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Enzyme-Modified Particles for Selective Biocatalytic Hydrogenation by Hydrogen-Driven NADH Recycling

Holly A. Reeve,[a] Lars Lauterbach,[b] Oliver Lenz,[b] and Kylie A. Vincent*[a]

We describe a new approach to selective H_2-driven hydrogenation that exploits a sequence of enzymes immobilised on carbon particles. We used a catalyst system that comprised alcohol dehydrogenase, hydrogenase and an NAD^+ reductase on carbon black to demonstrate a greater than 98% conversion of acetophenone to phenylethanol. Oxidation of H_2 by the hydrogenase provides electrons through the carbon for NAD^+ reduction to recycle the NADH cofactor required by the alcohol dehydrogenase. This biocatalytic system operates over the pH range 6–8 or in un-buffered water, and can function at low concentrations of the cofactor (10 μM NAD^+) and at H_2 partial pressures below 1 bar. Total turnover numbers >130000 during acetophenone reduction indicate high enzyme stability, and the immobilised enzymes can be recovered by a simple centrifugation step and re-used several times. This offers a route to convenient, atom-efficient operation of NADH-dependent oxidoreductases for selective hydrogenation catalysis.

Introduction

The ability to achieve high selectivity in catalytic hydrogenation reactions using H_2 as the reducing equivalent remains a major challenge in chemical production[1] in which 10–20% of chemical steps are catalytic hydrogenations.[2] Noble-metal complexes are well established as catalysts in this area,[1,3] but high H_2 pressures (>80 bar) are often required. Improvements in enantioselectivity typically require trial-and-error screening,[4] and chemoselectivity is still a key issue.[5] Oxidoreductase enzymes catalyse a wide range of hydrogenation reactions, which include the reduction of ketones, alkenes and imines as well as the reductive amination of ketones,[6] and it is becoming increasingly accepted that biocatalysts offer significant advances in chemo- and enantioselectivity. This is particularly valuable in the fine chemicals and pharmaceutical sectors in which, for example, 75% of new drugs in 2002 were single enantiomers.[7] However a major drawback to the majority of oxidoreductase enzymes is their dependence on hydride transfer from NADH or NADPH.[8] The cost of these cofactors means that stoichiometric addition is not a viable option for the application of enzymes in synthesis, and recycling systems for the reduced cofactors are, therefore, required. Established methods for cofactor recycling are biocatalytic, rely almost exclusively on the super-stoichiometric addition of a sacrificial reductant and the appropriate enzyme for its oxidation (typically glucose dehydrogenase with glucose or alcohol dehydrogenase (ADH) with isopropanol) and generate substantial carbon-based waste.[9] The ability to use H_2 as a reducing equivalent for NAD(P)H recycling would solve the poor atom economy of enzyme-catalysed hydrogenation reactions.[9–10] NAD(P)-linked hydrogenases that naturally couple H_2 oxidation to the reduction of the oxidised cofactors, NAD^+ or $NADP^+$, have been demonstrated for H_2-driven NAD(P)H recycling with several biocatalysed hydrogenations[10–11] but these enzymes tend to have limited stability and so have not become established for cofactor recycling.[12]

Previously, we have described a new, modular route to H_2-driven cofactor recycling in which a robust hydrogenase and an NAD^+ reductase moiety are adsorbed on electronically conductive graphite particles. The direct transfer of electrons from H_2 oxidation by the hydrogenase into the particle provides a source of electrons for NAD^+ reduction by the co-immobilised NAD^+ reductase moiety. Importantly, both enzymes possess an electron relay chain of iron-sulfur clusters that facilitate the rapid movement of electrons between the active site and the conducting particle. We demonstrated this approach for H_2-driven NADH production and to supply an NADH-dependent lactate dehydrogenase in solution for reduction of pyruvate to lactate.[13] The lactate dehydrogenase is unable to use electrons directly and, therefore, only operated if supplied with reducing equivalents in the form of NADH generated by the enzyme-modified particles.

Here, we demonstrate advances to this system of enzyme-modified particles for H_2-driven biocatalysis and provide a detailed characterisation of how the particles function over

[a] Dr. H. A. Reeve, Prof. K. A. Vincent
Department of Chemistry, University of Oxford
Inorganic Chemistry Laboratory
South Parks Road, Oxford, OX1 3QR (UK)
E-mail: kylie.vincent@chem.ox.ac.uk

[b] Dr. L. Lauterbach, Dr. O. Lenz
Institute of Chemistry, Technische Universität Berlin
Strasse des 17. Juni 135, 10623 Berlin (Germany)

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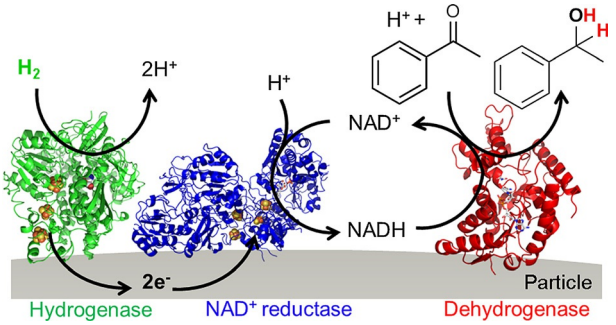
a range of reaction conditions. We select as a test system the robust and reversible NADH-dependent alcohol dehydrogenase 105 from Johnson Matthey Catalysis and Chiral Technologies that accepts a broad range of substrates, which includes linear and cyclic ketones and acetaldehyde. We use the reduction of acetophenone to phenylethanol as a test reaction. We show that the co-immobilisation of the NADH regeneration enzymes with the ADH on particles (Scheme 1) leads to faster ki-



