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Review

Electrocatalysis by Hydrogenases: Lessons for Building Bio-Inspired Devices

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Um grande número de enzimas podem funcionar como excelentes eletrocatalisadores quando colocados sobre eletrodos ou superfícies de materiais condutores ou semicondutores. Em particular, nessa revisão serão abordadas as hidrogenases, que são enzimas com centros catalíticos contendo dois átomos de ferro ou um de ferro e outro de níquel para fazer a interconversão entre $2H^+$ e H_2 com altas frequências de *turnover*, entretanto os conceitos aqui mostrados podem também ser igualmente aplicados a outras enzimas redox. Utilizando as hidrogenases como exemplo, examinamos como a compreensão detalhada do comportamento eletrocatalítico de uma enzima pode servir de modelo para o desenvolvimento de dispositivos nos quais a enzima troca elétrons diretamente com uma variedade de materiais inorgânicos, incluindo grafite, semicondutores e pontos quânticos (*quantum dots*). Nesta revisão mostramos descobertas recentes de catalisadores compósitos de enzimas-materiais inorgânicos, e alguns dos desafios encontrados para a construção de um dispositivo enzimático, e as oportunidades advindas de sistemas baseados em catalisadores biológicos, incluindo células à combustível, foto-reatores de combustíveis e catalisadores acoplados para síntese química.

A number of redox enzymes function as excellent electrocatalysts when attached to electrodes or conductor/semi-conductor surfaces. A particular focus of this review is on hydrogenases, enzymes which use a di-iron or nickel-iron center to interconvert $2H^+$ and H_2 at extremely high turnover frequencies, although the concepts we highlight apply to a wider range of redox enzymes. Taking hydrogenases as our main case study, we examine how a detailed electrochemical understanding of the electrocatalytic behaviour of an enzyme can inform the development of devices in which the enzyme exchanges electrons directly with a range of inorganic materials, including graphite electrodes and particles, semi-conductor electrodes and quantum dots. We review recent developments in composite enzyme-inorganic catalysts, some of the biological and materials challenges in building devices based on enzymes, and the future opportunities for devices based on biological catalysts, including fuel cells, light-driven fuel production and coupled catalysis for chemical synthesis.

Keywords: electrocatalysis, bioelectrochemistry, hydrogenase, enzyme fuel cell, hydrogen production

1. Introduction

This review examines how electrochemical studies of biological redox catalysts provide insight into opportunities for intelligent assembly of devices in which enzymes are interfaced with inorganic materials. We take hydrogenases as our main case study because they have been widely investigated as immobilised electrocatalysts using electrochemical methods, and a large number of interesting applications for hydrogenases have been demonstrated. These range from energy applications in hydrogen fuel cells or light-driven hydrogen production to hydrogen-driven chemical synthesis. Many of the design principles and challenges described for hydrogenases are common to other bio-electrocatalysts, and we indicate parallels where appropriate.

Nature uses enzymes as modular catalysts, combining redox half reactions in different ways in biological cells for specific purposes.[1] For example, hydrogenase modules incorporating a H_2-oxidising nickel-iron catalytic center are found as components in bacterial proton-pumping respiratory chains where they are ultimately linked to the reduction of small molecules such as nitrate or O_2. They are also found as components of larger enzymes such as

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the NAD^+-reducing soluble hydrogenases which couple H_2 oxidation to regeneration of the biological cofactor, NADH, Figure 1a.[2] Hydrogenases differ in their sensitivity to attack or inhibition by small molecules such as O_2, CO and sulphides and often exhibit interesting intrinsic mechanisms for recovery from inactive or poisoned states.[3] The soluble hydrogenases are one example: although their main role is to couple H_2 oxidation to production of the reduced cofactor NADH, it is likely that reverse electron transfer is also important in providing low-potential electrons from NADH to re-activate oxidised inactive forms of the [NiFe]-hydrogenase module.[2] A fascinating bifunctional role was recently proposed for *Escherichia* (*E*) *coli* hydrogenase I (Hyd-1) by Volbeda *et al.*[4] (see Figure 1b). They suggested that crystals containing the hydrogenase dimer complexed with cytochrome b may represent a physiologically relevant construct which *in vivo* rapidly removes O_2 from cells by coupling H_2 oxidation at one [NiFe]-hydrogenase site to O_2 reduction at the [NiFe] site of its partner hydrogenase molecule via internal electron transfer through iron-sulfur cluster relay chains in the hydrogenase molecules. The ability of hydrogenases to deal with O_2 is covered in more detail in section 2 on interpretation of enzyme electrochemistry because it is critical to applications of hydrogenases.

As shown in Figure 1c, the activity of enzymes can be tuned by plugging them in to functional surfaces: these include electrodes at which the potential can be varied continuously, photo-sensitive semiconductor nanoparticles, and conducting particles of graphite at which the potential is set by the catalytic activity of a second enzyme. These various configurations are represented schematically in Figure 2. In each case, the hydrogenase accepts electrons from, or donates electrons to, the surface on which it is attached. The modular nature in which enzymes are exploited within biological cells is immediately conducive to their use as building blocks alongside inorganic materials in hybrid devices. For example, hydrogenases attached to a carbon cathode,[5] a dye-sensitised metal oxide nanoparticle,[6] quantum dot,[7,8] or a molecule of photosystem I,[9,10] have been shown to be efficient electrocatalysts for production of H_2 from protons in water, using electrons provided by a series of sacrificial donors, Figure 2a-d. The reverse reaction of hydrogenases has been exploited in fuel cells in which electrons from H_2 oxidation are taken up by a carbon-based anode and channelled via an external circuit to a cathode, usually for O_2 reduction, Figure 2e.[11-13] Electrons transferred through a graphite particle from carbon monoxide dehydrogenase to hydrogenase have been used to couple oxidative biocatalysis (CO oxidation to CO_2) to H_2 production: the water-gas shift reaction,

