Enzyme-Modified Particles for Selective Bio-Catalytic Hydrogenation *via* H_2-driven NADH Recycling

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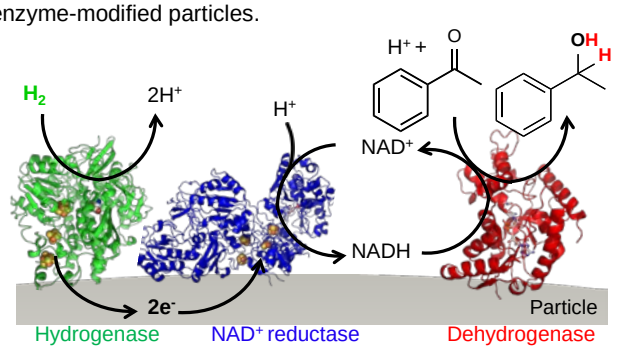
Abstract: We describe a new approach to selective H_2-driven hydrogenations that exploits a sequence of enzymes immobilised on carbon particles. Using a catalyst system comprising alcohol dehydrogenase, hydrogenase and an NAD^+ reductase on carbon black, we demonstrate greater than 98% conversion of acetophenone to phenylethanol. Oxidation of H_2 by the hydrogenase provides electrons, via 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, and also in un-buffered water, can function at low concentrations of the cofactor (10 μM NAD^+) and at H_2 partial pressures below 1 bar. Total turnover numbers >130,000 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] where 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, including reduction of ketones, alkenes and imines as well as reductive amination of ketones,[6] and it is becoming increasingly accepted that biocatalysts offer significant advances in chemo- and enantio-selectivity. This is particularly valuable in the fine chemicals and pharmaceutical sectors where, 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 the reduced nicotinamide adenine dinucleotide cofactor, NADH, or its phosphorylated derivative NADPH.[8] The cost of these cofactors means that stoichiometric addition is not a viable option for application of enzymes in synthesis, and recycling systems for the reduced cofactors are therefore required. Established methods for cofactor recycling are biocatalytic, and rely almost exclusively on super-stoichiometric addition of a sacrificial reductant and the appropriate enzyme for its oxidation (typically glucose dehydrogenase with glucose, or alcohol dehydrogenase 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 reduction of the oxidized cofactors, NAD^+ or $NADP^+$, have been demonstrated for H_2-driven NAD(P)H recycling with several bio-catalysed hydrogenations,[10-11] but, these enzymes tend to have limited stability and so have not become established for cofactor recycling.[12]

We have previously 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. 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 which facilitate 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 when supplied with reducing equivalents in the form of NADH generated by the enzyme-modified particles.



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 alcohol dehydrogenase (red) is supplied with NADH which is used for catalytic reduction of a ketone (here, acetophenone). Both hydrogen atoms of H_2 are incorporated into the product (here phenylethanol), allowing 100 % atom-efficient chemical synthesis.

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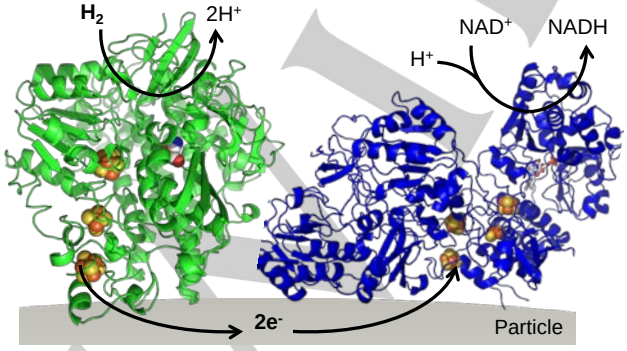
Here, we demonstrate advances on this system of enzyme modified particles for H_2-driven biocatalysis, and provide a detailed characterisation of how the particles function over a range of reaction conditions. We select as a test system the robust and reversible NADH-dependent alcohol dehydrogenase 105 from Johnson Matthey Catalysis and Chiral Technologies (ADH) which accepts a broad range of substrates, including linear and cyclic ketones and acetaldehyde. We use the reduction of acetophenone to phenylethanol as a test reaction. We show that co-immobilisation of the NADH regeneration enzymes with the ADH on particles, as shown in Scheme 1, leads to faster kinetics in comparison with use of the ADH in solution and allows use of low cofactor concentrations. Additionally, co-immobilisation makes it possible to handle all enzymes required for a H_2-driven hydrogenation as a single heterogeneous catalyst which can be readily separated from a reaction mixture and re-used. The use of H_2 gas for driving 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)), using H_2 as the reducing equivalent for reduction of NAD^+ to NADH, Scheme 2. 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 adsorption of the enzymes onto the carbon surface. Table S1 in the Supporting Information (SI) provides details of the quantities of enzyme and carbon used for each experiment. Due to the small scale of the experiments, and the difficulty in conducting individual enzyme activity assays on the carbon-immobilised enzymes, it was not possible to accurately quantify the amount or activity of each enzyme that adsorbed onto the carbon, but washing of the particles ensured that only adsorbed enzyme molecules contribute to the activity 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 *via* a chain of FeS clusters (spheres shown in elemental colours). These electrons can be transferred to the co-immobilised NAD^+ reductase (blue) for reduction of NAD^+ to NADH.

