The development and evaluation of a conducting matrix for the electrochemical regeneration of the immobilised co-factor NAD(H) under continuous flow†

B. Ngamsom,[a] A. M. Hickey,[b] G. M. Greenway,*[a] J. A. Littlechild,[b] T. McCreedy,[a] P. Watts[a] and C. Wiles[a,c]

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Through the preparation of a novel controlled pore glass-poly(pyrrole) material we have developed a conducting support that is not only suitable for the co-immobilisation of enzymes and co-factors, but also enables the facile electrochemical regeneration of the co-factor during a reaction. Employing the selective reduction of (*rac*)-2-phenylpropionaldehyde to (*S*)-phenyl-1-propanol as a model, we have demonstrated the successful co-immobilisation of the HLADH enzyme and co-factor NAD(H); with incorporation of the material into a continuous flow reactor facilitating the *in situ* electrochemical regeneration of NAD(H) for in excess of 100 h. Using this approach we have developed a reagent-less, atom efficient system applicable to the cost-effective, continuous biosynthesis of chiral compounds.

Introduction

Oxidoreductases are an important class of enzyme which have found application in the biosynthetic preparation of chiral compounds due to the wide variety of selective redox reactions that they promote. However, like many other enzymes in order for them to act catalytically, reactions must be perfomed in the presence of a stoichiometric quantity of co-enzyme.[1] Co-enzymes or co-factors, which include nicotinamide adenine dinucleotide ($NAD(P)^+/NAD(P)H$), flavin adenine dinucleotide ($FAD/FADH_2$), flavin mononucleotide (FMN) and pyrroloquinoline quinone (PQQ), are however generally more expensive than the target product which precludes their use in large-scale transformations.[2] Co-factor regeneration is one approach that can address this problem, along with (i) preventing the accumulation of the co-factor by-product (which has the potential to inhibit the desired reaction), (ii) influencing the equilibrium position (useful for driving thermodynamically unfavorable reactions) and (iii) simplifying post reaction processing.

One facile approach that has enabled the use of NAD(H) **1** for preparative scale reductions is that of ethanol coupled regeneration;[3] whereby NAD^+ **2** is regenerated *via* the oxidation of ethanol to afford a constant supply of NAD(H) **1** to the reaction mixture. Whilst this approach is simple to implement and relatively inexpensive it is however disadvantageous as it is atom inefficient, requiring a large proportion of co-solvent and generating significant quantities of acetaldehyde. Alternatively, electrochemical, photochemical, and even, microbial techniques have been developed; with the latter preferred for industrial processes due to its high selectivity and efficiency.[1-3]

Although these approaches have enabled users access to reasonable reaction rates and co-factor turnover numbers, when performed in batch reactors, these strategies can still suffer from problems with product inhibition, substrate solubility, product isolation and enzyme/co-factor recovery.

To promote biocatalyst recovery, it has become desirable to immobilise both the enzyme and co-factor;[2] however regeneration of these co-factors whilst enabling a continuous reaction to occur has again proved challenging, with users focusing on alcohol and electrochemical mediated regeneration.[3] Whilst the latter technique is advantageous as no by-products are generated and neither a second enzyme nor co-substrate/solvent is required, current systems only enable regeneration to occur within the immediate vicinity of the electrodes surface.[4] As such, system throughputs and efficiencies are low due to diffusion limitations, making this approach more suited to bioanalytical applications rather than synthesis of bulk materials. A method of increasing the conducting surface area was recently investigated by Park and co-workers,[5] who developed a silica-vanadium oxide hybrid xerogel and demonstrated its use for the electrochemical formation of NAD(H) **1** from a starting solution of NAD^+ **2**; however approaches such as these have not been applied to systems employing co-immobilised enzymes and co-factors.