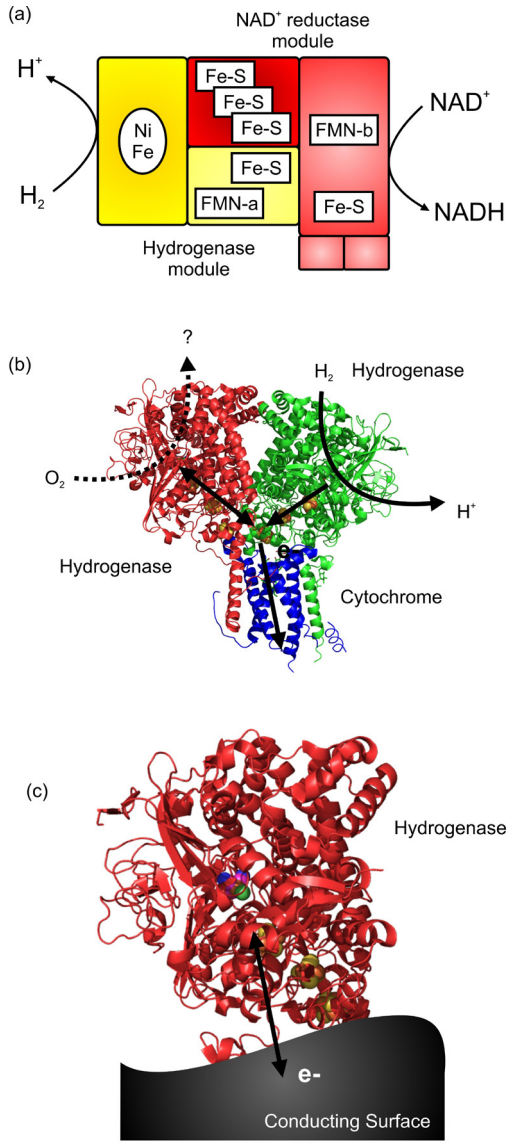

Figure 1. (a) Soluble NAD^+-reducing hydrogenase from *Ralstonia eutropha*. (b) X-ray crystallographic structure of *E. coli* hydrogenase I (Hyd-1) with a membrane-anchor cytochrome b attached (only half of the unit cell is shown; PDB code 4GD3).[4] Possible routes for electron transfer are shown by black arrows. (c) Schematic representation of the hydrogenase interfaced with an electrode such that the electrode surface replaces the physiological electron acceptor or donor and the enzyme can work in either direction. Enzyme structures were generated using Pymol (DeLano Scientific).

Figure 2f.[14] In reverse, electrons released into graphite from H_2 oxidation by an immobilised hydrogenase have been utilised to drive reductive catalysis by co-immobilised enzymes such as NAD^+-reduction for NADH supply to dehydrogenases, Figure 2g.[15] The inorganic components of these hybrid devices are mostly inexpensive materials: graphite or other carbons, metal oxides or metal sulfides. Although electrochemistry of proteins is well-developed on gold electrodes, use of a costly metal support for enzymes detracts from the benefits offered by biocatalysts built from

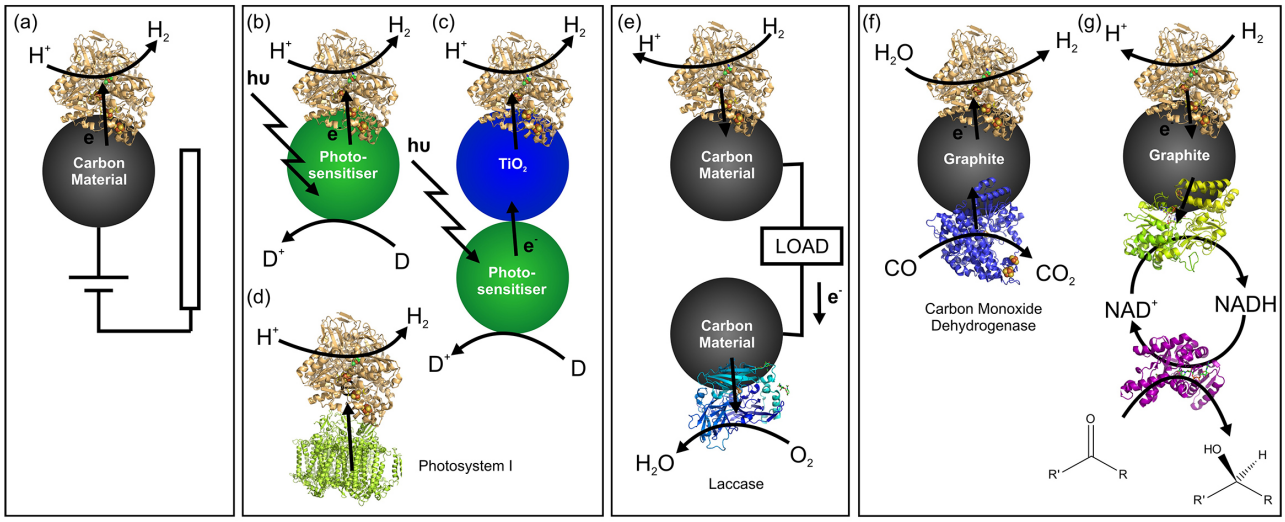

Figure 2. Modular combination of hydrogenases with electronically conducting particles and surfaces allows assembly of a variety of devices: (a) H_2 production at an electrode; light-driven H_2-production for hydrogenase on (b) a quantum dot, (c) a dye-sensitised TiO_2 nanoparticle or (d) coupled to photosystem I, (e) oxidation of H_2 in fuel cells; and hydrogenase catalysis coupled to other enzyme redox reactions on graphite particles: (f) the water gas shift reaction by hydrogenase and carbon monoxide dehydrogenase or (g) H_2-driven recycling of the cofactor NADH used to supply cofactor to an NADH-dependent dehydrogenase.

abundant metals such as iron or nickel/iron (hydrogenases and carbon monoxide dehydrogenase for CO oxidation) and copper (in enzymes for O_2 reduction).

Development of hydrogenase-based devices has been driven largely by curiosity or the desire to demonstrate or benchmark what is possible with impressively selective electrocatalysts that do not depend on precious metals. A number of advances in the longevity of hydrogenase electrodes and particle assemblies have been reported recently, but it remains unlikely that hydrogenase-based devices will make a significant contribution to high-level power generation or large-scale fuel production. Enzymes are more likely to be attractive catalysts in environments where their selectivity gives them strong advantages over metal catalysts. Real advances are being made in synthesis of functional bio-inspired catalysts or part-biological catalysts which may help to bridge the gap towards larger-scale applications. Enzymes are equipped with acidic and basic groups ready to donate or accept protons, hydrophobic channels for gas delivery and water-lined channels for rapid proton transport. A metal active site mimic alone is often not sufficient for effective electrocatalysis, but there are now interesting examples of catalysis by systems which incorporate some of the additional functional features of enzymes. These include synthetic catalysts with built-in proton acceptor or electron donor arms, as well as hybrid catalysts comprising synthetic cofactors assembled into apo-proteins, peptide fragments, or polymer coatings.[16-19] The boundaries between native enzyme catalysts and bio-synthetic catalysts are thus being bridged, and we can expect the range of enzyme-inspired catalysts for future devices to continue to develop. In parallel, the lessons learnt from efficient electrocatalysis by hydrogenases are important in defining requirements for the behaviour of new catalysts across a range of demanding conditions. Entrapment of enzymes in porous structures or covalent attachment may be necessary to improve their lifetime in devices.[20,21] In the arena of biocatalysis for chemical synthesis, enzyme longevity may not be such a problem since crude enzyme extracts are already used as essentially disposable catalysts in some areas of chemical synthesis. Hydrogenase-based systems may offer interesting benefits over currently-used enzyme-based methods for NADH cofactor recycling and catalysis for chemical synthesis, Figure 2g.[15]