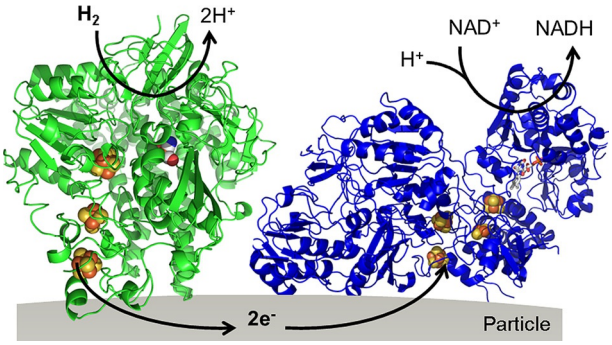
Scheme 1. Schematic representation of the H_2-driven particle system for NADH-dependent biocatalysis. Oxidation of H_2 by a hydrogenase (green) transfers electrons (e^-) into an electronically conducting particle and these are then used by an NAD^+ reductase (blue) for selective NAD^+ reduction. A co-immobilised NADH-dependent AHD (red) is supplied with NADH that is used for catalytic reduction of a ketone (here, acetophenone). Both hydrogen atoms of H_2 are incorporated into the product (here phenylethanol) to allow a 100% atom-efficient chemical synthesis.

netics in comparison with the use of the ADH in solution and allows the use of low cofactor concentrations. Additionally, co-immobilisation makes it possible to handle all the enzymes required for a H_2-driven hydrogenation as a single heterogeneous catalyst, which can be separated readily from a reaction mixture and re-used. The use of H_2 gas to drive NADH recycling means that overall the hydrogenation is 100% atom efficient.

Results

H_2-driven NADH production

We first evaluate the performance of carbon black particles modified with hydrogenase 2 (Hyd-2) from *E. coli* and NAD^+ reductase (SH^{I64A} protein from *R. eutropha* HF210 (pGE749)) with H_2 as the reducing equivalent for the reduction of NAD^+ to NADH (Scheme 2). The enzyme-modified particles were prepared by pre-mixing the required enzyme solutions and then adding carbon black particles, dispersed in aqueous buffer solution, to allow the adsorption of the enzymes onto the carbon surface. Details of the quantities of enzyme and carbon used for each experiment are given in Supporting Information Table S1. As a result of the small scale of the experiments and the difficulty to conduct individual enzyme activity assays on the carbon-immobilised enzymes, it was not possible to quantify the amount or activity of each enzyme that was adsorbed onto the carbon accurately, but the washing of the particles



Scheme 2. Schematic representation of the enzyme-modified particle system for H_2-driven NADH production. A hydrogenase (green) is able to oxidise H_2 and transfer the electrons into the particle through a chain of FeS clusters (spheres shown in elemental colours). These electrons can be transferred to the co-immobilised NAD^+ reductase (blue) for the reduction of NAD^+ to NADH.

ensured that only adsorbed enzyme molecules contribute to the activity of the particles.

Results for NADH production by particles assembled as shown in Scheme 2 are provided in Figure 1. The percentage conversion of NAD^+ to NADH is determined from the change

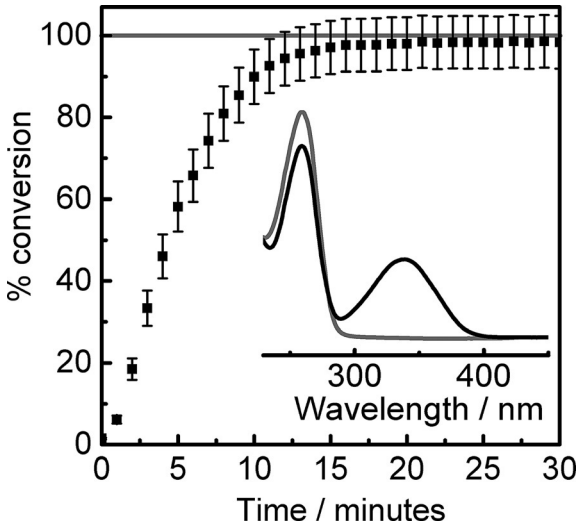


Figure 1. Time course for the conversion of NAD^+ to NADH using carbon particles modified with NAD^+ reductase and a hydrogenase suspended in a stirred, H_2-saturated solution of Tris-HCl pH 8.0 buffer at 30 °C; initial NAD^+ concentration: 70 μM. Inset: initial (grey) and final (black) UV/Vis absorbance spectra measured in situ during the reaction.

in the UV/Vis spectrum (inset in Figure 1), which shows >97% conversion of 70 μM NAD^+ after 30 min. The reaction rate is fairly linear up to ≈80% conversion and then it starts to plateau. A decrease in the rate during the course of the reaction is expected because of the depletion of substrate for the NAD^+ reductase, which has a Michaelis–Menten constant, $K_M(NAD^+)$, of 197 μM.[14] Additionally, the decrease in the ratio of NAD^+ to NADH during the reaction will cause the redox potential of the NAD^+/NADH couple to become more negative

according to the Nernst equation, which thus decreases the thermodynamic driving force for H_2-driven NADH generation (if we assume that the H_2 partial pressure and pH do not change significantly). The build-up of NADH during the reaction is also likely to lead to product inhibition of the NAD^+ reductase,[14] which further decreases the overall activity of the particles. These effects are largely eliminated if the particles are used in conjunction with an NADH-dependent ADH (see later) because the reduced cofactor is then consumed rapidly by re-oxidation.