We therefore proposed that through the co-immobilisation of an enzyme/co-factor onto a conductive support, electrochemical co-factor regeneration could be performed alongside the facile separation of reaction products and re-use of the biocatalyst; affording a reagent-less system. Using the enzymatic reduction of (*rac*)-2-phenylpropionaldehyde **3** to (*S*)-2-phenyl-1-propanol **4** and (*R*)-2-phenylpropionaldehyde **5** as a model reaction, we evaluated the development of a reagent-less system based on the co-immobilisation of horse liver alcohol dehydrogenase (HLADH) **6** and NAD(H) **1** (Scheme 1) onto a conducting support.

Over the past five years, packed-bed continuous flow reactors have been shown to offer the researcher a rapid, efficient and

[a]*Department of Chemistry, The University of Hull, Cottingham Road, Hull, HU6 7RX, UK. E-mail: g.m.greenway@hull.ac.uk*
[b]*School of Biosciences, Henry Wellcome Building for Biocatalysis, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK*
[c]*Chemtrix BV, Burgemeester Lemmensstraat 358, 6163 JT, Geleen, The Netherlands*
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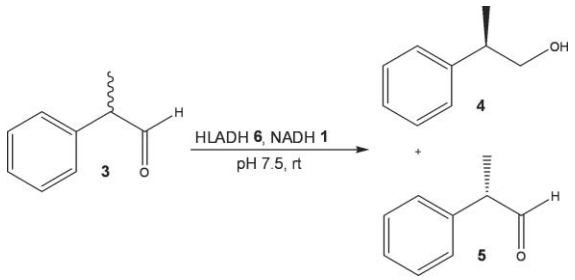


Scheme 1 Illustration of the model reaction used to demonstrate the selective reduction of (*rac*)-2-phenylpropionaldehyde **3** to (*S*)-2-phenyl-1-propanol **4** in the presence of HLADH **6** and NAD(H) **1**.

facile method of evaluating both chemical[6] and biochemical catalysts.[7] With the data generated from such systems readily scaled to afford access to production quantities of material *via* parallelisation/numbering-up[8] of reaction units or through increases in the packed-bed size,[9] this approach represents a cost effective method for system evaluation.

It was therefore envisaged that by packing the conducting biocatalytic material into a continuous flow reactor, product isolation and biocatalyst re-use would be more efficient compared to the use of a batch reactor; where biocatalytic material could be lost upon filtration and vessel transfer. It was also postulated that as substrates and products are constantly supplied to, and more importantly removed from, the reactor that this would prevent product accumulation and a thermodynamic resistance against the forward reaction.[2]

Results and discussion

As Fig. 1 illustrates, the proposed reaction set-up involved placing a conducting material into a packed-bed, through which a solution of (*rac*)-2-phenylpropionaldehyde **3** would be passed in order to undergo selective reduction. Maintaining the system under a potential would therefore ensure *in situ* electrochemical conversion of the spent NAD^+ **2** to NAD(H) **1**, increasing regeneration efficiency *cf.* electrode based systems. Using this approach, initial investigations centred on the use of a silica-vanadium oxide hybrid xerogel, as it had been shown to be electrically conducting by Park *et al.*[5] and contained a silica structure suitable for enzyme immobilisation. Unfortunately, when the unfunctionalised material was evaluated within a continuous flow reactor, leaching of vanadium was observed; rendering the material electrically inactive. As such an alternative material was sought that was both