2. Interpreting Electrochemistry of Hydrogenases

Two groups of hydrogenases are relevant in discussions of bio-electrocatalytic H_2 oxidation and H^+ reduction: the [NiFe]- and the [FeFe]-hydrogenases; their active sites are shown in Figure 3a and b.[3] Although these enzyme groups are unrelated in sequence and result from convergent evolution, they have surprisingly similar structural features. Both classes of hydrogenases incorporate relay chains of iron-sulfur clusters that facilitate the long-range electron transfer between the surface of the protein and the buried active site required for catalysis. These clusters are spaced about 10 Å apart in NiFe hydrogenases, as shown in Figure 3c. Moser, Dutton and co-workers have argued that since the rate of electron tunnelling has an exponential dependence on distance, natural selection has optimised

the distance between clusters to ensure that intramolecular electron transfer is not rate limiting in catalysis; a distance of less than about 14 Å between redox centres in a biological relay chain is common.[22,23] Iron-sulfur relay clusters are also important in allowing hydrogenases to exchange electrons with an electrode or other conductive support, meaning that electrons can be transferred to/from the buried active site for catalysis by imposing a potential at the electrode or surface. Interfacial electron transfer between an electrode and an enzyme such as hydrogenase has been successfully modelled using Butler Volmer kinetics, although for many enzymes the relationship had to be modified to account for a dispersion in orientation of enzyme molecules.[24,25] A mixture of orientations means that there is a range of distances between the outermost electron relay center and the electrode, leading to a range of interfacial electron transfer rate constants. It is possible to immobilise many hydrogenases such that interfacial electron transfer does not significantly limit catalysis, and the overall current observed is dictated by the rate of steps in catalysis. An exception is the [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* which has only a single Fe_4S_4 cluster attached to the [FeFe] active site and does not possess additional relay clusters.[25]

Consideration of a redox enzyme exchanging electrons with a solid support is not so different from the *in vivo* situation where an enzyme exchanges electrons with its partner donor or acceptor, such as *E. coli* Hyd-1 with cytochrome b shown in Figure 1b.[4] *In vivo*, Hyd-1 is presumed to be a H_2 oxidiser, consistent with the relatively positive potential of the haem centre in the electron acceptor cytochrome (–86 mV is reported for the related cytochrome b of *Ralstonia* (*R*) *eutropha* membrane bound hydrogenase[26]). At an electrode, the potential is controllable over a continuous range and hydrogenases can be driven to exhibit net oxidation of H_2 or reduction of H^+ (see below). For Hyd-1, the non-physiological H^+-reduction half reaction is strongly inhibited by the product H_2 and thus there is no net H^+ reduction detected under a H_2 atmosphere,[27] but other hydrogenases are highly active H_2 producers.[3]

The intricate dependence of enzyme redox catalysis on the potential at which electrons are delivered or removed means that electrochemical study of enzymes such as hydrogenases is a useful route into applications, contributing a 'voltage road-map' of the reactions of the enzymes at different potentials. We highlight some of the information that can be extracted from electrochemical studies of hydrogenases and its relevance in understanding how the enzymes will function in devices. A very wide range of electrocatalytic behaviours are exhibited across the ranks of the hydrogenases, offering diverse possibilities

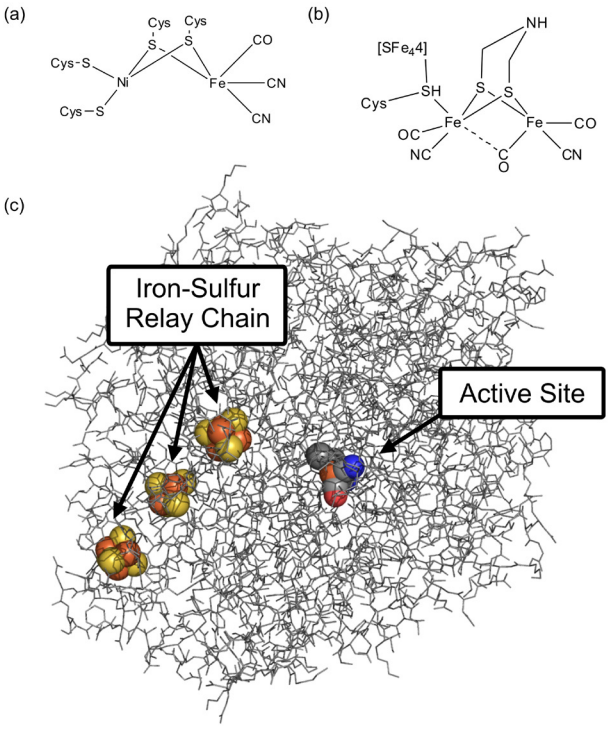

Figure 3. Hydrogenase active sites in catalytically active states: (a) [NiFe]-hydrogenase, (b) [FeFe]-hydrogenase. (c) Representation of the X-ray structure of *Desulfovibrio fructosovorans* [NiFe]-hydrogenase (PDB code: 1YRQ) showing the active site and iron-sulfur clusters as dark spheres.

for device development. Equipped with a detailed understanding of the catalytic function of hydrogenases, it is then possible to select 'circuit components' to suit a desired function, combining biological fragments from different organisms together with synthetic materials. In this way, larger electrocatalytic systems can be produced to fulfil specific tasks.

Electrocatalysis by hydrogenases adsorbed or covalently attached onto electrodes has become a well-established area, and voltammograms (current-potential traces) provide an immediate read-out of the catalytic behaviour of an enzyme over a given potential landscape under a given set of conditions (including pH, temperature and partial pressure of H_2 or O_2). Many of these diagnostic studies have been carried out with the hydrogenase directly adsorbed onto the edge surface of pyrolytic graphite with no further attachment chemistry. Voltammograms recorded under H_2 for several hydrogenase electrodes prepared in this way are shown in Figure 4. In each case, the scan commences at a negative potential, in a fairly reducing regime, and the electrode is then swept to positive potentials and finally back to the starting potential. Arrowheads indicate the direction of scan. In Figure 4a, the [NiFe] hydrogenase-2 (Hyd-2) from *E. coli* (pale gray line) is seen to be a good H_2 producer at low potentials: reduction of H^+ at the hydrogenase active site leads to electron flow from the

electrode into the enzyme and is defined as a negative current.[27] At higher potentials, the enzyme is also seen to be a good oxidiser of H_2; electrons released to the enzyme active site flow into the electrode giving a positive current. At potentials more positive than about –0.1 V, the enzyme begins to switch into a catalytically inactive state (anaerobic oxidative inactivation) and only switches back on when the potential is swept back towards more negative values; reductive activation is observed on the reverse scan in which the current recovers below about –0.1 V (see * in Figure 4a) and eventually rejoins the current level from the forward sweep. In order to avoid depletion of H_2 and build-up of H^+ in the solution region close to the electrode surface during high rates of catalysis by immobilized hydrogenases, the electrode is typically rotated rapidly.

Although the term 'bidirectional' is typically reserved for a special class of hydrogenases which are believed to operate in both the H_2 oxidation and H^+ reduction direction *in vivo* (such as *R. eutropha* NAD^+-reducing soluble hydrogenase, Figure 1a), many isolated hydrogenases are useful electrocatalysts in both directions. However, Hyd-1 from *E. coli* (Figure 4a, dark grey line) is a very poor H_2 producer due to inhibition of this reaction by the product, H_2. Lukey *et al.* show that even at just 0.3% H_2, the H^+ reduction current for Hyd-1 is negligible.[27] Figure 4a also shows that Hyd-1 commences H_2 oxidation at a small overpotential relative to the thermodynamic potential $E(2H^+/H_2)$. In practical terms, this slightly compromises the required operating voltage for immobilised Hyd-1 as an electrocatalyst for H_2 oxidation, and in a fuel cell, this detracts slightly from the zero current voltage (open circuit voltage) as well as the voltage during power production. In contrast, Hyd-2 behaves more like platinum, operating at no detectable overpotential in either the H^+ reduction or H_2 oxidation directions. Figure 4b shows a voltammogram for *Desulfovibrio* (*D*) *desulfuricans* [FeFe]-hydrogenase; like *E. coli* Hyd-2, this enzyme is a good H_2 producer at low potentials, and a good H_2 oxidiser at more positive potentials with no detectable overpotential. The [FeFe]-hydrogenase also converts reversibly to an oxidised inactive state at higher potentials, switching back on during the reverse sweep towards more negative potentials (* in Figure 4b).