Fig. 1 shows results for NADH production by particles assembled as shown in Scheme 2. The percentage conversion of NAD^+ to NADH is determined from the change in the UV-visible spectrum (inset to Fig. 1), which shows >97% conversion of 70 µM NAD^+ after 30 minutes. The reaction rate is fairly linear up to *ca* 80% conversion and then starts to plateau. A decrease in rate during the course of the reaction is expected due to depletion of substrate for the NAD^+ reductase which has a Michaelis Menten constant, $K_M(NAD^+)$, of 197 µM.[14] Additionally, the drop in 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, thus lowering the thermodynamic driving force for H_2-driven NADH generation (assuming that 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] further lowering the overall activity of the particles. These effects are largely eliminated when the particles are used in conjunction with an NADH-dependent alcohol dehydrogenase (see later), because the reduced cofactor is then rapidly consumed by re-oxidation.

Control experiments confirming 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 Fig. S1 of the SI. Data are also presented in the SI (Fig. S2) showing 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 conversion of NAD^+ to NADH.

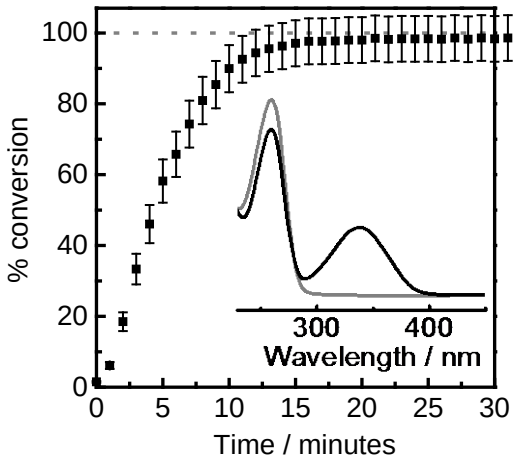


Fig. 1: Time course for conversion of NAD^+ to NADH using carbon particles modified with NAD^+ reductase and a hydrogenase suspended in a stirred, H_2-saturated solution of Tris-HCl pH 8.0 buffer at 30 °C; initial NAD^+ concentration: 70 µM. Inset: initial (grey) and final (black) UV-vis spectra,

measured *in situ* during the reaction.

Table 1 summarises 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. 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 of operating the cofactor recycling system. Initial activities were calculated from the rate of NADH generation during the starting linear phase of the reaction. 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.

Results from the experiment shown in Fig. 1 are summarised as 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 when the cofactor concentration is low. The TTN appears modest in this experiment because the cofactor to NAD^+ reductase ratio is low. Entry 2 showcases 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}. The NADH concentration was measured *in situ*, spectrophotometrically, as for the experiment shown in Fig. 1, and measurement was stopped after just 35 minutes due to saturation of the absorption from NADH, and hence a high conversion percentage was not reached. Entry 3 showcases the TTN that can be achieved with a set of enzyme-modified particles with low enzyme loading and high initial cofactor concentration (5 mM). 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, 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 using HPLC in comparison to authentic β-NADH and β-NAD^+ standards, and this confirmed that only the correct, bioactive, form of NADH was generated by the particles (SI Fig. S3).

