taken forward over probe 2 for further optimisation due to its increased reactivity and the greater ΔE_{ox} observed between substrate and product, which is likely caused by its increased hydrophobicity.

**Assay optimisation**

In order to improve the sensitivity of the assay, a number of reaction parameters were investigated. First, a range of different alkaline buffers were tested to determine if the buffer type had any effect on the reactivity of the probe. Unfortunately, any diversion away from the originally chosen Tris buffer either led to the appearance of significant artefacts.

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**Fig. 4** Differential pulse voltammogram (DPV) overlays of probe 1 after exposure to 10 eq. of H₂O₂.

**Fig. 5** DPV of probe 1 (50 μM) and aminoferrocene 6 (50 μM) in 50 mM pH 8.1 Tris buffer.

**Fig. 6** Conversion of probe 1 (100 μM) to aminoferrocene 6 in 50 mM pH 8.1 Tris buffer in the presence of various concentrations of H₂O₂.
on the voltammogram or caused substantial decomposition of the ferrocene probes. As such, ideal like-for-like comparisons could not be made since accurate peak integrations, and therefore precise reaction conversions, were unable to be obtained.

The pH of the assay was next to be studied since peroxide reactivity and linker elimination can both be affected through changes in pH. The useful working pH range of Tris buffer is 7–9 and as such, the assay was conducted at various pH values within this range (Fig. 7). As expected, decreasing the pH of the assay medium led to a significant reduction in conversion due to the shift in equilibrium from the hydroxide anion (HOO⁻) to its neutral species (H₂O₂) and also due to the increase in stability of the resultant phenol intermediate post-oxidation of probe 1. Increasing the pH above 8.1 had the desired effect of enhancing the reactivity of H₂O₂ towards probe 1, enabling near quantitative conversion to aminoferrocene 6 to be obtained within 20 minutes in the presence of 500 µM (5 eq.) H₂O₂.

Finally, in a bid to improve the sensitivity of the assay further, the temperature of the reaction was next to be looked at (see ESI†). Increasing the temperature of the assay from room temperature to 37 °C was found to only slightly increase reaction conversion. Increasing the temperature further led to a dramatic rate increase with near quantitative conversions being obtained within 20 minutes in the presence of only 250 µM (2.5 eq.) H₂O₂. However, at these elevated temperatures, fluctuating conversions can be seen thought to be due to the slow disappearance of the aminoferrocene 6 product peak and thus indicating decomposition of the product. This can be rationalised by the increased oxidation rate, and subsequent fragmentation, of aminoferrocene 6 at these high temperatures via known oxidation pathways. To minimise product oxidation, all subsequent assays were performed at room temperature. Importantly however, at all temperatures tested, minimal background conversions were observed (<2%), which reinforces the high stability of the carbamate functionality to undesired background hydrolysis.

Peroxide selectivity studies

To determine the selectivity of the probe for H₂O₂, a range of different peroxides, oxidants and salts were screened (Fig. 10). Specifically, a 100 µM solution of probe 1 was exposed to 5 equivalents of the oxidant and after 20 minutes of vigorous stirring, a sample of the assay was taken and subjected to DPV analysis. Of all oxidants screened, sodium percarbonate was the only oxidant other than H₂O₂ to give >15% conversion of

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**Fig. 7** Conversion of probe 1 (100 µM) to aminoferrocene 6, varying the pH of 50 mM Tris buffer in the presence of 500 µM of H₂O₂.

**Fig. 8** Conversion of probe 1 (100 µM) to aminoferrocene 6 in 50 mM pH 9 Tris buffer in the presence of various concentrations of H₂O₂.

**Fig. 9** Calibration curve for the conversion of probe 1 (100 µM) to aminoferrocene 6 after 20 minutes at varying H₂O₂ concentrations.
caused by behavioural and environmental risk factors.38 Left unregulated, elevated blood glucose levels can inflict significant capillary vessel damage and, depending on its location in the body, can go on to cause retinopathy, kidney failure and the onset of cardiovascular disease.29 Evidently, glucose concentrations in blood needs to be regularly measured reliably and to a high level of accuracy (±20% for concentrations above 5.6 mM or within ±0.83 mM for below).40 Surprisingly however, many currently commercially available glucose biosensors do not meet this standard.41 There is therefore still significant room for improvement regarding improving the accuracy and reliability of glucose detection.