Having established the basic operational voltage range for a given hydrogenase, and its ability to show net catalysis in the desired reaction direction (H_2 oxidation *vs.* H^+ reduction), the next important question is the robustness of the enzyme towards O_2. Electrochemistry has been particularly useful in probing the O_2 tolerance of hydrogenases, a phenomenon that has a complex dependence on potential.[29-31] Many of the first [NiFe]-hydrogenases to be isolated were found to be rapidly,

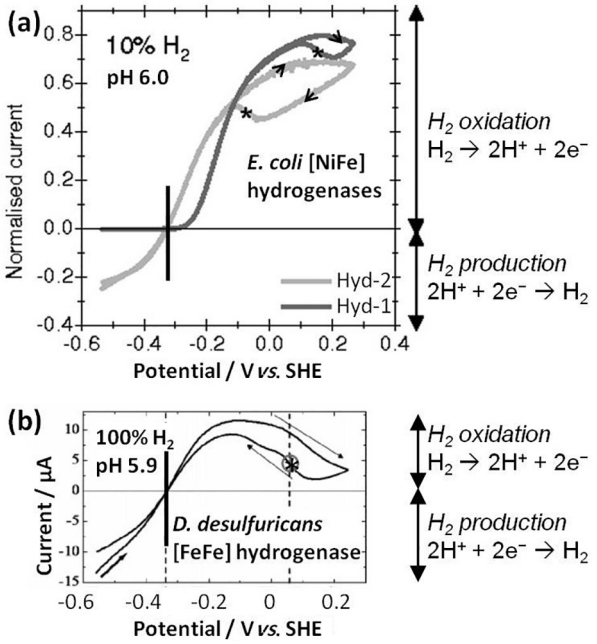

Figure 4. Cyclic voltammograms revealing electrocatalytic properties of hydrogenases adsorbed on a pyrolytic graphite edge electrode. (a) Two [NiFe] hydrogenases at 30 °C, a scan rate of 1 mV s^{-1} and an electrode rotation rate of 8500 rpm (adapted with permission from reference 27). (b) An [FeFe] hydrogenase at 10 °C, a scan rate of 10 mV s^{-1}, and an electrode rotation rate of 2500 rpm (adapted with permission from reference 28. Copyright (2006) American Chemical Society).

but reversibly, inactivated by O_2, requiring low-potential electrons for re-activation, while [FeFe]-hydrogenases were found to be inactivated irreversibly by O_2 under most conditions.[32] Demonstration that the [NiFe] membrane-bound hydrogenase from *R. eutropha* is able to sustain H_2 oxidation in the presence of O_2, albeit at a lowered catalytic rate,[33] led to a definition of O_2 tolerance in hydrogenases as their ability to function in the presence of O_2, with [NiFe]-hydrogenases being labelled as either O_2-sensitive or O_2-tolerant, and [FeFe]-hydrogenases being assumed to be all O_2-sensitive. Figure 5 shows an example of the current obtained during oxidation of H_2 at an electrode modified with *E. coli* Hyd-1 operating under different H_2/O_2 mixtures as the percentage O_2 in the mixture increases while the H_2 content is kept constant.[31] This gives an indication of the how the enzyme might function in a device operated on a mixed H_2/O_2 feed. At 6% O_2, 10% H_2, about half of the activity measured anaerobically at 10% H_2 remains.

Electrochemical examination of hydrogenase electrocatalysis over a range of potentials reveals the story to be more subtle and complex. Armstrong and co-workers have used cyclic voltammetry to investigate the activity of *E. coli* Hyd-1 at a range of safe H_2/O_2 mixtures as shown in Figure 6.[12] Also shown in Figure 6, are the responses of an electrode modified with the blue copper O_2-reducing enzyme, bilirubin oxidase. Figure 6a shows

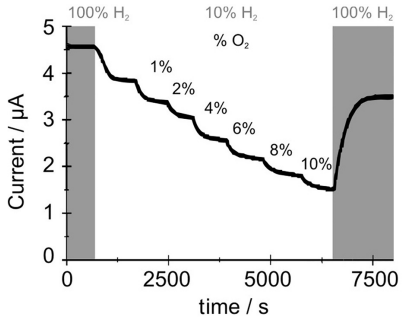

Figure 5. Electrochemical experiments on showing the ability of *E. coli* Hyd-1 to oxidise H_2 in the presence of O_2. The enzyme is adsorbed on a pyrolytic graphite edge electrode at pH 6, 30 °C and under the gas mixtures indicated, with an electrode rotation rate of 3000 rpm (adapted with permission from reference 31. Copyright (2013) American Chemical Society).

the electrodes functioning in separate compartments of a proton exchange membrane fuel cell, separated by a Nafion membrane. The hydrogenase electrode (the anode) is in a solution flushed with 1 bar H_2 while the bilirubin oxidase electrode (the cathode) is operating under 1 bar O_2. Under these conditions, the Hyd-1 electrode response resembles that in Figure 6a, while bilirubin oxidase is shown to be an excellent O_2 reduction catalyst, commencing catalysis at ca. +0.75 V, only just below the thermodynamic potential for the O_2/H_2O couple. Nafion is an imperfect barrier to O_2 transport, and a highly O_2 sensitive hydrogenase would be inactivated even by traces of O_2 crossing to the anode compartment from the cathode compartment,[33] so the O_2-tolerance of Hyd-1 is important even here.

Mixtures of H_2 and O_2 are safe at the extremes where the gas mix is either dilute in H_2 or dilute in O_2. Figures 6b and c show the response of the hydrogenase and bilirubin oxidase electrodes operating in the same compartment under high H_2 or low H_2 mixed in a safe ratio with O_2 or air. At 96% H_2/4% O_2 there is still substantial H_2 oxidation activity at the hydrogenase anode although O_2 causes a higher proportion of the enzyme to switch into an inactive state above about 0 V. The H_2 oxidation activity is observed against a background of direct O_2 reduction at bare sites on the graphite electrode which commences around 0 V. The fact that the net current is positive between about –0.2 and +0.1 V shows that there is a higher electron flux into the electrode from H_2 oxidation than the electron flow out of the electrode for direct O_2 reduction in this potential window. This would be the useful working potential window for the electrode in a fuel cell operated under a mixed feed of 96% H_2/4% O_2. The activity of the bilirubin oxidase electrode suffers under these conditions due to the lower availability of O_2. At 4% H_2/96% air, the bilirubin oxidase electrode fares somewhat better, but the current at the hydrogenase anode only shows net H_2 oxidation in a very small potential window with the hydrogenase being