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 minutes; [d] measured at 35 minutes; [e] measured at 20 h; N.D., not determined.

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

A requirement for using extensively purified enzymes is undesirable for most industrial applications. We therefore tested whether it is possible to use soluble cell extract of *R. eutropha* as a source of the NAD^+ reductase, which is known to make up ca. 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 containing NAD^+ reductase. On 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, showing the feasibility of using minimally purified enzyme preparations for immobilisation on the carbon particles.

Parameters affecting 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. Due to the difficulty in assaying individual enzyme activities on the particles, we were unable to determine whether the enzymes actually adsorb in the ratio initially supplied to the particles. Nevertheless, the results for H_2-driven NADH production by these particles, summarised in 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 *via* the electronically conducting particle. The activity of the particles is quoted as both an initial activity (in µmol min^{-1} per mg NAD^+ reductase) to aid comparison with enzyme data, and as a turnover frequency (in NADH per second per NAD^+ reductase catalyst).

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 / µmol 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

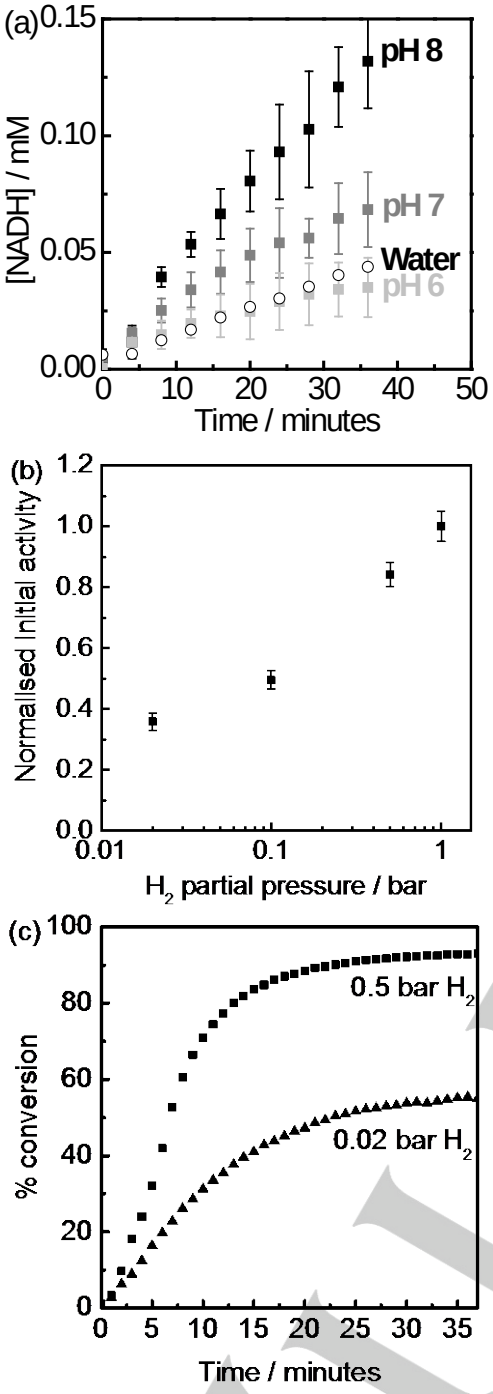


Fig. 2: Parameters affecting H_2-driven NADH generation by particles modified with hydrogenase and NAD^+ reductase. (a) Time series for H_2-driven NADH generation by identical aliquots of particles at a range of pH values; pH 6.0 (50 mM potassium phosphate, KBP), pH 7.0 (KPB), pH 8.0 (50 mM Tris-HCl), and un-buffered MilliQ water. Other conditions: 1 mM NAD^+, 33 ^oC, stirring, H_2 flowing through the headspace. Initial activities were 0.8, 1.9, 3.4 and 1.0 μmol 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 carried out in triplicate. (b) Initial activity of H_2-driven NADH generation under different H_2:N_2 mixtures (100, 50, 10 and 2 %) at overall atmospheric pressure to give different H_2 partial pressures. Other conditions: 0.1 mM NAD^+, 50 mM Tris-HCl pH 8, 30 ^oC. (c) Plot of NAD^+ to NADH conversion from (b) at 0.5 and 0.02 bar H_2.

