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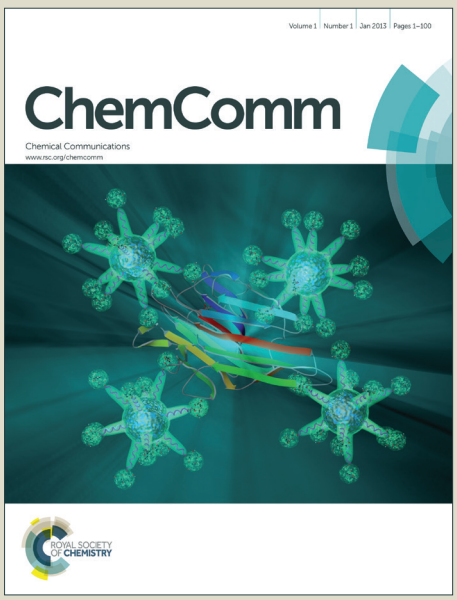

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COMMUNICATION

Ratiometric Electrochemical Detection of Alkaline Phosphatase†

Sean Goggins,[a] Christophe Naz,[b] Barrie J. Marsh,[b] and Christopher G. Frost*[a]

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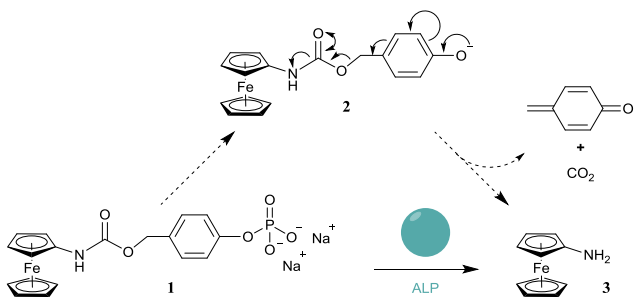
A novel ferrocene-derived substrate for the ratiometric electrochemical detection of alkaline phosphatase (ALP) was designed and synthesised. The substrate was demonstrated to be an excellent electrochemical substrate for ALP-labelled enzyme-linked immunosorbent assays (ELISA).

Alkaline phosphatase (ALP, EC 3.1.3.1) is one of the most commonly assayed enzymes as abnormal levels in serum can be used to preliminarily diagnose several diseases such as bone disease,[1] liver dysfunction,[2] breast and prostatic cancer,[3] and diabetes.[4] Moreover, ALP is extensively used as an enzyme-label within enzyme-linked immunosorbent assays (ELISA) due to being easily conjugated to antibodies, inexpensive and has high catalytic activity.[5] Typically, ALP is detected photometrically using *p*-nitrophenyl phosphate (*p*NPP),[6] or fluorimetrically using 4-methylumbelliferyl phosphate (4-MUP).[7] Chemiluminescence has also been employed to determine ALP activity.[8] However, to circumvent problems associated with these spectroscopic techniques, such as the use of expensive optical equipment and the need for transparent samples, there has been considerable demand for the development of electrochemical enzyme substrates. Importantly, these substrates have potential application within the development of electrochemical biosensors for point-of-care medical diagnostics.[9] In recent years, there has been tremendous progress made in this area of research and in particular, in the development of electrochemical enzyme-labelled immunoassays.[10] The main focus in improving the sensitivity of electrochemical immunoassays has been aimed towards immunosensor fabrication or electrode modification.[11] However, an area in which there is still a considerable opportunity for progress, is in the development of electrochemical substrates for enzymes.

Phenyl phosphate can be used as an electrochemical substrate for ALP but the electro-active product, phenol, requires separation, either by liquid chromatography (LC) or flow injection analysis (FIA), prior to electrochemical analysis.[12] Electron-rich phenyl phosphates can give improved signals but the electron-rich phenol products are prone to electro-polymerisation, which contributes to undesirable electrode fouling.[13] A commonly used electrochemical substrate for ALP is L-ascorbic acid 2-phosphate (AA2P) as it is inexpensive and electro-inactive.[14] In the presence of ALP, AA2P is converted to ascorbic acid which is electro-active, giving a 'turn-on' electrochemical detection system. Sensitivity is compromised however, as the product requires acidic media to deliver optimal current, which conflicts with the ideal pH of the enzyme.