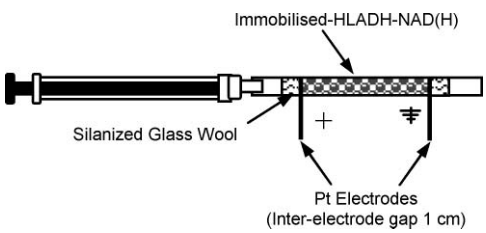


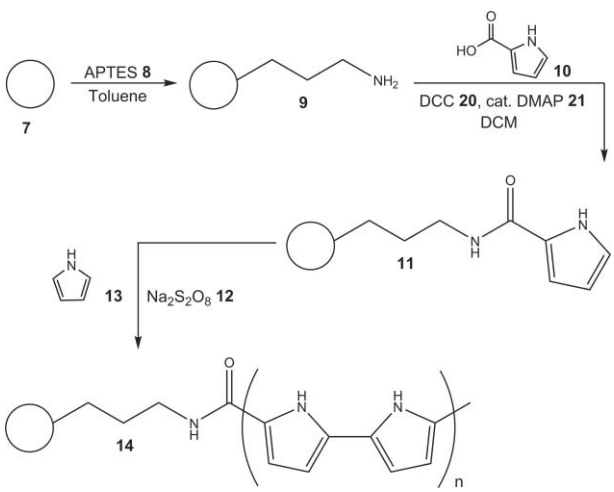
Fig. 1 Schematic illustrating the flow reactor set-up used to evaluate the *in situ* electrochemical regeneration of an immobilised NAD(H) **1** in the presence of an immobilised enzyme.

conducting and provided a suitable surface for enzyme/co-factor immobilisation.

Of the conducting polymers reported, polypyrrole (PPy) is the most thoroughly investigated material for biological applications due to its high electrical conductivity, flexible method of preparation and *in vitro* compatibility;[10-12] the material however provided little means of functionalisation suitable for the co-immobilisation of HLADH **6** and NAD(H) **1** or application in a continuous flow system; as PPy is more conventionally applied as films directly onto electrodes.[13,14] In comparison, controlled pore glass (CPG) **7** is widely employed as a solid-support for the immobilisation of enzymes due to a plethora of well characterised derivatisation techniques, also its uniform particle size and shape is ideal for use in packed-beds.

Preparation and evaluation of poly(pyrrole) functionalised controlled pore glass

Consequently, in order to achieve our target of developing a novel material suitable for application towards a reagent-less biocatalytic system, a combination of these materials was required to afford a conducting matrix onto which the enzyme and co-factor could be readily immobilised and packed within a continuous flow reactor. As Scheme 2 illustrates, to achieve this CPG **7** (120-200 mesh, 500 Å) was initially functionalised with 3-aminopropyl triethoxysilane **8**. Under standard coupling reaction conditions, the aminopropyl-functionalised CPG **9** was derivatised with pyrrole-2-carboxylic acid **10** to afford a pale yellow powder **11**. Due to the involatility of the solid support, the material could not be evaluated using conventional elemental analysis and the maximum loading was assumed to be 4.0 mmol g^{-1}. At this stage the material **11** was non-conducting however upon treatment with $Na_2S_2O_8$ **12**, in the presence of pyrrole **13**, the immobilised pyrrole moieties polymerised to afford a dark grey (indicative of polypyrrole formation) conducting powder **14**.



Scheme 2 Synthetic protocol employed for the preparation of a conducting CPG-PPy matrix **14** suitable for the co-immobilisation of HLADH **6** and NAD(H) **1**.

Prior to incorporation into a continuous flow reactor, the material **14** was evaluated as a conducting surface for the formation of NAD(H) **1** from NAD^+ **2** in a conventional batch system,

consisting of a cuvette and two Pt electrodes (interelectrode distance = 1 cm, applied field = 12 V cm^{-1}). Fig. 2 shows NAD(H) **1** generated from 2.5 mM NAD$^+$ **2** in phosphate buffer pH 7.5 and compares two systems, (a) without CPG-PPy matrix **14** and (b) with CPG-PPy matrix **14**; illustrating enhanced NAD(H) **1** generation in the presence of the conducting CPG-PPy matrix **14**.

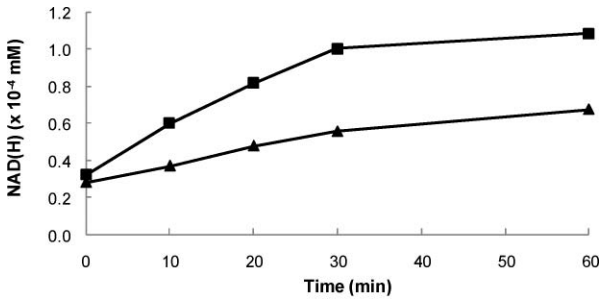
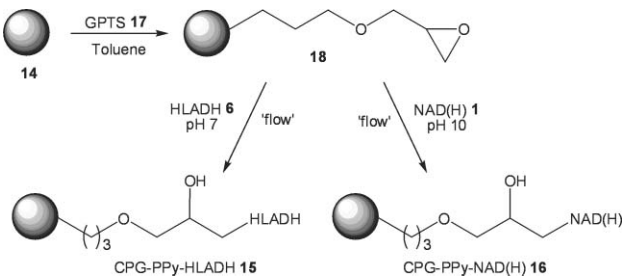