Fig. 2(a) shows 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 containing NAD^+ (1 mM) at pH 6.0, 7.0 and 8.0. The initial activities increase with pH, consistent with the net release of one proton during NADH production with H_2 as reductant, Scheme 2, and the 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 (Fig. 2(a), open circles) was conducted in pure water (MilliQ) with no buffer present and also showed a significant rate of conversion of NAD^+ to NADH, between that of the pH 6 and pH 7 series.

The effect of H_2 partial pressure was then examined via 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 Fig. 2(b) whereas panel 2(c) shows a plot of percentage conversion over the course of 40 minutes at both 2 and 50 % H_2 in N_2. Although the initial activity and overall conversion decrease as the partial pressure of H_2 is lowered, these results show feasibility of operating 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 immobilising the cofactor recycling enzymes is that they can be readily recovered from a batch reaction and re-used. Fig. 3 shows 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. The reaction was first allowed to proceed to ca 25 % conversion to allow determination of an initial rate from the linear reaction phase. Then, three times, the particles were separated from the reaction mixture by centrifugation and re-suspended in fresh H_2-saturated NAD^+ solution (again 1 mM), and a new initial rate determination was carried out. The particles retained 76 %, 64 %, and 46 % of the original activity on the second, third and fourth cycles, respectively. This represents an average of 77 % activity retained on each re-use cycle, and this value was reproduced in separate experiments carried out in a similar way. These activities are likely to be underestimates since some of the particles are lost during each separation step when working on such a small scale.

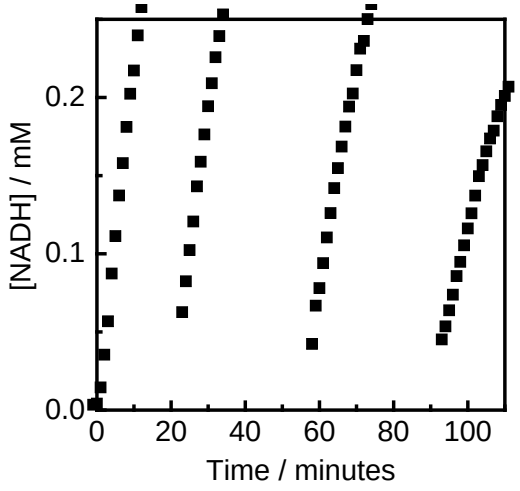


Fig. 3 The enzyme-modified particles can be removed from solution by centrifugation and reused. Each cycle was performed in fresh H_2-saturated buffer solution (50 mM Tris-HCl, pH 8, 30 ^{o}C) containing NAD^+ (1 mM). The enzyme-modified particles were removed from solution and separated by centrifugation (7000 × *g*).

H_2-driven ketone reduction

The cofactor recycling system was then applied to supply NADH to a NADH-dependent enzyme, an alcohol dehydrogenase (ADH), for the hydrogenation of acetophenone to phenylethanol, with the ADH 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 added to 1 mL of H_2-saturated reaction mixture (1 mM NAD^+, 10 mM acetophenone, 2% dimethyl sulfoxide, 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 hour, and the enzyme-modified particles were washed twice and separated by centrifugation, before being 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 hours to determine the initial activity, and again after 21.5 hours to analyse the extent of acetophenone to phenylethanol conversion. Over the first 6 hours 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, thus indicating 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 due to removal of any unadsorbed enzyme in the washing step. For both experiments, the conversion of acetophenone to phenylethanol measured at 21.5 hours was 98 %, showing 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, showing that the H_2-driven cofactor recycling system is stable over a large number of catalytic cycles.