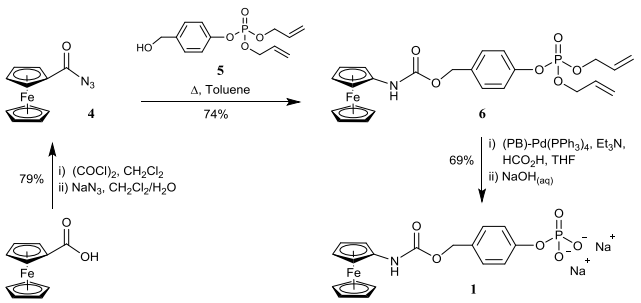
Ferrocene is often used as the redox-active moiety of electrochemical probes due to its easy derivatisation and favourable electrochemical properties.[15] Moreover, ferrocene exhibits excellent stability in aqueous media and its derivatives have therefore been widely used in biological applications.[16] Despite this, few ferrocene phosphate derivatives have been reported in the literature designed for use as electrochemical substrates for ALP. Bannister *et al.* describe the amperometric determination of ALP activity using [*N*-ferrecenoyl]-4-aminophenyl phosphate.[17] Unfavourably, this system relies on the product generating a greater signal than the substrate as both substrate and product have similar oxidation potentials. Degrand *et al.* describe the use of modified-electrodes in order to distinguish between a similar ferrocene-phenyl phosphate substrate and the corresponding ferrocene-phenol product.[18] However, the fabrication of modified electrodes is time-consuming and the electrodes are not reusable. To the best of our knowledge, a ferrocene-derived electrochemical substrate for ALP in which substrate and product have significantly different oxidation potentials is yet to be described.



Scheme 1 Structure of substrate **1** and proposed mechanism of ALP-catalysed breakdown with subsequent release of compound **3**.

Continuing our interest in ferrocene-based electrochemical sensing,[19] we designed ferrocenylphenyl phosphate **1** as an electrochemical substrate for ALP inspired by trigger-linker-effector sensing methodologies.[20] We proposed that in the presence of ALP, dephosporylation would occur giving rise to an unstable phenolate intermediate **2**, which would undergo 1,6-elimation and release ferrocenylamine **3**, along with an equivalent of quinone methide and CO_2 (Scheme 1). In principle, ferrocenylamine would be oxidised at a lower potential than that of the substrate due the increased electron density around the iron centre.[21] Therefore, **3** would be electrochemically distinguishable

from **1** and as a result, we would be able to monitor ALP activity ratiometrically using electrochemical analysis. Electrochemical ratiometric analysis has the benefit of being able to obtain reaction conversions, thus minimising sampling errors or errors that occur through instrument/electrode variations.



Scheme 2 Synthesis of substrate **1**.

The synthesis of substrate **1** (Scheme 2) began with ferrocenecarboxylic acid being converted to ferrocenoyl azide **4**, which was synthesised according to a literature procedure.[22] Curtius rearrangement of **4** in the presence of benzyl alcohol **5** (see SI for synthesis) generated the desired protected phosphate **6**. Polymer-bound palladium-catalysed deallylation followed by sodium salt formation gave phosphate **1** in 40% overall yield. Once synthesised, substrate **1** is a bench-stable solid with no decomposition observed for several weeks. Also, high concentrations of substrate **1** in buffer solution are stable to hydrolysis for a week at room temperature. With substrate **1** in hand, its electrochemical behaviour was analysed *via* differential pulse voltammetry (DPV) and compared to that of ferrocenylamine **3**.

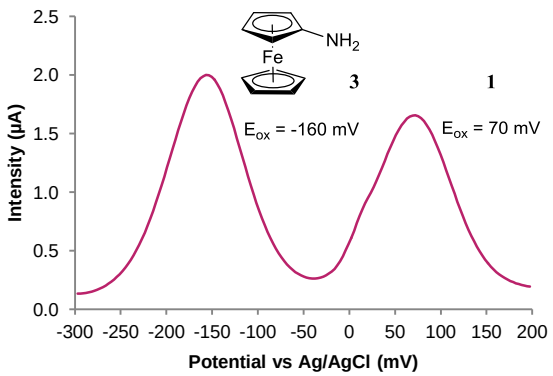


Fig. 1 Differential pulse voltammogram obtained for substrate **1** (100 μM) and ferrocenylamine **3** (100 μM) in 0.05 M pH 9 Tris buffer.