Fig. 2 Graph illustrating the enhanced electrochemical generation of NAD(H) **1**, from NAD$^+$ **2**, in the absence (▲) and presence (■) of the CPG-PPy **14**.

Evaluation of CPG-PPy 14 for enzyme immobilisation

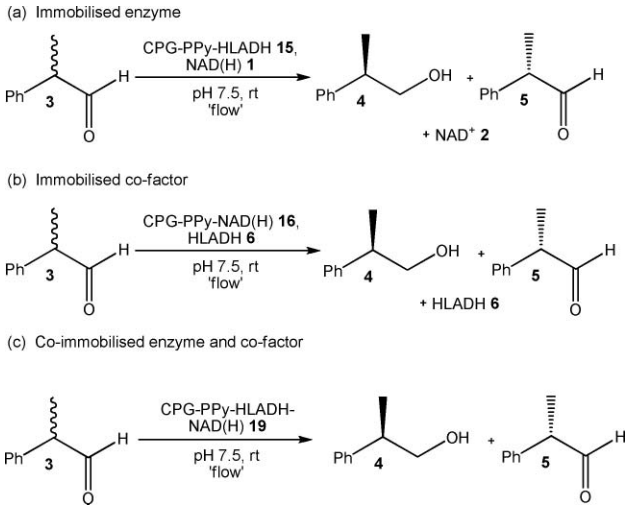
Having demonstrated an enhancement in the formation of NAD(H) **1** in the presence of CPG-PPy **14**, the material was subsequently evaluated as a solid support for the separate immobilisation of HLADH **6** and NAD(H) **1**, affording CPG-PPy-HLADH **15** and CPG-PPy-NAD(H) **16** respectively, prior to investigating the co-immobilisation of HLADH **6** and NAD(H) **1**.

As Scheme 3 illustrates, enzyme **6** and co-factor **1** immobilisation was achieved utilising CPG-PPy **14**, derivatised with 3-glycidoxypropyltrimethoxysilane (GPTS) **17** to afford the epoxide-functionalised CPG-PPy **18**. The material **14** (0.08 g) was subsequently dry-packed into a flow reactor (5 mm i.d. x 5 cm (long) Fig. 1) and in the first instance a solution of HLADH **6** in phosphate buffer (pH 7) pumped through the reactor (1 µl min^{-1}) for a period of 24 h. Any unbound enzyme was then removed from the reactor upon purging with phosphate buffer (pH 7.0, 1 µl min^{-1}, 24 h) to afford a packed-bed containing CPG-PPy-HLADH **15**.



Scheme 3 Schematic illustrating the protocols used to immobilise HLADH **6** and NAD(H) **1**.

To evaluate the immobilised enzyme **15**, the continuous flow reduction of (*rac*)-2-phenylpropionaldehyde **3** (2.6 × 10^{-4} mM) was performed in the presence of a solution of free NAD(H) **1** (8.5 × 10^{-4} mM) in phosphate buffer (pH 7.5) (Scheme 4a). Off-line HPLC analysis was used to determine the conversion to (*S*)-2-phenyl-1-propanol **4**, with quantification achieved using internal standardisation.



Scheme 4 Schematic illustrating the stepwise approach taken to determine the effectiveness of the immobilisation strategies described herein.

As Fig. 3 illustrates, employing a flow rate of 2 µl min^{-1}, and continuously supplying a solution NAD(H) **1** to the reactor, an average 43% conversion of (*rac*)-2-phenylpropionaldehyde **3** was obtained over a 20 h period; confirming the successful immobilisation of HLADH **6**. Owing to the selective nature of the enzyme **6**, a maximum conversion of 50% **4** was attainable and would signify 100% reduction of the reactive enantiomer.

