A modular system for regeneration of NAD cofactors using graphite particles modified with hydrogenase and diaphorase moieties†‡

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Pyrolytic graphite particles modified with hydrogenase and an NAD+/NADH cycling enzyme provide a modular heterogeneous catalyst system for regeneration of oxidised or reduced nicotinamide cofactors using H₂ and H⁺ as electron source or sink. Particles can be tuned for cofactor supply under different conditions by appropriate choice of hydrogenase.

Nicotinamide adenine dinucleotide (NAD) cofactors are essential redox agents for a range of oxidoreductases, utilised at stoichiometric levels to transfer reducing equivalents in the form of hydride. Regeneration systems for these expensive cofactors are required for many industrial biocatalysis applications, including stereoselective generation of chiral alcohols from prochiral precursors or resolution of racemic alcohols by dehydrogenases.1–4 Direct electrochemical regeneration is impractical at most electrodes because of high overpotential requirements and generation of bio-inactive products from single-electron transfer steps.1,5 Enzyme-based regeneration systems are thus attractive, in which H⁺ (⇌H² + 2e⁻) is transferred directly. A commonly employed NADH regeneration method couples NAD⁺ reduction to the oxidation of formate to CO₂ by formate dehydrogenase.1,2 Glucose-6-phosphate dehydrogenases have been used for reduction of either NAD⁺ or its phosphate derivative NADP⁺, and a range of coupled alcohol dehydrogenase reactions involving transformation of sacrificial substrates have been trialed.1,2,6

A class of heteromeric, bidirectional cytoplasmic hydrogenases reduce NAD⁺ or NADP⁺ at the expense of H₂, or link H⁺ reduction to NADH or NADPH oxidation (eqn (1)).7 Linking cofactor regeneration to the H⁺/H₂ couple is attractive since separation of desired products is simplified.

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\begin{align*}
2\text{H}^+ &\rightarrow 2\text{e}^- & \text{NAD}(\text{P})^+ + \text{H}^+ \\
\text{H}_2 &\rightarrow 2\text{e}^- & \text{NAD}(\text{P})H
\end{align*}
\]

Schneider and coworkers showed that the NAD⁺-reducing soluble hydrogenase from Ralstonia eutropha (SH; formerly Alcaligenes eutrophus) provides a convenient means to regenerate NADH using electrons from H₂.8 NADPH regeneration using hydrogenase-1 from hyperthermophile Pyrococcus furiosus has also been demonstrated.9 The limited stability of these multi-subunit hydrogenases has so far precluded practical utilisation in industrial biocatalysis.1

Here we uncouple the NAD⁺/NADH and H⁺/H₂ cycling moieties of the SH, and then re-couple these activities on electronically conducting particles to generate a modular, heterogeneous catalyst system for cofactor regeneration.

We showed recently that the NAD⁺-reducing (diaphorase) subunits of the SH, HoxFU, can be purified separately and function as an efficient electrocatalyst for NAD⁺ reduction and NADH oxidation when adsorbed on a pyrolytic graphite (PG) electrode, exchanging electrons directly with the graphite with no requirement for mediators and working very close to the theoretical potential E(NAD⁺/NADH) at each set of conditions.10 Pyrolytic graphite has also been used widely in direct electrochemical studies on H₂/H⁺ cycling by hydrogenases,11 and micron-scale particles of PG have been exploited to couple catalysis by pairs of redox enzymes.1,2,13

We build upon these concepts to develop a reversible regeneration system for NADH or NAD⁺ involving PG particles modified with a mixture of HoxFU and a hydrogenase, Fig. 1. The hydrogenase moiety of the SH is unstable towards loss of its flavin mononucleotide (FMN) cofactor and easily inactivated by O₂.14

By using PG particles as an electron ‘wire’, we are able to link HoxFU with more robust hydrogenases, Fig. 1 Schematic representation of NADH regeneration for an NADH-dependent oxidoreductase by pyrolytic graphite (PG) particles modified with HoxFU and a hydrogenase; iron sulfur electron relay cluster; catalytic active site. By appropriate choice of enzymes and conditions, the direction of catalysis can be reversed.
and to select the hydrogenase according to the required reaction conditions.

The thermodynamic potentials $E(H^+/H_2)$ and $E(NAD^+/NADH)$ are very closely spaced, meaning that the favoured direction of reaction (1) is finely balanced (see also ESI Fig. S1)). Providing that the half reactions can be catalysed at negligible overpotentials, the electrochemical driving force (at 25 °C, pH 7.0) for reduction of NAD$^+$ by H$_2$ at a 1000-fold excess of NAD$^+$ over NADH is ca. 180 mV at 1 bar H$_2$ and remains favourable down to a few ppm H$_2$. A similar driving force is available for oxidation of NADH by protons at a 1000-fold excess of NADH and 1 ppm H$_2$ (10$^{-6}$ bar). Many hydrogenases operate reversibly, and very close to $E(H^+/H_2)$.11

For bidirectional cofactor recycling we select *Escherichia coli* Hydrogenase 2 (Hyd-2), a good H$_2$ oxidiser and producer that adsorbs well on PG and operates at negligible overpotential.15 Cyclic voltammetry (see ESI Fig. S2A, B) confirms that HoxFU and Hyd-2 on PG should be capable of transferring electrons from H$_2$ to NAD$^+$ or NADH to H$_2$, under suitable conditions.