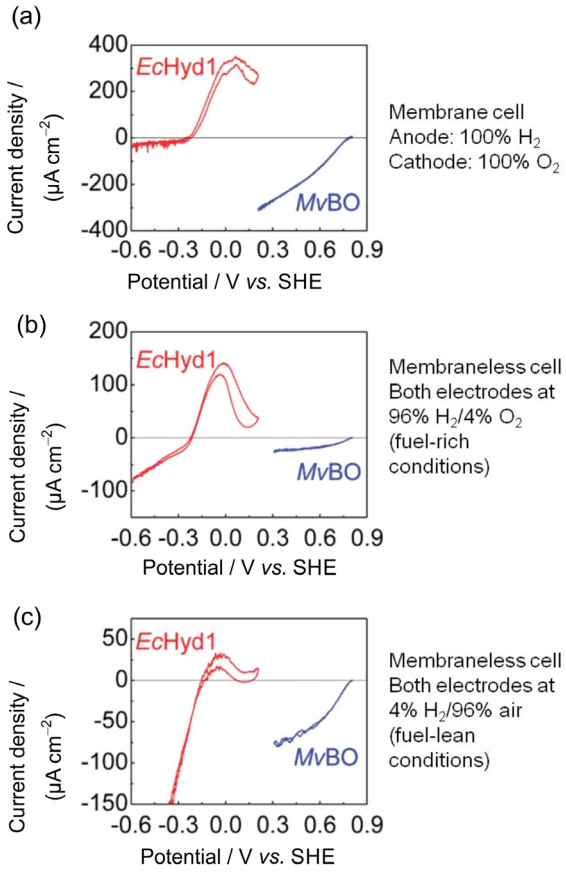

Figure 6. Practical demonstration of how O_2 and H_2 levels affect electrocatalysis of H_2 oxidation by a [NiFe]-hydrogenase (*E. coli* Hyd-1) and O_2 reduction by bilirubin oxidase immobilised on separate graphite electrodes. The electrodes are examined separately in a fuel cell configuration. (a) Hydrogenase electrode under 1 bar (100%) H_2 and bilirubin oxidase electrode under 1 bar (100%) O_2, with the electrodes separated by a Nafion membrane; (b) both electrodes under a mixed gas atmosphere in the fuel-rich safe regime: 96% H_2 / 4% O_2; and (c) both electrodes in the fuel-lean safe regime: 4% H_2/96% air (adapted with permission from reference 12. Copyright (2010) American Chemical Society).

completely inactive above about 0 V. Clearly *E. coli* Hyd-1 and bilirubin oxidase are able to function in a mixed H_2/O_2 feed fuel cell at either the low H_2 or the high H_2 safe extremes of the gas concentration ranges, but operational current will be very much dependent on the gas availability at either electrode.

Electrochemical investigations of the tolerance of H_2 oxidation by hydrogenases to O_2 have been more extensive than investigations in the H^+ reduction potential regime because of the practical problems of direct O_2 reduction at lower potentials at most electrodes which generates a complicating background current contribution (and probably also contributes reactive oxygen species to the solution). Pyrolytic graphite has a particularly large overpotential for O_2 reduction, but even here the reaction commences at about 0 V. Nevertheless, Armstrong and co-workers designed an elaborate protocol of multiple inhibition steps to investigate

H^+ reduction in the presence of O_2. Their experiments showed that H_2 oxidation by the [NiFeSe]-hydrogenase from *Desulfomicrobium* (*Dm*) *baculatum* (having a selenocysteine replacing one of the active site cysteines) is completely inactivated by O_2 (ca. 5 μM) at 0 V under 5% H_2 in N_2, but the enzyme maintains its ability to reduce H^+ at ca. 10 μM O_2 (1%) at –0.45 V.[34] This ability was exploited by Reisner *et al.* in light-driven H_2 production by *Dm baculatum* hydrogenase after O_2 exposure,[6] as discussed in section 4.2. *E. coli* Hyd-2 is also O_2-sensitive as far as its H_2 oxidation ability is concerned, but has been shown to reduce H^+ under 1.25% O_2 at –0.56 V.[27] Even the [FeFe]-hydrogenases from bacteria *D. desulfuricans* and *Clostridium* (*C*) *acetobutylicum*, and green alga *Chlamydomonas* (*Ch*) *reinhardtii* show some ability to reduce H^+ under 1% O_2.[35] These findings demonstrate that there is no simple definition of O_2 tolerance, but that a range of hydrogenases show activity for either H_2 oxidation or H^+ reduction in the presence of sub-atmospheric levels of O_2, and some, including the relatively robust [NiFe]-hydrogenases from *A. aeolicus* and *E. coli* are able to oxidise H_2 in air. A more sophisticated description of O_2 effects on hydrogenase activity is beyond the scope of this review.

Effects of O_2 need not necessarily be seen as a barrier to applying hydrogenases in devices; careful selection of 'the right enzyme for the job' should be possible in many cases. As discussed in the following section, the relatively robust *E. coli* Hyd-1 is well suited for applications in H_2 oxidation and has been exploited in membraneless H_2/O_2 fuel cells,[12] and in H_2-driven chemical synthesis,[15] whereas Hyd-2 has attracted attention for its abilities in H^+ reduction.[15] The [FeFe]-hydrogenases have been exploited primarily for H_2 production applications under strictly anaerobic conditions.[10,36]

Beyond tolerance to O_2, the ability of hydrogenases to select H_2 over other small gaseous molecules may also be important depending on the application. Again, electrochemistry provides an immediate picture of the voltage roadmap for inhibition if particular redox states of the active site are more susceptible to inhibition. A notable example of potential-dependent inhibition is that of [NiFe]-hydrogenases by H_2S which acts only in a high-potential window. Practically, this means that the hydrogenase is an effective electrocatalyst for H_2 oxidation even in the presence of sulfides providing it does not experience potentials above about 0 V (Figure 7).[37] It is interesting to compare the mild and reversible 'poisoning' of hydrogenases by small molecules such as sulfides and CO with the serious effects of these molecules on Pt for which inhibition is difficult to reverse. The sulfur tolerance of a [NiFe]-hydrogenase from *Thiocapsa* (*T*) *roseopersicina* has been exploited in a H_2/O_2 fuel cell operating on sulfur-contaminated fuel produced from microbial fermentation of paper pulp waste.[38]

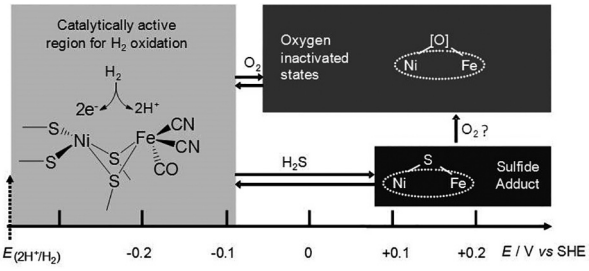

Figure 7. Scheme showing the potential window in which *Desulfovibrio vulgaris* Miyazaki F [NiFe]-hydrogenase is catalytically active for H_2 oxidation (light gray box); reversibly inactivated by O_2 (dark gray box) and reversibly inhibited by H_2S (black box). (Adapted with permission from reference 37. Copyright (2006) American Chemical Society).

The wide variety of hydrogenases now accessible in isolated form provides a versatile library of catalysts for device development: it is often possible to use electrochemical data to assist in the selection of enzymes that show the highest activity under a required set of conditions. This has led to many different hydrogenase-based devices, and materials and engineering challenges now stand alongside chemical and biochemical challenges in defining which applications may become viable on a larger scale in the future.