Expectedly, **3** has a much lower oxidation potential than that of **1**, a difference of approximately 230 mV, which is considerable enough that both peaks do not overlap (Figure 1). As a result, the peaks can be individually integrated and the conversion of substrate to product can be calculated using equation 1.

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

Substrate **1** (100 μM) was then exposed to varying concentrations of ALP in 50 mM pH 9 tris buffer at room temperature (~25 °C) and sampled every 3 minutes for 30 minutes (Figure 2). DPV was performed on each sample and the reaction conversion calculated by equation 1. At ALP concentrations higher than 500 UL^{-1}, quantitative conversions were observed within 10 minutes. Full conversions were also seen within 30 minutes at ALP concentrations as low as 175 UL^{-1}. Pleasingly, a minimal background reaction is observed and as such, this allows for ALP concentrations as low as 5 UL^{-1} to be detected in 30 minutes under these conditions.

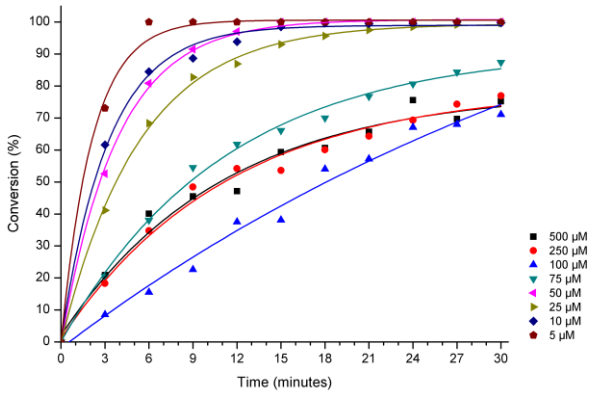


Fig. 2 Conversion of substrate (100 μM) to product after addition of different concentrations of ALP at room temperature.

Next, the optimal concentration of substrate **1** was investigated (Figure 3). An ALP concentration of 100 UL^{-1} was chosen as this would allow for both positive and negative effects of changing substrate concentration to be clearly identified. Increasing the concentration of substrate **1** from 100 μM did not elicit discernible increases in the rate of reaction. However, halving the concentration of substrate **1** allowed for quantitative conversion to be observed in 15 minutes. Further decreases of substrate concentration provide slightly increased reaction rates but due to both a lower current return and an increase in background hydrolysis, we decided to continue our investigation using a substrate concentration of 50 μM.

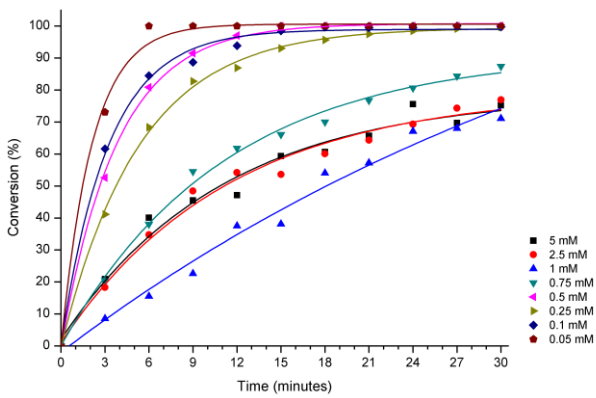


Fig. 3 Conversion of substrate to product after addition of ALP (100 UL^{-1}) using different concentrations of substrate at room temperature.