Control experiments that confirm that the hydrogenase and NAD^+ reductase are both required for H_2-driven NADH production and that the two enzymes must be co-immobilised on particles are shown in Table S1 and Figure S1. Data are also presented in Figure S2 that show that it is possible to couple hydrogenase and NAD^+ reductase in the same way on other electronically conductive carbon materials (pyrolytic graphite particles, single-walled carbon nanotubes and carbon paper) to give high levels of the conversion of NAD^+ to NADH.

The results from a collection of experiments designed to highlight specific catalytic properties of the enzyme-modified carbon particles, percentage conversion of NAD^+ to NADH, initial activity and total turnover number, are summarised in Table 1. The results from each run are not completely comparable because experiments were conducted under different conditions and favour certain aspects of the catalysis, which are relevant to different ways to operate the cofactor recycling system. Initial activities were calculated from the rate of NADH generation during the starting linear phase of the reaction. The total turnover number (TTN) is calculated as moles of NADH generated at the time specified per mole of NAD^+ reductase applied to the carbon particles. It is likely that some of the enzyme sample remains unadsorbed and is removed during the particle-washing step, so the amount of NAD^+ reductase is likely to be overestimated.

Table 1. Results from different H_2-driven NADH generation experiments designed to evaluate specific operational parameters of the enzyme-modified particles.

Entry	[NAD^+] [mM]	Conversion [%]	Initial activity[a]	Total turnover number[b]
1	0.07	>97[c]	1.4±0.1	1250[c]
2	2.00	9.3[d]	7.8±0.5	53 100[d]
3	5.00	75[e]	N.D.	>94 800[e]

[a] Initial activity [μmol min^{-1} per mg NAD^+ reductase] measured during the initial linear reaction phase; [b] Total turnover number [moles of NADH generated per mole of NAD^+ reductase exposed to the carbon particles]; [c] Measured after 30 min; [d] Measured at 35 min; [e] Measured at 20 h; N.D., not determined.

The results from the experiment shown in Figure 1 are summarised in entry 1 of Table 1. The specific reaction conditions selected for this experiment show that a high conversion can be achieved in a short timeframe if the cofactor concentration is low. The TTN appears modest in this experiment because the cofactor to NAD^+ reductase ratio is low. Conditions that give a high initial activity, equating to a turnover frequency (TOF) of 24 s^{-1} (NADH produced per second per NAD^+ reductase applied to the carbon) or 84 400 h^{-1} are given in Entry 2. The NADH concentration was measured in situ spectrophotometrically, as in the experiment shown in Figure 1, and the measurement was stopped after just 35 min because of the saturation of the absorption from NADH, and hence a high conversion was not reached. The TTN that can be achieved with a set of enzyme-modified particles with low enzyme loading and high initial cofactor concentration (5 mM) is shown in Entry 3. For this experiment, only a starting and final measurement of NADH concentration were recorded, and hence the initial activity was not determined. At this high cofactor concentration, the conversion of NAD^+ to NADH remained incomplete even after 20 h of reaction. The resulting product solution from the experiment summarised in Entry 3 was analysed by HPLC in comparison to authentic β-NADH and β-NAD^+ standards, and this confirmed that only the correct bio-active form of NADH was generated by the particles (Figure S3).

Experiments shown in Entries 2 and 3 were conducted using the NiFe hydrogenase from *Desulfovibrio* (*D.*) *vulgaris* Miyazaki F in place of *E. coli* Hyd-2 (Table S1). These enzymes have very similar properties in terms of their catalytic bias, O_2 sensitivity and electro-activity (see Figure S4 and Ref. [15]).

For most industrial applications it is undesirable to use enzymes that have been extensively purified. Therefore, we tested whether it is possible to use the soluble cell extract of *R. eutropha* as a source of the NAD^+ reductase, which is known to make up ≈5% of the total soluble protein in this microorganism.[16] The enzyme-modified particles were prepared by adding a suspension of the carbon particles to a mixture of purified hydrogenase and the soluble extract that contained NAD^+ reductase. Upon the addition of a sample of these particles to a H_2-saturated solution of NAD^+ (1 mM), 50% conversion of NAD^+ to NADH was observed spectrophotometrically, which shows the feasibility of the use of minimally purified enzyme preparations for immobilisation on the carbon particles.

Parameters that affect H_2-driven NADH generation

We next explore parameters that affect the rate of H_2-driven NADH generation by enzyme-modified particles.