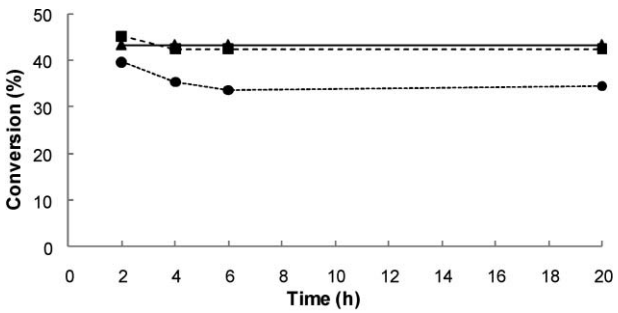


Fig. 3 Graph illustrating the results obtained for the continuous flow evaluation of CPG-PPy-HLADH **15** (▲), CPG-PPy-NAD(H) **16** (●) and CPG-PPy-HLADH-NAD(H) **19** (■) towards the selective reduction of (*rac*)-2-phenylpropionaldehyde **3** as a model reaction; room temperature, flow rate 2 µl min^{-1}.

Evaluation of CPG-PPy 14 for co-factor immobilisation

Having demonstrated the successful immobilisation of HLADH **6**, the conducting support **14** was investigated for the immobilisation of NAD(H) **1**. In 1980, Fuller *et al.*,[15] reported a procedure for the covalent immobilisation of NAD(H) **1** onto an epoxide derived polymer, a process that was found to take 8-10 days. Herein, the process depicted in Fig. 3 was achieved in 72 h by pumping a solution of NAD(H) **1** (0.08 mM) in carbonate buffer (pH 10.0, 0.2 M) through the flow reactor containing epoxide-functionalised CPG-PPy **18** at a flow rate of 1 µl min^{-1}. Using this approach, the

co-factor **1** was successfully immobilised *via* the aromatic amino group on the adenosine moiety and unlike the work of Fuller *et al.* no additional steps were required to obtain the co-factor **1** in the desired oxidation state; affording a rapid route to co-factor **1** immobilisation.

Employing a solution of (*rac*)-2-phenylpropionaldehyde **3** and free HLADH **6** in phosphate buffer (pH 7.5), the CPG-PPy-NAD(H) **16** was evaluated (Scheme 4b) at a flow rate of 2 μl min^{-1} (residence time = 1 h), with measurements initiated after a 2 h period of equilibration; after which time the reactor reached a steady state. As Fig. 3 illustrates, in the absence of co-factor regeneration 40% conversion to (*S*)-2-phenyl-1-propanol **4** was obtained over the first 4 h of use, decreasing to 34% **4** for the remaining 14 h of continuous evaluation. This result therefore shows that in the absence of co-factor regeneration, sufficient NAD(H) **1** has been immobilised to catalyse the selective reduction of (*rac*)-2-phenylpropionaldehyde **3** for a short period of time.

Co-immobilisation of HLADH 6 and NAD(H) 1 onto a conducting matrix 14

Gratified by the results obtained thus far, the third step was to co-immobilise HLADH **6** and NAD(H) **1** onto the conducting support. Again this was achieved *via* packing of the flow reactor with epoxide-functionalised CPG-PPy **18** (0.08 g) and firstly pumping a solution of HLADH **6** in phosphate buffer (pH 7.0, 0.2 M) through the reactor at 1 μl min^{-1} for 24 h.

The reactor was then purged with carbonate buffer (pH 10.0, 0.2 M) to remove any free HLADH **6**, prior to the introduction of NAD(H) **1** in carbonate buffer (pH 10) at a flow rate of 1 μl min^{-1} for 72 h. The system was subsequently purged with phosphate buffer (pH 7.5, 0.2 M), prior to evaluating the resulting co-immobilised CPG-PPy-HLADH-NADH **19** towards the model reaction.