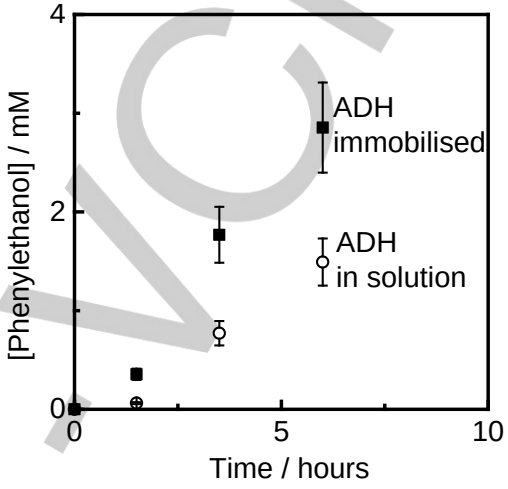


Fig. 4: Comparison of the rate of phenylethanol generation using particles modified with hydrogenase, NAD^+ reductase and ADH (■) or hydrogenase and NAD^+ reductase supplying ADH in solution (o). Each experiment was performed in H_2-saturated buffer solution (50 mM Tris-HCl, pH 8, 2 % DMSO, 22 ^{o}C) containing 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 using HPLC.

HPLC traces for the reaction solution at 1.5 hours and 21.5 hours for the particles with all three enzymes co-immobilised are shown in Fig. 5, and demonstrate the product purity that can be achieved with the H_2-driven cofactor recycling system.

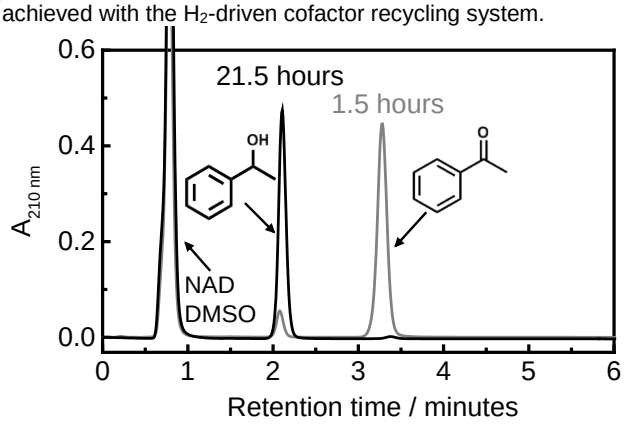


Fig. 5: HPLC confirms the purity of the reaction product, phenylethanol after H_2-driven acetophenone reduction by particles with co-immobilised hydrogenase, NAD^+ reductase and ADH; traces correspond to 1.5 hours (grey) and 21.5 hours (black), reaction conditions: 10 mM acetophenone, Tris-HCl buffer, pH 8, 25 ^{o}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 acetophone and 2 % DMSO, containing 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 hours 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^+. Reactions were stopped after 21.5 hours, 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 biotransformations 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.

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

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

Biocatalysed hydrogenations using H_2 gas as the reductant should lead to no change in solution pH since 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), containing 1 mM NAD^+, and 10 mM acetophenone, showed 90 % conversion after 3 hours, showing that it is even possible to generate product without contamination from buffer ions (SI Fig 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 containing acetophenone (10 mM) and NAD^+ (1 mM). A conversion of 60 % was determined after 6 hours. The particles were then recovered by centrifugation (7000 x g, 5 minutes), and replaced in a fresh solution of acetophenone and NAD^+. On the second cycle, conversion of 90% was determined after 20 hours, demonstrating 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 for recycling the biological cofactor NADH involving 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. High conversion of NAD^+ to NADH (>98%) is achieved under an atmospheric pressure of H_2. When 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 (ca 1 mM at 1 bar) it is possible to drive a reaction involving 10 mM acetophenone almost to completion under a H_2 atmosphere. 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, 10 µM, and cofactor turnover numbers of >650 under these conditions show that the NAD^+ and NADH are very stable when used with this enzyme particle system.

Immobilisation of enzymes via direct adsorption onto carbon beads provides a straightforward and novel way to handle enzymes as heterogeneous catalysts for organic synthesis. Carbon is widely used 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 widely used for electrochemical studies of redox enzymes, where the carbon surface seems to provide scope for adsorption of a range of different enzymes. This suggests that the carbon particle approach should be extendable to a wide range of enzymes. Co-immobilisation of the cofactor recycling enzymes with an enzyme carrying out a specific chemical transformation means that the entire biocatalyst system can be easily separated from the chemical product (for example 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 when using the particles for 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 when it is used as the reductant for biocatalysed hydrogenations. These factors, together with the ability to operate in water with no buffer ions present, means that the resulting reaction solution contains only product and trace cofactor in the solvent.