Experiments were conducted to explore the effect of changing the ratio of hydrogenase to NAD^+ reductase in the solution used to prepare the particles. As a result of the difficulty to assay individual enzyme activities on the particles, we were unable to determine whether the enzymes actually adsorb in the ratio supplied initially to the particles. Nevertheless, the results for H_2-driven NADH production by these particles (Table 2) show that the initial rate of NADH produced per mg of NAD^+ reductase is improved by increasing the relative loading of hydrogenase in the enzyme solution. An excess of hydrogenase on the particles is expected to increase the availability of electrons from H_2 for the NAD^+ reductase through the electronically conducting particle. The activity of the particles is quoted as both an initial activity [μmol min^{-1} per mg

Table 2. Effect of changing the relative quantity of immobilised hydrogenase and NAD^+ reductase on the rate of H_2-driven NADH generation.

Mole fraction of hydrogenase/ NAD^+ reductase	Initial activity[a]	Turnover frequency[b]
2.5	2.0 ± 0.1	6.3 ± 0.4
7.6	4.9 ± 0.3	14.5 ± 1.0
22.7	7.8 ± 0.5	24.6 ± 1.6

[a] Initial activity [$\mu mol\,min^{-1}$ per mg NAD^+ reductase] measured during the starting linear phase of the reaction; [b] Moles of NADH per second per mole of NAD^+ reductase.

NAD^+ reductase] to aid the comparison with enzyme data and as a turnover frequency [NADH per second per NAD^+ reductase catalyst]. The effect of pH on H_2-driven NADH production by sets of particles all taken from the same preparation and operated in H_2-saturated buffer solutions that contain NAD^+ (1 mM) at pH 6.0, 7.0 and 8.0 is shown in Figure 2a. The initial activities increase with pH, consistent with the net release of one proton during NADH production with H_2 as the reductant (Scheme 2), and the activity optimum of pH 8.0 reported for NAD^+ reduction by the NAD^+ reductase.[14] This shows that the enzyme-modified particle system should be applicable across a range of pH values relevant to biocatalysis. A further experiment (Figure 2a, open circles) was conducted in pure water (MilliQ) with no buffer present that also showed a significant rate of conversion of NAD^+ to NADH, between that of the conversion rates achieved at pH 6 and 7.

The effect of H_2 partial pressure was then examined in a series of experiments conducted at atmospheric pressure with different percentages of H_2 maintained throughout each run. The relative initial activities at 2, 10, 50 and 100% H_2 in N_2 are compared in Figure 2b, and a plot of the percentage conversion over the course of 40 min at both 2 and 50% H_2 in N_2 is shown in Figure 2c. Although the initial activity and overall conversion decrease as the partial pressure of H_2 is lowered, these results show the feasibility of the operation of NADH recycling at low H_2 levels, consistent with the low K_M for H_2 of the hydrogenase, *E. coli* Hyd-2, of 17 μM.[15a]

Re-use of particles

A key advantage of the immobilisation of the cofactor recycling enzymes is that they can be recovered readily from a batch reaction and re-used. Measurements of the concentration of NADH produced during a sequence of batch reactions in H_2-saturated NAD^+ solution (1 mM initial concentration) using a single set of particles are shown in Figure 3. The reaction was first allowed to proceed to ≈25% conversion to allow the determination of an initial rate from the linear reaction phase. Then the particles were separated from the reaction mixture by centrifugation and re-suspended in fresh H_2-saturated NAD^+ solution (again 1 mM) three times, and a new initial rate determination was performed. The particles retained 76, 64, and 46% of the original activity in the second, third and fourth cycles, respectively. This represents an average of