3. Materials Challenges

We focus attention in this review on direct exchange of electrons between hydrogenases and conductive surfaces. For device development, the use of enzymes in solution is generally unfavourable as this usually necessitates small soluble mediator molecules for electron transfer between the protein and conducting surfaces; this can lead to energy losses and slower kinetics, and mediators such as viologens are toxic. In order for immobilised enzymes to function as effective electrocatalysts, there must be rapid interfacial electron transfer between the protein and support, in addition to rapid electron transfer through the protein itself. This requires the protein to be orientated such that its outermost electron relay centres are close to the support. Attempts to improve orientation go hand-in-hand with developments in the stability of enzyme attachment on surfaces and the design of new electrode materials. Fortuitously, many hydrogenases adsorb spontaneously onto graphitic surfaces, in particular the edge surface of pyrolytic graphite, to give highly electroactive films.[3] It is very difficult to assess accurately the proportion of enzyme molecules able to engage in direct electron

transfer since the total coverage and electroactive coverage are rarely known. It is presumably advantageous that the rough surface of pyrolytic graphite provides cavities that allow enzyme molecules to adsorb with multiple contact points. Furthermore, the heterogeneous chemical nature of a freshly polished or abraded graphite surface probably provides a variety of carbon/oxygen functionalities for electrostatic and hydrophobic interactions with protein molecules. However, in moving from fundamental, diagnostic electrochemical studies of enzymes towards development of devices, it has become desirable to explore a wider range of materials and attachment strategies.

The triple challenge of (*i*) stabilising protein attachment whilst (*ii*) retaining direct electron transfer and (*iii*) even improving the number of molecules orientated for direct electron transfer has been tackled for both [FeFe] and [NiFe]-hydrogenases by taking advantage of charged patches on the protein surfaces close to the outer FeS relay cluster. Once an appropriately charged patch has been identified on the protein surface, usually of surface lysines or glutamic and aspartic acid residues, an opposite surface charge is introduced onto the electrode via a versatile diazonium coupling strategy that allows introduction of carboxylic acid or amine functionalities. The protein is allowed to orientate according to these charges, and is then covalently linked to the electrode via peptide bonds formed by a carbodiimide coupling reaction. Electrodes modified with [NiFe]-hydrogenase from *Desulfovibrio gigas* using this method led to current densities of almost 0.2 mA cm^{-2} and retained 90% of their activity after a week of continuous use.[21] To demonstrate that the orientation is dominated by electrostatic interactions with the surface, both the pH and the ionic strength were increased to disrupt the interactions.[21] This approach was extended by Léger and co-workers to [FeFe]-hydrogenases from *C. acetobutylicum* and *Ch reinhardtii* to give current densities of over 1 mA cm^{-2}.[39] Importantly, this study also confirmed that parameters such as the affinity of the enzymes for their substrate H_2 and the rate of entry of inhibitory CO into the enzymes are unchanged for direct adsorption *vs.* the covalent attachment on electrodes.[39] Direct adsorption of the hydrogenases still gives the highest initial activity, although poorer stability results in a faster drop-off in activity compared to that for covalently immobilised hydrogenase electrodes. A limitation of this attachment strategy is that the hydrogenase must possess a charged surface patch close to its electron-entry point, and this is not always the case. For example, in hydrogenase I from *Aquifex* (*A*) *aeolicus*, a hydrophobic helix is believed to help in anchoring the enzyme to the perisplasmic membrane, and it has been suggested that this accounts for the adsorption of this particular hydrogenase onto hydrophobic carbon nanotubes.[40]

A second major challenge for enzyme electrodes is the low coverage per geometric area dictated by the large footprint of protein catalysts (ca. 20 nm^2 for most [NiFe]-hydrogenases). This is partly compensated by very fast turnover rates for hydrogenases, estimated at 1,000-20,000 s^{-1}.[5,41-43] Nevertheless, planar electrodes modified with hydrogenase offer relatively low current densities, and to obtain currents suitable for practical devices, the electroactive area must be increased through the development of three-dimensional structures, just as with conventional fuel cell electrodes. One way that this has been achieved is via adsorption of hydrogenase onto conducting graphite platelets or other particulate carbon materials which are subsequently assembled into an electrode. Healy *et al.* compared the current response for electrocatalytic H_2 oxidation by *E. coli* Hyd-1 adsorbed directly onto a pyrolytic graphite edge electrode with the response at an electrode assembled from pyrolytic graphite particles exposed to approximately the same quantity of hydrogenase and attached using pH-neutral Nafion as binder; the current increased by almost 10 times at the particle electrode.[44] De Lacey and co-workers prepared high surface area electrodes by direct growth of carbon nanotubes onto a gold surface; amine groups were introduced onto the nanotubes by diazonium coupling, followed by carbodiimide coupling to a localised patch of carboxylic acid groups on *D. gigas* [NiFe]-hydrogenase surface, as discussed above.[45] Such an electrode produced a H_2 oxidation current of over 1 mA cm^{-2} for more than a month of continuous operation. Rather than covalently modifying the nanotubes themselves, Armstrong and coworkers exploited π-π stacking of pyrene derivatives on nanotubes to introduce carboxylic acid groups for coupling to surface amines of *E. coli* Hyd-I.[46] This electrode also gave currents greater than 1 mA cm^{-2} for 100 h. Kihara *et al.* tested direct immobilisation of *Thiocapsa* (*T*) *roseopersicina* [NiFe]-hydrogenase onto the hydrophobic surface of a carbon nanotube forest for H_2 production, but their electrodes exhibited sluggish H^+-reduction behaviour.[47]

Electrodes for enzymatic devices are not limited to carbon-based materials and, spurred on by the possibilities offered by visible light-driven catalysis, semiconducting materials have been investigated as conducting supports for hydrogenases and as photoactive materials, in particular anatase TiO_2 and CdX where X=S or Te. Metal oxide films are promising materials for protein (ad)sorption as they offer a highly functionalised surface whose charge can be modified according to the pH. For example, the

most commonly used metal oxide, TiO_2, has an isoelectric point of ca. 6.3 and can exhibit positive or negative surface charges in a biologically relevant pH window, equation 1.

$$Ti–O^- \leftrightarrow Ti–OH \leftrightarrow Ti–OH_2^+ \quad (1)$$

In addition, the band gap of TiO_2 is a function of pH (decreasing by 0.059 V for every increase in pH unit) and so consideration of the potential of electrons provided by the material is also essential. Solution conditions must also be finely tuned, as ions such as phosphate can adsorb strongly on TiO_2 and either help or hinder protein adsorption. Hydrogenase devices have also been built from 'quantum dots', semiconducting materials with dimensions between 1-50 nm for which absorption spectra are determined solely by size. Sustained enzyme turnover has been demonstrated, although in some cases the stability of biomolecules in contact with the relatively reactive surface of these materials may limit their effectiveness.[48]

These approaches to functionalising materials with hydrogenases have been used to make electrodes for use in fuel cells and hydrogen production devices, and the concept has been taken further by coupling multiple enzymes together to catalyse the formation or regeneration of useful chemicals. Next we review several examples of these applications and the future outlook for a range of bio-hybrid devices.