Conclusion

The system of enzyme-modified particles that we describe here offers a new way of working for biocatalysed hydrogenations, dealing 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 mole % cofactor, and the possibility to recover and reuse the immobilised biocatalyst particles. Although we have focused on hydrogenation of a ketone catalysed by an alcohol dehydrogenase, the approach we describe here should be applicable to many other NADH-dependent biotransformations. Further work will explore the applicability of this H_2-driven NADH recycling system to alcohol dehydrogenases operating on a range of substrates, and to other NADH-dependent enzymes such as C=C bond reductases, and compare performance to established cofactor recycling systems. 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 of operating this biocatalysis system at elevated pressures and temperatures. Work is also underway to demonstrate H_2-driven biotransformations using enzyme-modified particles at greater scale.

Experimental

All experiments were performed anaerobically in a N_2 glove box (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 previously described methods.[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 SI Table 1. 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} *via* a UV-visible spectrophotometric assay. The schematic enzyme structures used in Schemes 1-3 were prepared 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 has similarity with *R. eutropha* HoxFU; and ADH is represented by rabbit muscle lactate dehydrogenase, 3H3F.

Chemicals

The 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 experiments in Fig 2(a)) or buffer (for all other experiments, 50 mM Tris-HCl, pH 8) by sonication for at least 15 minutes. The required enzyme solutions were pre-mixed before addition of an aliquot of the carbon particle suspension. The enzyme particle mixtures were left at 4 ^oC for a minimum of 1 hour 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 in order to sustain catalysis (since they must each exchange electrons with the particle to provide each half reaction with an electron source or sink); control experiments shown in the SI confirm that there is no activity for unadsorbed hydrogenase and NAD^+ reductase, and therefore that they do not contribute to the activity recorded. The enzyme-modified particles for H_2-driven NADH supply to ADH were washed to prevent ADH in solution contributing to acetophenone reduction. Washing was achieved via repeated (x 2) centrifugation steps (7000 × *g*, 5 minutes) followed by addition of fresh buffer solution.

H_2-driven NADH generation followed by *in situ* UV-vis spectroscopy

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

In experiments where the particles were re-used, the solution was removed from the UV-Vis cuvette after *ca* 20 minutes and the particles collected by centrifugation (7000 × *g*, 5 minutes). The supernatant was replaced with fresh H_2-saturated solution containing NAD^+ and monitored using 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 (containing 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 in 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 approx. 20 % v/v) and the supernatant 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 in 4 with Milli Q water. Detection of acetophenone to phenylethanol conversion was carried out by HPLC (Prominence, Shimadzu). Acetophenone and

phenylethanol were separated using a Chromolith® Performance, 100-3 mm column with a mobile phase of 80 % water and 20 % acetonitrile, run at 1 mL min^{-1} with column oven maintained at 40 ^{o}C. The substrate and product were observed at 3.28 and 2.21 minutes, respectively. The concentration of product was determined by comparison to concentration standards; the conversion of acetophenone to phenylethanol was determined by comparison to ratio standards. No background reaction of acetophenone was detected over 20 hours in either water or Tris-HCl buffer, in the absence of the enzymes (SI Fig. S7).

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Notes and references

Keywords: biotransformations • oxidoreductase • heterogeneous catalysis • catalytic hydrogenation • atom economy