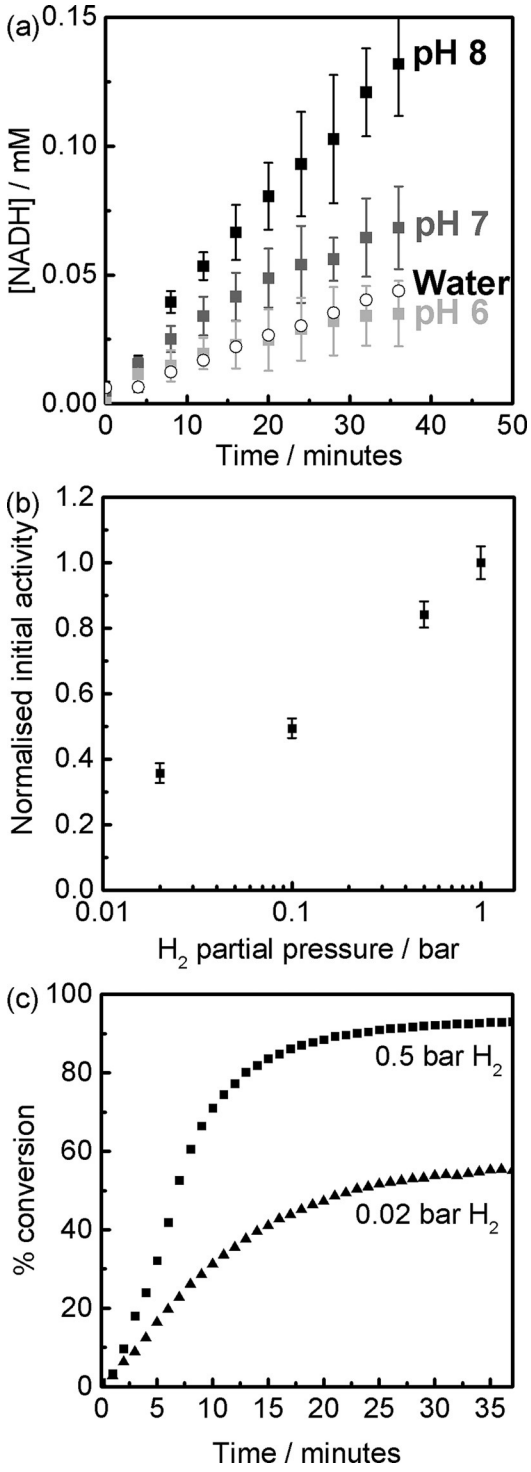


Figure 2. Parameters that affect H_2-driven NADH generation by particles modified with hydrogenase and NAD^+ reductase. a) Time series for H_2-driven NADH generation by identical aliquots of particles at a range of pH values; pH 6.0 (50 mM potassium phosphate, KBP), pH 7.0 (KPB), pH 8.0 (50 mM Tris-HCl) and un-buffered MilliQ water. Other conditions: 1 mM NAD^+, 33 °C, stirring, H_2 flow through the headspace. Initial activities were 0.8, 1.9, 3.4 and 1.0 $\mu mol\,min^{-1}$ per mg NAD^+ reductase respectively for the pH 6, 7, 8 and water series. Error bars reflect the average value ±1 standard deviation calculated from measurements performed in triplicate. b) Relative initial activity (normalised to the value at 100% H_2, 1 bar) of H_2-driven NADH generation under different H_2/N_2 mixtures (100, 50, 10 and 2% H_2) at overall atmospheric pressure. Other conditions: 0.1 mM NAD^+, 50 mM Tris-HCl pH 8, 30 °C. c) Plot of NAD^+ to NADH conversion from b) at 0.5 and 0.02 bar H_2.

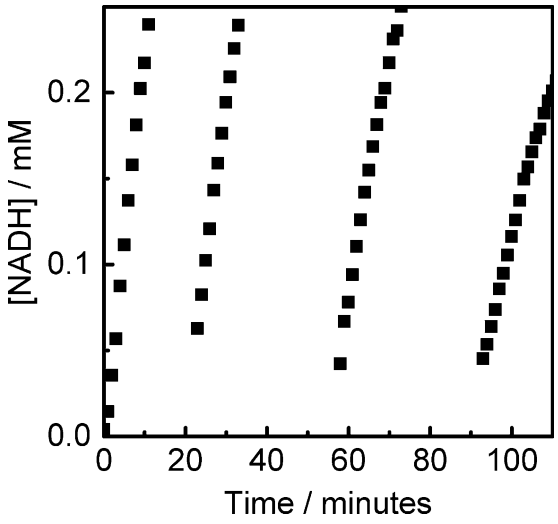


Figure 3. The enzyme-modified particles can be removed from solution by centrifugation and re-used. Each cycle was performed in fresh H_2-saturated buffer solution (50 mM Tris-HCl, pH 8, 30 °C) that contained NAD^+ (1 mM). The enzyme-modified particles were removed from solution and separated by centrifugation (7000×*g*).

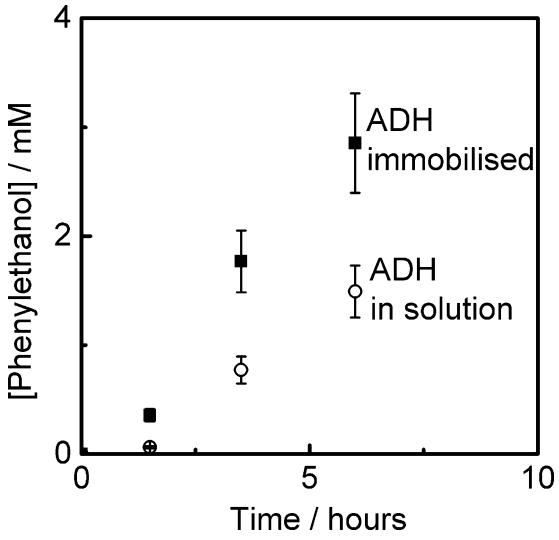


Figure 4. Comparison of the rate of phenylethanol generation using particles modified with hydrogenase, NAD^+ reductase and ADH (■) or hydrogenase and NAD^+ reductase supplying ADH in solution (○). Each experiment was performed in H_2-saturated buffer solution (50 mM Tris-HCl, pH 8, 2% DMSO, 22 °C) that contained NAD^+ (1 mM) and acetophenone (10 mM). The samples were left in a shaker vessel (500 rpm) with H_2 flowing through the headspace. At specific time points aliquots were removed and analysed by HPLC.

77% activity retained for each re-use, which was reproduced in separate experiments performed in a similar way. These activities are likely to be underestimates as some of the particles are lost during each separation step if the reactions are performed on small scale.

H_2-driven ketone reduction

The cofactor recycling system was then applied to supply NADH to an NADH-dependent enzyme, an ADH, for the hydrogenation of acetophenone to phenylethanol. The ADH was either used in solution with the NADH recycling particles or co-immobilised on the particles as shown in Scheme 1.