4. Hydrogenase-Based Devices

4.1 Hydrogen fuel cells

A conventional proton exchange membrane H_2/O_2 fuel cell consists of two high surface area electrodes comprising finely divided Pt catalysts, separated by the membrane, usually Nafion. Humidified gases are flowed into the two electrode compartments and the current is collected at graphite plates contacting the electrodes. Blue copper oxidases and hydrogenases have attracted attention for both the cathode and anode catalysts respectively because they can potentially be produced more cheaply than precious metal catalysts and they are relatively insensitive to poisoning by trace contaminants such as CO and H_2S which arise during production of H_2 from steam reforming of methane. Furthermore, O_2 reduction commences at a lower overpotential at electrodes modified with blue copper oxidases (laccase or bilirubin oxidase) than at platinum.[49] While developments that would allow enzymes to compete with platinum on the scale of large power generation have not occurred, an understanding of how enzymes function in fuel cell devices is providing insight into what can be achieved in fuel cells with the best possible functional catalyst, albeit they are short-lived. If nothing else, the ability of enzymes to catalyse H_2 oxidation and O_2 reduction efficiently using only base metals such as Ni, Fe and Cu is providing inspiration to chemists seeking to develop new fuel cell catalysts.

The existence of [NiFe]-hydrogenases with sufficient tolerance to O_2 that they are able to operate in H_2/O_2 mixtures, has allowed demonstration of membraneless fuel cells operating on safe H_2/O_2 mixtures. The absence of a membrane greatly simplifies fuel cell design. The better the O_2-tolerance of the hydrogenase, the greater the current under mixed fuel/oxidant conditions. The first demonstration of a membraneless enzyme H_2/O_2 fuel cell utilised the substantially O_2-tolerant membrane bound hydrogenase from *R. metallidurans* and a laccase, both adsorbed onto pyrolytic graphite strip electrodes, operated under fuel-lean conditions (3% H_2 in air, a safe, non-flammable, mix).[11] This cell showed a high open circuit voltage (0.95 V compared to a theoretical maximum of 1.23 V) although, unsurprisingly under these demanding conditions, the power density was small (5.2 μW cm^{-2}). Addition of 1% CO had no detectable effect on the current, neatly showcasing the selectivity benefits of hydrogenases over Pt.[11]

The behaviour of membrane-less enzyme H_2/O_2 fuel cells was examined in more detail by Wait *et al.* who compared identical cells with and without a membrane, and with different fuel/oxidant ratios.[12] This time the anode was constructed by adsorbing the O_2-tolerant Hyd-1 from *E. coli* onto graphite, while the cathode had bilirubin oxidase covalently attached to the graphite surface. Voltammograms for these electrodes in the fuel cell under 3 fuel/oxidant scenarious were presented in Figure 6: (*i*) the electrodes separated by a membrane and each compartment receiving pure gas (H_2 or O_2); (*ii*) no membrane, and a fuel-lean regime (4% H_2/21% O_2); (*iii*) no membrane, and a fuel-rich regime (96% H_2/4% O_2). Scenario 1 gave the highest open circuit voltage and power density (0.99 V and 63 μW cm^{-2}, respectively), as expected from the high fuel/oxidant concentrations permitted with membrane-separated compartments. However, a small amount of O_2 crossover led to reversible inactivation of Hyd-1 at anode potentials above 0 V. Under fuel cell operation, the anode activity could be recovered by a short period at open circuit conditions, which presumably imposes a low enough anode potential to re-activate the hydrogenase. As expected, in the absence of a membrane and with a mixed H_2-O_2 feed, the inactivation of Hyd-1 was more pronounced. Interestingly, in fuel-rich conditions the inactivation experienced on running at high anode potential could be reversed by a period at open circuit voltage, but

under fuel-lean conditions inactivation was substantial and could not be reversed by returning to open circuit conditions. This is understood by reference to the individual anode-cathode voltammograms in Figure 6c. Under the fuel-lean/O_2-rich conditions the cathode exhibits much greater electrocatalytic current with respect to the anode, so under fuel cell operation, the anode is forced to high potential to match the cathode current, thus readily tipping the hydrogenase over into its oxidatively inactivated state which cannot easily be re-activated at low H_2 levels. The hydrogenase anode could be 'jump-started' by connecting it to an identical hydrogenase electrode which had been exposed to the same fuel/oxidant mix, but had not been connected to the cathode. This exemplifies the complex relationship between hydrogenase activity, potential and O_2 concentration, and makes an interesting link to the physiological situation where it has been hypothesised that pairs of *E. coli* Hyd-1 molecules may supply electrons to re-activate one-another (see section 1).[4]

The issues of hydrogenase inactivation in fuel cells were addressed again by Ciaccafava *et al.* in analysis of a Nafion membrane-based fuel cell involving *A. aeolicus* Hyd-1 and bilirubin oxidase covalently attached to single walled carbon nanotube electrodes.[13] The anode environment was nominally 100% H_2 in this case, although some O_2 crossover from the cathode (supplied with 100% O_2) is likely. Even under anaerobic conditions, *A. aeolicus* Hyd-1 exhibits inactivation at potentials above about 0 V,[29] and the enzyme is reported to be damaged irreversibly at potentials higher than about +0.4 V.[13] Ciaccafava *et al.* demonstrated the importance of balancing the electrocatalytic half reaction rates at the anode and cathode in order to avoid driving the anode to high potentials; the most stable fuel cell operation was observed when the anode half reaction current outcompetes the cathode current, and the anode achieves sufficient current at potentials close to the $2H^+/H_2$ couple potential. The Hyd-1 of hyperthermophilic *A. aeolicus* has maximum activity at 85 °C, and thus it was possible to operate this fuel cell with the anode maintained at 60 °C, although the cathode was kept at 20 °C. The elevated anode temperature, in combination with the high surface area offered by the carbon nanotube electrodes, led to much larger power densities than observed for planar graphite electrode cells, and a power density of 300 μW cm^{-2} was achieved with well-matched cathode/anode current densities.[13] The covalent enzyme attachment also meant the fuel cell exhibited relatively good stability (retaining 60% of its activity after 24 h use).

The possibility of electricity generation from H_2 and O_2 using enzyme catalysts is firmly established and it is likely that continued advances will yield sufficiently optimised current and stability for working cells for specialised, low power applications. Competing O_2 reduction by hydrogenases remains a challenge: although a number of substantially O_2 tolerant [NiFe] hydrogenases have been identified and demonstrated in fuel cell devices, the mechanism for hydrogenase recovery from O_2-inactivation necessarily draws on electrons generated from H_2 oxidation thus lowering the power output. Even with the most O_2 tolerant hydrogenases, operation on mixed H_2/O_2 feeds thus leads to lowered anode performance via short circuiting at the anode. Direct O_2 reduction at bare regions of the graphite will add to this effect below about 0 V. Some headway has been made recently in stabilisation of enzyme electrodes and construction of high surface area electrodes for enzyme-based H_2 fuel cells, with carbon nanotube electrodes looking promising in this context.[13,46] What is needed now is sustained and rational efforts to improve bio-electrodes with a focus on stability, enzyme orientation and coverage, as well as high conductivity throughout the electrode material.