Selective enzymatic reactions are often employed within electrochemical analyte detection methods to minimise noise from the possible presence of electrooxidisable interferences in the sample matrix.42 For electrochemical glucose sensing, glucose oxidase (GOx) is most commonly chosen,43 and in the process of oxidising glucose to D-glucono-δ-lactone, the mechanism of action for GOx also reduces molecular oxygen to hydrogen peroxide ($H_2O_2$).44 As such, we hypothesised that we could apply probe 1 under our previously optimised conditions towards the ratiometric electrochemical detection of glucose.

Initially, we sought to investigate the level of GOx activity needed to achieve full conversion of probe 1 in the shortest time (see ESI†), to help facilitate implementation of the methodology into a point-of-need device in the future. The concentration of glucose within the assay was chosen to be 5 mM (50 eq.) to ensure that enough $H_2O_2$ would be released to deliver full conversion of 1. As expected, high levels of GOx activity (>50 U mL$^{-1}$) delivered full conversion of 1 to 6 within the 20-minute assay time. Lowering the concentration of the enzyme led to a much slower conversion since the rate of glucose oxidation, and therefore $H_2O_2$ production, would be greatly reduced. Importantly, in the absence of GOx, neither conversion of 1 to aminoferrocene 6, or any shift in oxidation potential,45 was observed showing that conversion of the probe was not occurring through a supramolecular interaction between the boronic acid ester and glucose.46

As both high sensitivity for the analyte and a quick time-to-response are both critical factors in the implementation of diagnostic assays within point-of-need biosensors, we chose to take forward the highest concentration of GOx previously tested. Thus, to attain the sensitivity of the ratiometric electrochemical assay towards glucose, a range of glucose concentrations were screened in the presence of 100 U mL$^{-1}$ GOx (Fig. 11).

Similar to previous, high concentrations of glucose (>5 mM) were able to successfully achieve full conversion of probe 1 to 6 within the 20-minute timeframe. Again, in the absence of the sugar, no conversion was observed which highlights the selectivity of the probe towards the enzyme-catalysed production of $H_2O_2$ and not through any undesired interaction with the enzyme. The assay also demonstrated a good dynamic range over two orders of magnitude allowing for glucose concentrations between 10 µM and 1 mM to be easily distinguishable after just 10 minutes (Fig. 12). Glucose concentrations in blood, and other bodily fluids, are typically found within this range,47 which lends this ratiometric detection method towards such application if desired. As all ratiometric electrochemical conversions have been determined through the use

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**Fig. 10** Conversion of probe 1 (100 µM) to aminoferrocene 6 in 50 mM pH 9.0 Tris buffer in the presence of 500 µM of varying oxidants at room temperature. Abbreviations: mCPBA = meta-chloroperbenzoic acid, Oxone® = potassium peroxydisulfate, TEMPO = 2,2,6,6-tetramethyl-1-piperidinyloxy, CHP = cumene hydroperoxide, NMO = 4-methylmorpholine N-oxide, Luperox® = di-tert-butyl peroxide, TBHP = tert-butylhydrogen peroxide.
aminoferrocene carbamate linker was found to give a larger di-

tive linkers for comparison. The probe containing a benzyl

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meters found that pH 9 tris(hydroxymethyl)methylamine (Tris)
carbamatelinker. An optimisation of the diagnostic assay para-

dation potential between probe and product and exhibited

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the ratiometric electrochemical detection of hydrogen peroxide

probedoctorthe background rate within 20 minutes and the assay

also exhibited a good dynamic range over 2 orders of

magnitude.

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Conclusions

In conclusion, two ferrocene-derived probes were designed for

the ratiometric electrochemical detection of hydrogen peroxide

(H2O2) and synthesised to contain two different self-immola-

tive linkers for comparison. The probe containing a benzyl

carbamate linker was found to give a larger difference in oxida-

tion potential between probe and product and exhibited

faster elimination kinetics than the probe containing an allyl

carbamate linker. An optimisation of the diagnostic assay para-

meters found that pH 9 tris(hydroxymethyl)methylamine (Tris)

buffer at room temperature afforded efficient conversion of

of screen-printed carbon electrodes, subsequent work will

involve the implementation of the methodology into a point-
of-need biosensor with the aim of developing a device with an

improved accuracy over those which are currently commercially

available.