Performing the flow reaction, depicted in Scheme 5 (no applied field), CPG-PPy-HLADH-NAD(H) **19** was found to reduce (*rac*)-2-phenylpropionaldehyde **3** to (*S*)-phenyl-1-propanol **4** in an average conversion of 44% over a period of 20 h (Fig. 4) and although this confirmed the successful co-immobilisation of HLADH **6** and NAD(H) **1**, this configuration is only useful until the NAD(H) **1** is consumed. Consequently, the next step of the investigation was to evaluate the electrochemical regeneration of the immobilised NAD(H) **16**.

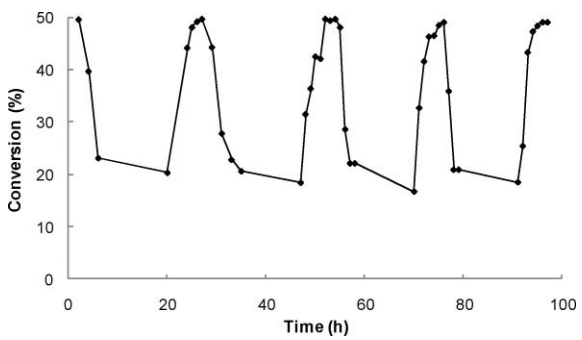
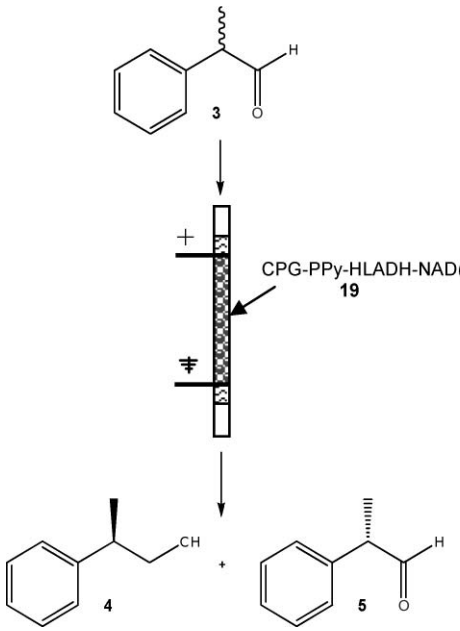


Fig. 4 *In situ* electrochemical regeneration of NAD(H) **1** using CPG-P-Py-HLADH-NAD(H) **19** in a continuous flow reactor; room temperature, flow rate 2 μl min^{-1}.



Scheme 5 Schematic illustrating the reaction set-up used for the continuous flow synthesis of (*S*)-phenyl-1-propanol **4** utilising *in situ* co-factor regeneration.

Electrochemical regeneration of CPG-PPy-HALDH-NAD(H) 19

In order to initially promote consumption of the NAD(H) **1**, the reactant concentration delivered to the flow reactor was increased from 2.6×10^{-4} mM to 2.6×10^{-3} mM **3**; this resulted in the desired 30% decrease in aldehyde **4** conversion within 6 h. After stabilisation of the system, achieved after 20 h, a voltage of 12 V (12 V cm^{-1}) was applied to the system restoring the biocatalytic materials **19** activity in 2 h. As Fig. 4 illustrates, repeating the cycle of enzymatic reduction in the absence of a field, followed by electrochemical regeneration a further three times (mean = 49.4% **4**, 0.57% RSD ($n = 5$)), we were able to demonstrate the stability of the CPG-PPy-HLADH-NAD(H) **19** over a period of 100 h.

However as the overall aim was to develop a continuous system, we subsequently investigated the uninterrupted application of a lower voltage. Using this approach, it was found that performing the reaction in the presence of a continuous applied field (3 V cm^{-1}) constant regeneration of the immobilised NAD(H) **1** was achieved affording (*S*)-2-phenyl-1-propanol **4** in 48% conversion out of a possible 50%. Having observed reduced efficacy of the co-factor after 2 h, in the absence of an applied field (Fig. 4), the system was monitored every hour over a period of 6 h to confirm constant regeneration of the immobilised NAD(H); at this stage no reduction in conversion was obtained, monitoring was simply stopped.