The optimum working temperature of ALP is 37 °C,[6a] and as such, the effect of temperature on the reaction of ALP with substrate **1** was studied next. Accordingly, the reaction was screened against varying concentrations of ALP at 37 °C with the

optimised substrate concentration (Figure 4). As desired, the reaction rates increased from previous results as quantitative conversions were now observed for ALP concentrations of 50 UL^{-1} or higher in 21 minutes. The limit of detection (LOD) for ALP was determined (see SI) to be 0.4 UL^{-1} after 27 minutes at 37 °C under these reaction conditions, which compares favourably to other electrochemical substrates.[23]

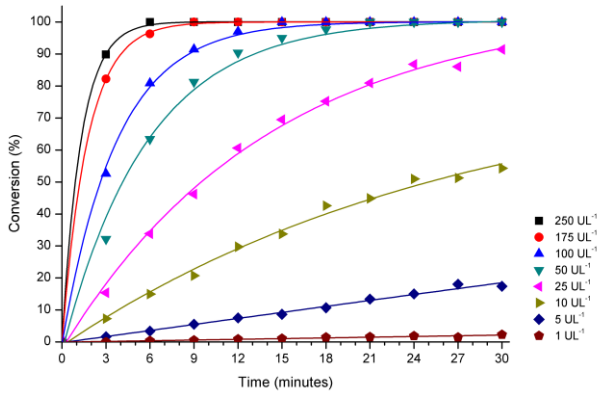


Fig. 4 Conversion of substrate (50 µM) to product after addition of different concentrations of ALP at 37 °C.

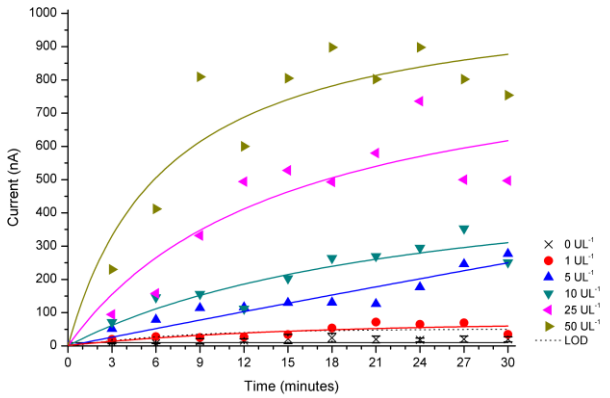


Fig. 5 Amperometric response of ferrocenylamine released from substrate 1 (50 µM) after the addition of ALP in different concentrations. Error bars indicate standard deviations (n = 3). LOD calculated as 3 × standard deviations higher than the mean.

With our reaction conditions now optimised, we next looked towards the application of substrate **1** within immunoassays. Prior to this, tests with substrate **1** with conjugated derivatives of ALP were needed. Streptavidin-conjugated ALP is often used in immunoassays to couple the enzyme to biotinylated antibodies, taking advantage of the high binding affinity between streptavidin and biotin.[24] However, enzyme conjugation can often lead to denaturisation and consequently a loss of enzyme activity.[5] As such, we applied our substrate to commercially-available streptavidin-ALP (Figure 5). Pleasingly, enzyme–protein conjugation does not lead to deactivation of the enzyme towards our substrate. Concentrations of conjugated ALP higher than 25 ngL^{-1} delivers full conversion of the substrate within 30 minutes. A concentration of 0.5 ngL^{-1} can also be distinguished from the background rate within this time period.

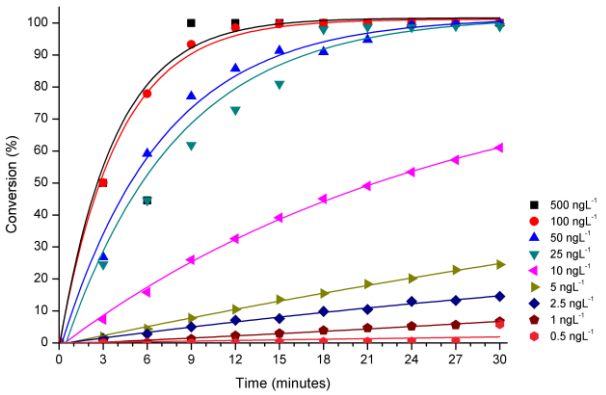
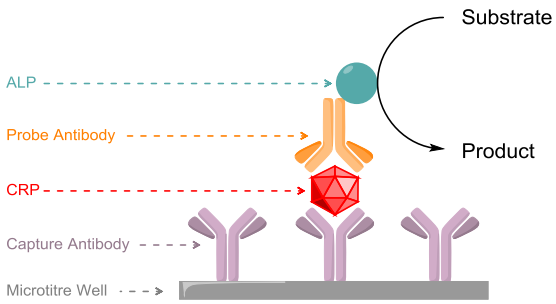


Fig. 6 Conversion of substrate (50 µM) to product after addition of different concentrations of ALP at 37 °C.