A batch of particles modified with hydrogenase and NAD^+ reductase was split into two fractions. One set of particles was washed and added to 1 mL of H_2-saturated reaction mixture (1 mM NAD^+, 10 mM acetophenone, 2% DMSO) with 0.34 mg ADH in solution, (Figure 4 ○). The other fraction of particles was combined with 0.34 mg ADH to allow the ADH to adsorb with the hydrogenase and NAD^+ reductase over 1 h, and washed before they were introduced into 1 mL H_2-saturated reaction mixture (Figure 4 ■). For each set of particles, the concentration of the reaction product, phenylethanol, was quantified by HPLC at three time points during the first 6 h to determine the initial activity and again after 21.5 h to analyse the extent of acetophenone to phenylethanol conversion. Over the first 6 h, the initial activity measured in moles of phenylethanol per second per mole of NAD^+ reductase was 1.41 s^{-1} for the particles with all three enzymes co-immobilised compared with 0.74 s^{-1} for the system with ADH used in solution, which thus indicates an approximate doubling of the rate of reaction by co-immobilising the NADH-dependent enzyme with the cofactor recycling system. This is particularly significant given that the amount of ADH is likely to be lower in the co-immobilised system because of the removal of any non-adsorbed enzyme in the washing step. For both experiments, the conversion of acetophenone to phenylethanol measured at 21.5 h was 98%, which shows that the H_2-driven NADH recycling system demonstrated here gives high levels of reactant to product conversion for an NADH-dependent hydrogenation with H_2 at atmospheric pressure. In both cases the total turnover number was >130 000 phenylethanol per NAD^+ reductase, which shows that the H_2-driven cofactor recycling system is stable over a large number of catalytic cycles.

HPLC traces for the reaction solution at 1.5 and 21.5 h for the particles with all three enzymes co-immobilised demonstrate the product purity that can be achieved with the H_2-driven cofactor recycling system (Figure 5).

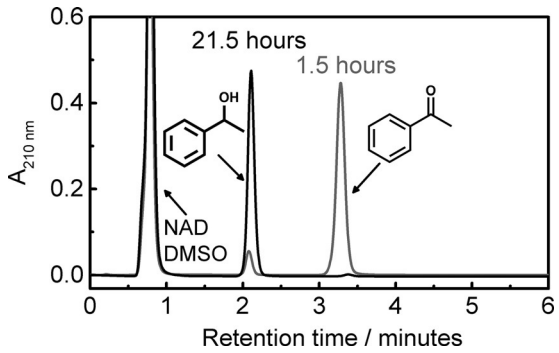


Figure 5. HPLC confirms the purity of the reaction product, phenylethanol, after H_2-driven acetophenone reduction by particles with co-immobilised hydrogenase, NAD^+ reductase and ADH; traces correspond to 1.5 (grey) and 21.5 h (black), reaction conditions: 10 mM acetophenone, Tris-HCl buffer, pH 8, 25 °C, 1 mM NAD^+, 2% DMSO.

Experiments were then conducted to examine the effect of changing the cofactor concentration for particles with NAD^+ reductase, hydrogenase and ADH co-immobilised. Identical aliquots of enzyme-modified particles were added to a H_2-saturated solution of 10 mM acetophenone and 2% DMSO that contained 1, 0.1 or 0.01 mM NAD^+ respectively. Initial activities (reported in Table 3 as turnover frequency: phenylethanol per second per NAD^+ reductase) measured over the first 6 h of the reaction decrease as the cofactor concentration is lowered but show that H_2-driven acetophenone hydrogenation is still possible even at just 0.01 mM NAD^+. The reactions were stopped after 21.5 h and had not necessarily reached completion at this time. The high turnover number of 655 moles of phenylethanol per mole cofactor achieved at 0.01 mM NAD^+, demonstrates that the cofactor is very stable with the H_2-driven NADH recycling system. Thus bio-transformations should be possible at a mole percentage of cofactor as low as 0.1%—a sufficiently small quantity that it would not be necessary to separate the cofactor from the chemical product.

Table 3. Comparison of the activity of H_2-driven acetophenone reduction using particles modified with hydrogenase, NAD^+ reductase and ADH in the presence of different cofactor concentrations.

Entry	[NAD^+] [mM]	Turnover frequency[a]	Turnover number[b]	Total turnover number[c]	Conversion [%][d]
1	1	1.41	9.85	135 000	>98
2	0.1	0.88	84.4	116 000	84
3	0.01	0.20	655	90 000	66

[a] Moles of phenylethanol per second per mole NAD^+ reductase recorded over the first 6 h; [b] Moles of phenylethanol per mole cofactor after 21.5 h; [c] Moles of phenylethanol per mole NAD^+ reductase after 21.5 h; [d] Conversion [%] after 21.5 h. Conditions: samples were shaken during reaction, with H_2 (1 bar) flowing through the headspace; 10 mM acetophenone, Tris-HCl buffer, pH 8, 25 °C.