Using this approach, we achieved our target of developing a reagent-less system suitable for the biocatalytic reduction of (*rac*)-2-phenylpropionaldehyde **3** to (*S*)-2-phenyl-1-propanol **4** and (*R*)-2-phenylpropionaldehyde **5**.

Conclusions

A conducting CPG-PPy material **19** was successfully synthesised and utilised as a solid-support for the immobilisation of

HLADH **6** and NAD(H) **1**. The biocatalytic material **19** was subsequently evaluated for the enzymatic reduction of (*rac*)-2-phenylpropionaldehyde **3** to (*S*)-2-phenyl-1-propanol **4** under continuous flow, with excellent conversions obtained for 20 h in the absence of electrochemical co-factor regeneration. Upon exhaustion of the immobilised NAD(H) **1**, *in situ* electrochemical regeneration was found to restore enzyme activity within the biocatalyst **19**, with repetition of this cycle enabling continued operation for in excess of 100 h.

Whilst it can be viewed as an advantage, the small volumes of biocatalytic material employed in such systems, place demands on analytical methodology and as such, further advances are required within the field to develop suitably accurate methods for the determination of enzyme loading within micro fabricated reactors.[16]

In conclusion, the CPG-PPy matrix **14** described herein has the potential to afford a facile and cost effective solution to the challenge associated with immobilising enzymes that require a NAD(H) **1** or related co-factors. Further work will therefore be directed towards the co-immobilisation of other oxidoreductase enzymes and their co-factors along with the development of a micro fluidic system for the high throughput screening of potential substrates.

Experimental

Experimental/Materials

Chemicals were purchased from the sources indicated and used as supplied; 3-aminopropyl triethoxysilane **8** (99%, Aldrich), 3-glycidoxypropyl trimethoxysilane **17** (98%, Aldrich), pyrrole-2-carboxylic acid **10** (99%, Aldrich), pyrrole **13** (98%, Aldrich), *N*,*N*′-dicyclohexylcarbodiimide (DCC) **20** (99%, Aldrich), 4-dimethylaminopyridine (DMAP) **21** (98%, Fluka), sodium persulfate **12** (98%, Aldrich), (*rac*)-2-phenylpropionaldehyde **3** (98%, Aldrich), (*S*)-2-phenyl-1-propanol **4** (97%, Aldrich), controlled pore glass (CPG) **7** (Sigma), horse liver alcohol dehydrogenase (HLADH) **6** (Sigma) and NAD(H) **1** (Sigma). Acetonitrile, used as HPLC mobile phase was of HPLC grade, dichloromethane (99%) and tetrahydrofuran (99.5%) were all purchased from Fisher Scientific as general purpose reagent grade. The HPLC column used was a BDS Hypersil C18, particle size 5 μm (150 mm × 4.6 mm) (Phenomenex).

Preparation of conducting CPG-PPy 14. CPG **7** (0.50 g) was added to a solution of 3-aminopropyltriethoxysilane **8** (0.47 ml, 2.0 mmol) in toluene (5 ml) and stirred overnight at room temperature. The resulting aminopropyl-functionalised CPG **9** was subsequently washed with toluene (20 ml), then dichloromethane (20 ml) and dried under suction to afford a white free flowing powder **9**. Pyrrole-2-carboxylic acid **10** (0.22 g, 2.0 mmol), DCC **20** (0.62 g, 3.0 mmol) and DMAP **21** (0.02 g, 0.2 mmol) were then added to a solution of aminopropyl-functionalised CPG **9** in DCM (20 ml) and the resulting reaction mixture stirred at room temperature, under N_2 for 3 days. The reaction mixture was filtered under suction, washed with DCM (50 ml), then THF (50 ml) and dried to afford a pale yellow flowing powder **11**. To enable polymerisation of the immobilised pyrrole moieties, pyrrole **13** (0.05 mL, 0.72 mmol) was added to the CPG-Py **14** and stirred with aq. $Na_2S_2O_8$ **12** (3.0 ml, 4.0 mM) for 2 h. The CPG-PPy **14** was filtered under suction, washed with DI water (50 ml), THF (50 ml) and finally DCM (50 ml) to afford a free flowing grey powder **14**. Analysis of the material by IR spectroscopy ($\upsilon = 1675$ cm^{-1}) confirmed the presence of pyrrole functionality.