Conversion of NAD$^+$ to NADH is readily quantified by UV spectroscopy via the decrease in the ratio of absorbances, $A_{340 \text{ nm}}$/$A_{410 \text{ nm}}$ (see ESI Fig. S3)). We make use of this to determine initial rates for the activity of particles (prior to substantial substrate depletion and product build-up) using a method akin to a biochemical assay for H$_2$-driven NAD$^+$ reduction activity.16 Fig. 2A (■) shows that particles modified with HoxFU and Hyd-2 operating in buffer containing 2 mM NAD$^+$ produced NADH at an initial rate of 6.6 ± 0.9 nmol min$^{-1}$ per mg of PG. This represents an initial rate of 2.2±0.3 μmol min$^{-1}$ per mg of total HoxFU, almost certainly an underestimate of the rate per mg of electroactive HoxFU since not all the enzyme applied to particles is adsorbed or in a correct orientation for electron uptake. This rate therefore compares favourably with established values for formate dehydrogenase of 4–6 μmol min$^{-1}$ per mg of enzyme.4 A total conversion of 61% was achieved with an identical set of particles operating on 1 mM NAD$^+$ (inset). Control experiments in which aliquots of identical particles were incubated in the presence and absence of H$_2$ (ESI Fig. S4A) confirm that H$_2$ is essential for the NADH generation activity. Electrochemical studies have shown that NAD$^+$ reduction by HoxFU is NADH-inhibited, with an inhibition constant of ca. 0.2–0.3 mM.10 This is likely to explain the plateau in NADH production at ca. 50% conversion in Fig. 2. Note that in a coupled regeneration system used to supply NADH to a cofactor-dependent oxidoreductase, excessive build-up of NADH is prevented by concurrent consumption of the reduced cofactor, so product inhibition of HoxFU will have a much smaller effect on activity of the particles under cofactor recycling conditions. Particles are readily separated from a reaction mixture and washed, and can be reused for further cycles of NADH production (ESI Fig. S4Bj). Generation of NADH by particles modified with HoxFU and Pt (in place of a hydrogenase) is also possible (ESI Fig. S5)).8

Fig. 2A (○) also shows results for NAD$^+$ generation by PG particles modified with HoxFU and Hyd-2 operating in buffer containing 1 mM NADH under N$_2$, confirming that cofactor generation by the particles works in both directions.

![Fig. 2](image-url)
The concept of NAD(H) regeneration using electronically conducting particles modified with a diaphorase and a hydrogenase which are able to exchange electrons directly with the conducting surface provides a promising alternative to homogeneous enzyme regeneration systems, with H2 as a clean, cheap electron donor or easily separated product. Other particulate systems reported have made use of light-driven electron transfer from photo-sensitised Pt nanoparticles or titanium dioxide-carbon with a rhodium catalyst, with various sacrificial electron donors.

The modular approach to cofactor regeneration employed here allows a selection of appropriate enzyme moieties as catalysts for the H+/H2 and NAD+/NADH half reactions over a wide range of conditions, providing scope for tailoring catalytic ‘beads’ for cofactor supply to many oxidoreductases. Many different hydrogenases have been isolated and studied electrochemically, each found to have subtly different pH and temperature optima and O2-tolerance.\(^{7,11,14–19}\) Of particular interest, particles modified with an O2-tolerant hydrogenase such as R. eutropha MBH enable NADH generation in the presence of O2, providing opportunities for cofactor supply to the important family of cytochrome P450 mono-oxygenases.\(^{1,3}\) A diaphorase subcomplex of bovine Complex I (NADH:Ubiquinone Oxidoreductase) has also been shown to be an excellent electrocatalyst for NAD+/NADH cycling on a graphite electrode.\(^{22}\) Reports of hydrogenase catalysis on carbon nanotubes suggest that substitution of pyrolytic graphite particles with carbon nanotubes could extend the concept down to the nanoscale. Enzyme-modified particles may be a convenient way to introduce enzyme-catalysed cofactor regeneration into non-aqueous solvents, and particles can be readily separated from a reaction mixture by filtration or centrifugation for re-use. Modifying electronically conducting particles with hydrogenase and diaphorase moieties thus provides a versatile platform for recycling biological cofactors.

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Notes and references
§ Experiments were set up under anaerobic conditions in a glove box (MBraun, N<sub>2</sub> < 1 ppm O2, room temperature) and were carried out at room temperature. Solutions were prepared from purified water (MilliQ). HoxFU and MBH were isolated and purified as described in ref. 10 and 17 respectively. Enzyme-modified particles were prepared by sonicating ca. 4 mg of particles (from freshly sanded PG) in 100 µL of buffer for 10 s, followed by addition of the enzymes (HoxFU: ca. 10 pmol; and Hyd-2: ca. 1 pmol or MBH: ca. 1 pmol) to 10 µL aliquots of the particle suspension (i.e. 0.4 mg particles); based on a typical electrocative enzyme coverage on PG of 1 pmol cm\(^{-2}\) (ref. 12) this would support ca. 2 pmol of protein. Enzymes were allowed to adsorb over 30 min at 4°C. A H2-saturated solution of NAD\(^{+}\) was added to initiate NADH generation, and the reaction headspace was flushed with H2. For NAD\(^{3+}\) generation, the reaction was initiated by injection of particles into a N2-flushed solution of NADH. Aliquots were removed and the particles separated by bench-top centrifugation (7000 g) before spectroscopic analysis (Varian, Cary 50 spectrophotometer). For re-use the particles were collected by centrifugation and immersed in fresh H2 saturated NAD\(^{+}\) solution.

IR difference spectra were recorded using a Bio-Rad FTS-6000 FTIR spectrometer equipped with a diamond ATR accessory (DurasampIR II, SensIR Technologies).