Bio-catalysed hydrogenations using H_2 gas as the reductant should lead to no change in the solution pH as both H atoms of H_2 are incorporated into the final product and there is no additional byproduct from the reaction (Scheme 1). An additional experiment conducted in un-buffered pure water (MilliQ) that contained 1 mM NAD^+ and 10 mM acetophenone showed 90% conversion after 3 h, which shows that it is even possible to generate product without contamination from buffer ions (Figure S5).

Finally, the possibility to recover and re-use particles with co-immobilised NAD^+ reductase, hydrogenase and ADH was examined. A batch of particles was added to a solution that contained acetophenone (10 mM) and NAD^+ (1 mM). A conversion of 60% was determined after 6 h. The particles were then recovered by centrifugation (7000×*g*, 5 min) and added to a fresh solution of acetophenone and NAD^+. In the second cycle, a conversion of 90% was determined after 20 h, which demonstrates that the entire biocatalytic system can be handled as a composite heterogeneous catalyst and used more than once.

Discussion

We have demonstrated a H_2-driven system to recycle the biological cofactor NADH that involves carbon beads modified with hydrogenase and NAD^+ reductase over a range of reaction conditions both for NADH generation and for a H_2-driven hydrogenation reaction catalysed by an NADH-dependent ADH. The high conversion of NAD^+ to NADH (>98%) is achieved under an atmospheric pressure of H_2. If coupled with an NADH-dependent ADH, high levels of acetophenone to phenylethanol conversion (also >98%) are achieved with the ADH either in solution or co-immobilised on the carbon particles. The stability of the cofactor recycling system is demonstrated by a high total turnover number of >130 000 moles of product per mole of NAD^+ reductase. Despite the relatively low solubility of H_2 in water (≈1 mM at 1 bar) it is possible to drive a reaction that involves 10 mM acetophenone almost to completion under a H_2 atmosphere. The co-immobilisation of the ADH on the particles with the cofactor recycling enzymes leads to greater initial activity, and this is likely to arise from a high local concentration of reduced cofactor maintained at the surface of the particles. It is possible to operate the H_2-driven NADH recycling system at a low cofactor concentration of 10 μM, and cofactor turnover numbers of >650 under these conditions show that the NAD^+ and NADH are very stable if used with this enzyme particle system.

The immobilisation of enzymes by direct adsorption onto carbon beads provides a straightforward and new way to handle enzymes as heterogeneous catalysts for organic synthesis. Carbon is used widely as a support for molecular metal and nanoparticle catalysts, but there have been few industrial applications of enzymes on carbon beads or particles. One notable example is the use of bone charcoal as a support for invertase in industrial sugar processing.[17] Graphitic carbon electrodes are used widely for electrochemical studies of redox enzymes, in which the carbon surface seems to provide scope for the adsorption of a range of different enzymes. This suggests that the carbon particle approach should be extendable to a wide range of enzymes. The co-immobilisation of the cofactor recycling enzymes with an enzyme that performs a specific chemical transformation means that the entire biocatalyst system can be separated easily from the chemical product (e.g., by a simple filtration step) and re-used. Although there is a requirement for cofactor in solution, the ability to operate at low cofactor concentrations (as low as 10 μM) means that it may not even be necessary to separate the cofactor from the chemical product if the particles are used for the synthesis of fine chemicals. H_2 functions as a very clean reductant because it introduces no contaminants into the reaction solution, and there are no byproducts if it is used as the reductant for biocatalysed hydrogenations. These factors, together with the ability to operate in water with no buffer ions present, mean that the resulting reaction solution contains only product and trace cofactor in the solvent.

Conclusions

The system of enzyme-modified particles that we describe here offers a new way of working for biocatalysed hydrogenations, which deals with the challenges of enzyme immobilisation and cofactor recycling. Particular advantages of the H_2-driven approach to cofactor recycling that we demonstrate are the formation of a highly pure chemical product with low loading of cofactor and the possibility to recover and re-use the immobilised biocatalysts. Although we have focused on the hydrogenation of a ketone catalysed by an alcohol dehydrogenase, the approach we describe here should be applicable to many other NADH-dependent bio-transformations. Further work will explore the applicability of this H_2-driven NADH recycling system to alcohol dehydrogenases that operate on a range of substrates and to other NADH-dependent enzymes such as C=C bond reductases, and will compare the performance of these enzymes with H_2-driven and established cofactor recycling systems. The tolerance of the enzyme-modified particles to different organic solvents will need to be established to support operation on substrates with poor water solubility. It will be important to determine the feasibility to operate this biocatalysis system at elevated pressures and temperatures. Work is also underway to demonstrate H_2-driven biotransformations using enzyme-modified particles on a larger scale.

Experimental Section

All experiments were performed anaerobically in a N_2-filled glovebox (Glove Box Technology Ltd, <1 ppm O_2).