Immobilisation of HLADH 6. CPG-PPy **14** (0.08 g) was stirred with 3-glycidoxypropyl trimethoxysilane **17** (0.075 ml, 0.32 mmol) in toluene (20 ml) for 3 h, to afford the epoxide derived CPG-PPy **18**. Prior to use the material was washed with ethanol and dichloromethane and dried under suction to afford a grey free flowing powder. The epoxide functionalised CPG-PPy **18** was subsequently packed into a polymeric flow reactor (5 mm i.d. × 5 cm length), retained using silanized glass wool and prior to enzyme immobilisation, the material was washed with potassium phosphate buffer (pH 7.0, 0.2 M). HLADH **6** immobilisation was achieved by pumping a solution of HLADH **6** (2 mg) in 0.2 M potassium phosphate buffer (2.0 ml, pH 7.0, 0.2 M) through the reactor at 1 μl min^{-1} for 24 h. After this time, any unbound enzyme was washed from the system using potassium phosphate buffer.

Immobilisation of NAD(H) 1. CPG-PPy **14** (0.08 g) was stirred with 3-glycidoxypropyl trimethoxysilane **17** (0.075 ml, 0.32 mmol) in toluene (20 ml) for 3 h, to afford the epoxide derived CPG-PPy **18**. Prior to use the material was washed with ethanol (50 ml) and dried under suction to afford a grey free flowing powder. The epoxide-functionalised CPG-PPy **18** was subsequently packed into a polymeric flow reactor (5 mm i.d. × 5 cm length), retained using silanized glass wool and prior to enzyme immobilisation, the material was washed with carbonate buffer (pH 10.0, 0.2 M). NAD(H) **1** immobilisation was achieved by pumping a solution of NAD(H) **1** (0.08 M) in 0.2 M carbonate buffer through the flow reactor at 1 μl min^{-1} for 3 days. After this time, any unbound co-factor was washed from the reactor using 0.2 M carbonate buffer.

Co-immobilisation of HLADH 6 and NAD(H) 1. To attain co-immobilisation of HLADH **6** and NAD(H) **1**, the aforementioned procedures were performed in series, ensuring that between enzyme **6** (24 h) and co-factor **1** (72 h) immobilisation, the functionalised CPG was washed with 0.2 M carbonate buffer.

Enzymatic reduction of (*rac*)-2-phenylpropionaldehyde 3 to (*S*)-2-phenyl-1-propanol 4. Due to the relative insolubility of (*rac*)-2-phenylpropionaldehyde **3** in potassium phosphate buffer, a small amount of organic solvent was required to aid dissolution. The details of the solvent study are not reported here however 10% MeCN was found to provide comparable results without affecting the enzyme activity. Accordingly, (*rac*)-2-phenyl-propionaldehyde **3** (100 μl, 0.75 mmol) was dissolved in MeCN (25 ml) and the resulting mixture was diluted 10 folds with 10 mM potassium phosphate buffer pH 7.5 to afford the reactant feed stock. The feedstock was pumped through the flow reactor at a flow rate of 2 μl min^{-1} and the reaction products collected at the reactor outlet. To determine the conversion of (*rac*)-2-phenylpropionaldehyde **3** to (*S*)-2-phenyl-1-propanol **4**, the reaction products were collected and analysed off-line by HPLC using a BDS Hypersil C18 column (150 mm × 4.6 mm) (Phenomenex), with a flow rate of 1 ml min^{-1}, 35% acetonitrile in water as the mobile phase and detected at 215 nm; 2.5 mM *N*-benzoyl-L-phenylalanine was employed as the internal standard.

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