[1] H. U. Blaser, C. Malan, B. Pugin, F. Spindler, H. Steiner, M. Studer, *Adv. Synth. Catal.* **2003**, *345*, 103-151.
[2] F. Nerozzi, *Platinum Met. Rev.* **2012**, *56*, 236-241.
[3] R. Noyori, M. Kitamura, T. Ohkuma, *Proc. Natl. Acad. Sci.* **2004**, *101*, 5356-5362.
[4] M. Yoshimura, S. Tanaka, M. Kitamura, *Tetrahedron Lett.* **2014**, *55*, 3635-3640.
[5] L. Bonomo, L. Kermorvan, P. Dupau, *Chemcatchem* **2015**, *7*, 907-910.
[6] a) D. Monti, G. Ottolina, G. Carrea, S. Riva, *Chem. Rev.* **2011**, *111*, 4111-4140; b) B. M. Nestl, S. C. Hammer, B. A. Nebel, B. Hauer, *Angew. Chem.* **2014**, *53*, 3070-3095; c) P. Ramesh N, *Curr. Opin. Biotechnol.* **2001**, *12*, 587-604; d) A. Wells, H.-P. Meyer, *ChemCatChem* **2014**, *6*, 918-920.
[7] I. Agranat, H. Caner, J. Caldwell, *Nat. Rev. Drug Discov.* **2002**, *1*, 753-768.
[8] a) W. F. Liu, P. Wang, *Biotechnol. Adv.* **2007**, *25*, 369-384; b) W. A. van der Donk, H. M. Zhao, *Curr. Opin. Biotechnol.* **2003**, *14*, 421-426.
[9] Y. Ni, D. Holtmann, F. Hollmann, *ChemCatChem* **2014**, *6*, 930-943.
[10] L. Lauterbach, O. Lenz, K. A. Vincent, *FEBS* **2013**, *280*, 3058-3068.
[11] A. K. Holzer, K. Hiebler, F. G. Mutti, R. C. Simon, L. Lauterbach, O. Lenz, W. Kroutil, *Org. Lett.* **2015**, *17*, 2431-2433.
[12] a) L. Lauterbach, J. Liu, M. Horch, P. Hummel, A. Schwarze, M. Haumann, K. A. Vincent, O. Lenz, I. Zebger, *Eur. J. Inorg. Chem.* **2011**, *2011*, 1067-1079; b) E. van der Linden, B. Faber, B. Bleijlevens, T. Burgdorf, M. Bernhard, B. Friedrich, S. Albracht, *Eur. J. Biochem.* **2004**, *271*, 801-808; c) A. Weckbecker, H. Groeger, W. Hummel, in *Biosystems Engineering I: Creating Superior Biocatalysts, Vol. 120* (Eds.: C. Wittmann, W. R. Krull), **2010**, pp. 195-242.
[13] H. A. Reeve, L. Lauterbach, P. A. Ash, O. Lenz, K. A. Vincent, *Chem. Commun.* **2012**, *48*, 1589-1591.
[14] L. Lauterbach, Z. Idris, K. A. Vincent, O. Lenz, *Plos One* **2011**, *6*, e25939.
[15] a) M. J. Lukey, A. Parkin, M. M. Roessler, B. J. Murphy, J. Harmer, T. Palmer, F. Sargent, F. A. Armstrong, *J. Biol. Chem.* **2010**, *285*, 3928-3938; b) K. A. Vincent, N. A. Belsey, W. Lubitz, F. A. Armstrong, *J. Am. Chem. Soc.* **2006**, *128*, 7448-7449.
[16] L. Lauterbach, O. Lenz, *J. Am. Chem. Soc.* **2013**, *135*, 17897-17905.
[17] P. Monsan, D. Combes, *Biotechnol. Bioeng.* **1984**, *26*, 347-351.
[18] T. Burgdorf, A. L. De Lacey, B. Friedrich, *J. Bacteriol.* **2002**, *184*, 6280-6288.
[19] T. Yagi, K. Kimura, H. Daidoji, F. Sakai, S. Tamura, H. Inokuchi, *J. Biochem.* **1976**, *79*, 661-671.

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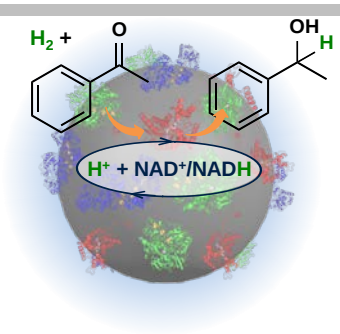
Heterogeneous hydrogenation bio-catalyst: a system of enzyme-modified particles allows H_2-driven NADH supply for NADH-dependent biocatalysed hydrogenations.



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Enzyme-Modified Particles for Selective Bio-Catalytic Hydrogenation *via* H_2-driven NADH Recycling