Encouraged by these results, we then applied substrate **1** to a commercial ELISA kit for the detection of the inflammation biomarker, C-reactive protein (CRP).[25] The sandwich immunoassay was constructed according to the manufacturer's procedure (see SI) and then exposed to substrate **1** under the optimised conditions (Scheme 3). Comparatively, we also used L-ascorbic acid 2-phosphate (AA2P), a literature electrochemical substrate for ALP,[14] as the immunoassay substrate to determine the benefits of using a ratiometric ferrocene-based electrochemical substrate over a 'switch-on' organic-based substrate. The substrate was incubated at 37 °C for 30 minutes prior to measuring the current obtained at the oxidation potential of the product.



Scheme 3 An illustration of the ELISA format for CRP immunoassay used. Electrochemical detection obtained using DPV with either **1** or AA2P as the substrate.

Gratifyingly, good responses were achieved using substrate **1** as the enzyme substrate for the CRP immunoassay with over 100 nA detected at CRP concentrations of 30 and 100 μgL^{-1}. This is over a 7-fold increase in current compared with AA2P at these concentrations. Positive current was still obtained at the lowest concentration (1 μgL^{-1}) of CRP tested. In comparison, no discernible signal was seen at this concentration when AA2P was used.

In conclusion, we have described the first example of ratiometric electrochemical detection for alkaline phosphatase activity. A novel ferrocene-based substrate, charged with a phosphate trigger, was synthesised and found to be electrochemically distinguishable from the product *via* differential pulse voltammetry. The compound synthesised was shown to be an efficient substrate for alkaline phosphatase and

demonstrated the detection of low concentrations of both protein-conjugated and unconjugated enzyme. The substrate was also successfully applied to an alkaline phosphatase-labelled ELISA for the ratiometric electrochemical detection of CRP, which delivered up to a 7-fold increase in current compared with that of commonly used electrochemical substrate.

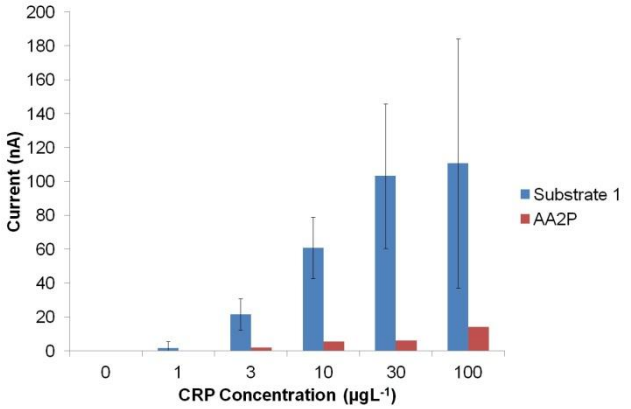


Fig. 7 Current measured from CRP ELISA using substrate **1** and AA2P as electrochemical. Error bars indicate standard deviations (n = 6).

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Notes and references

[a] *Department of Chemistry, University of Bath, Bath, BA2 7AY, UK. Fax: +44 (0)1225 386231; Tel: +44 (0)1225 386142; E-mail: c.g.frost@bath.ac.uk.*

[b] *Atlas, Derby Court, Epsom Square, White Horse Business Park, Trowbridge, Wiltshire, BA14 0XG, UK. Tel: +44 (0)1225 717932; E-mail: barrie.marsh@atlasgenetics.com.*

† Electronic Supplementary Information (ESI) available: Experimental procedures, characterisation data, copies of NMR spectra for synthesised compounds. See DOI: 10.1039/b000000x/

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