Enzymes

The NAD^+ reductase used is a construct of the NAD^+-reducing soluble hydrogenase from *R. eutropha* with inactive hydrogenase following a single amino acid substitution (I64A) in the hydrogenase large subunit;[18] this was purified similarly to methods described previously.[16] Two very similar hydrogenases were used and purified according to published methods; *Desulfovibrio vulgaris* Miyazaki F hydrogenase[19] and *Escherichia coli* hydrogenase 2.[15a] Details of the quantities used in each experiment are shown in Table S1. The ADH, alcohol dehydrogenase 105 (Johnson Matthey Catalysis and Chiral Technologies), was used as supplied without further purification; stock solutions were prepared in buffer (50 mM Tris-HCl, pH 8) at a concentration of 10 mg mL^{-1}. The activity of the ADH for NADH-linked reduction of acetophenone was determined to be 0.5 U mg^{-1} by a UV/Vis spectrophotometric assay. The schematic enzyme structures shown in Schemes 1 and 2 were prepared by using the PyMOL Molecular Graphics System, Schrödinger, LLC from crystallographic datasets: hydrogenase is represented by *E. coli* Hyd-1, PDB code 3USE; NAD^+ reductase is represented by ABC subunits of the soluble NADH-oxidising domain of *Thermus thermophilus* complex I, PDB code 2FUG, which is similar to *R. eutropha* HoxFU; and ADH is represented by rabbit muscle lactate dehydrogenase, 3H3F.

Chemicals

Acetophenone (Sigma), phenylethanol (Sigma), NAD^+ (Prozomix), acetonitrile (Sigma), carbon black particles (Black Pearls 2000, BP2000, Cabot Corporation) and buffer salts (Sigma) were used as received without further purification. All solutions were prepared with MilliQ water (Millipore, 18 MΩ cm).

Enzyme-modified particles

Stock solutions of carbon particles (20 mg mL^{-1}) were dispersed in water (for the experiments shown in Figure 2a) or buffer (for all other experiments, 50 mM Tris-HCl, pH 8) by sonication for at least 15 min. The required enzyme solutions were pre-mixed before the addition of an aliquot of the carbon particle suspension. The enzyme/particle mixtures were left at 4 °C for a minimum of 1 h for enzyme adsorption.

The enzyme-modified particles for H_2-driven NADH generation were used without washing. The hydrogenase and NAD^+ reductase must be co-immobilised on the carbon particles to sustain catalysis (as they must each exchange electrons with the particle to provide each half reaction with an electron source or sink); control experiments shown in the Supporting Information confirm that there is no activity for non-adsorbed hydrogenase and NAD^+ reductase, and therefore, they do not contribute to the activity recorded. The enzyme-modified particles for the H_2-driven NADH supply to ADH were washed to prevent ADH in solution from contributing to acetophenone reduction. Washing was achieved by repeated (×2) centrifugation steps (7000 × *g*, 5 min) followed by the addition of fresh buffer solution.

H_2-driven NADH generation followed by in situ UV/Vis spectroscopy

For the in situ detection of H_2-driven NADH generation, experiments were performed in a UV/Vis cuvette. An aliquot of H_2-saturated solution that contained NAD^+ was added to the UV/Vis cuvette (path length 1 cm, cell volume 1 mL, Hellma), which was then sealed with a rubber septum. An aliquot of enzyme-modified particles was injected with a gas-tight syringe. A cell holder (Agilent) with magnetic stirring and a Peltier element for temperature control was used, and time-course UV/Vis spectra were recorded by using a Cary 60 spectrophotometer (Agilent). The presence of particles in solution led to uniform light scattering across the entire spectral region monitored ($\lambda = 200$–800 nm), and a simple baseline correction was applied. The absorbance at $\lambda = 340$ nm or a ratio of absorbances at $\lambda = 260$ and 340 nm (Figure S6) was used to determine the concentration of NADH in solution at high and low cofactor concentrations, respectively.

In experiments in which the particles were re-used, the solution was removed from the UV/Vis cuvette after ≈20 min, and the particles collected by centrifugation (7000 × *g*, 5 min). The supernatant was replaced with fresh H_2-saturated solution that contained NAD^+ and monitored by UV/Vis spectroscopy in the same way. This was repeated multiple times.

H_2-driven acetophenone reduction with ex situ HPLC analysis

For H_2-driven acetophenone reduction, experiments were performed in a plastic vial (1.5 mL) with the lid removed. An aliquot of

enzyme-modified particles was added to a H_2-saturated reaction mixture (which contained NAD^+, acetophenone and 50 mM Tris-HCl buffer, pH 8, unless otherwise stated); the ADH was either co-immobilised on the particles or added into the reaction solution. A shaker vessel was designed and made in-house such that multiple tubes could be shaken at 500 rpm under a H_2 atmosphere with continuous H_2 flow through the headspace.

At well-defined time points, aliquots of solution were removed from each reaction vial, acetonitrile was injected (to approximately 20% v/v) and the supernatant was collected by centrifugation (10 min, 7000×*g*). The samples were passed through a centrifugal protein concentrator (Amicon) to remove any unadsorbed enzyme and then diluted 1:4 with MilliQ water. The acetophenone to phenylethanol conversion was detected by HPLC (Prominence, Shimadzu). Acetophenone and phenylethanol were separated by using a Chromolith® Performance 100–3 mm column with a mobile phase of 80% water and 20% acetonitrile at 1 mL min^{-1} with the column oven maintained at 40 °C. The substrate and product were observed at 3.28 and 2.21 min, respectively. The concentration of product was determined by comparison to concentration standards, and the conversion of acetophenone to phenylethanol was determined by comparison to ratio standards. No background reaction of acetophenone was detected over 20 h in either water or Tris-HCl buffer in the absence of the enzymes (Figure S7).

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