4.2 Hydrogen-production devices

Hydrogenases are also inspirational for their ability to produce H_2 from protons in water at high catalytic rates and close to the H^+/H_2 potential. A range of photo-hydrogen production devices involving hydrogenases or bio-inspired catalysts has been reviewed recently,[50] so we do not attempt to offer a complete survey of the developments in biohybrid H_2 production, but instead intend to highlight key challenges and opportunities relating to hydrogenase electrocatalysis. Hydrogenase-based devices for H_2 production rely upon electron transfer to the hydrogenase either from an electrode or from a sacrificial donor via a photo-activated electron transfer step. As with H_2 fuel cells, two key challenges in the development of devices are the size of hydrogenases, which leads to low surface coverage, and their limited stability over many hours. Additional materials and engineering challenges arise in photo-catalytic bio-devices in terms of the stability of the entire set of system components under sustained illumination.

Of the two main classes of hydrogenase, it is generally the [FeFe]-type which show the greatest catalytic bias towards H_2 production. A device demonstrated by Hambourger *et al.* retained the well-established hydrogenase-on-carbon motif for a H_2 producing cathode involving *C. acetobutylicum* [FeFe]-hydrogenase A (HydA) adsorbed on carbon felt, Figure 8a. High current densities are achieved at the hydrogenase electrode, with a waveshape closely resembling that at Pt.[5] The driving force for H_2 production at the high surface area

hydrogenase cathode was provided by photo-energised electrons supplied from the sacrificial donor, NADH via a porphyrin-sensitised TiO_2 photoanode. The high cost of NADH means that it is not viable to consume this cofactor as a sacrificial donor, and the authors note that a future goal of this research is to incorporate NAD^+-dependent biomass oxidation reactions into the anode compartment such that the net reaction of the cell is H_2 production from biomass. Oxidation of NADH without a catalyst requires a large overpotential relative to the NAD^+/NADH couple, hence the need for photo-activated electrons at the anode. The reduction of H^+ by NADH in the absence of illumination is actually a thermodynamically favourable process at pH 7, low level H_2 and a high ratio of NADH:NAD^+. For example, at atmospheric H_2 (0.5 ppm), and a 1000-fold excess of NADH, the cell potential for this reaction, E_{cell} = 182 mV at pH 7. In practice, this can only be achieved with ideal catalysts which operate reversibly at the thermodynamic potentials for each of the half reactions. Efficient NADH oxidation without a detectable overpotential has been

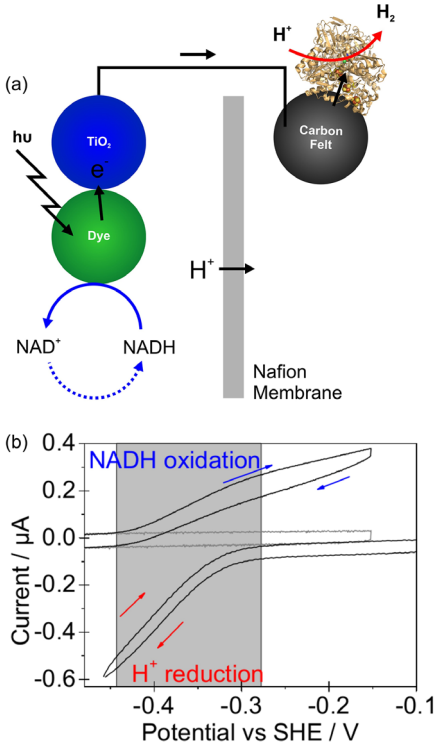

Figure 8. Coupling of NADH oxidation to H^+ reduction. (a) A device demonstrated by Hambourger *et al.* for photoactivated NADH oxidation at a porphyrin-sensitised TiO_2 anode coupled to H^+ reduction by an [FeFe]-hydrogenase carbon felt cathode.[5] A longer goal of this work was to achieve biomass oxidation at the photo-anode, recycling NADH (dotted arrow). (b) Cyclic voltammograms demonstrating that under appropriate conditions, H^+ reduction by NADH is thermodynamically favourable, and is feasible with ideal electrocatalysts, here the HoxFU NAD^+-reductase moiety of *R. eutropha* soluble hydrogenase (1 mM NADH), and Hyd-2 from *E. coli* (under N_2), both at pH 7. Figure 8b is reproduced with permission from reference 52.

demonstrated at several enzyme-modified electrodes,[51,52] indicating that ideal catalysts do exist and that it should be possible to assemble a H_2-producing fuel cell device (NADH oxidising anode and H^+ reducing cathode) from two enzyme electrodes (see Figure 8b),[15] without the need for photo-activation. Further challenges in the proof-of-concept photo-H_2 production device described by Hambourger *et al.* relate to increased electron recombination with the sensitiser at the anode as H_2 builds up and shifts the potential of the coupled cathode half cell.[5]

Other photo-H_2 production devices involve the hydrogenase directly adsorbed onto semi-conductor materials. Morra *et al.* report a high surface area electrode constructed from the [FeFe]-hydrogenase *C. acetobutylicum* HydA adsorbed on a TiO_2 film.[53] The electrode gives rise to a large reductive current (450 µA cm^{-1} at –0.714 V) although at this potential the stability is very poor and 50% of the activity is lost after just 10 min.

Devices utilising [FeFe]-hydrogenases have been demonstrated under strictly anaerobic conditions, because of the difficulty of handling these sensitive enzymes in the presence of O_2, although separate electrochemical studies have shown that H_2 production in air by these enzymes is possible over a limited timeframe, as mentioned in section 2.[35] The greater O_2 tolerance of [NiFe]-hydrogenases makes them more versatile catalysts. The Peters group attached a Ru(II) photosensitizer to a [NiFe]-hydrogenase from *T. roseopersicina* and although they were unable to achieve direct electron transfer from the Ru(II) to the hydrogenase under illumination, they were able to record photo-hydrogen production in the presence of methyl viologen as an electron transfer mediator under anaerobic and aerobic conditions. It was assumed that the proximity of the photo-reductant to the hydrogenase helps to maintain a reducing environment that further protects the hydrogenase from O_2 damage.[54] Most [NiFe]-hydrogenases suffer from strong product inhibition (by H_2; see for example the dark gray line in Figure 4a), and this is likely to be a limiting factor in their H_2 production. For example, Kihara *et al.* used the [NiFe]-hydrogenase from *T. roseopersicina* immobilised on hydrophobic carbon nanotubes as a H_2 production electrode, but found the current to be very sensitive to H_2 partial pressure.[47] Reisner *et al*. have made use of the [NiFeSe]-hydrogenase from *Dm baculatum* - an enzyme known to suffer much less from product inhibition and exhibit good O_2 tolerance.[55] Voltammetry of this enzyme on films of TiO_2 annealed onto indium tin oxide on glass showed promising H^+ reduction currents, even under low-level H_2 (at ca. –0.56 V, currents were ca. 140 µA cm^{-2} under N_2 and 50 µA cm^{-2} under 5% H_2). These electrodes retained 50% of their activity after

storage under N_2 for 1 month, demonstrating that sorption in TiO_2 films does not interfere with the enzyme structure, and may actually stabilise the protein although long term stability of the electrodes under electrocatalytic conditions has not been investigated. Reisner *et al.* then went on to exploit hydrogenase on nanoparticulate TiO_2, sensitised with a ruthenium dye, for visible light-driven H_2 production.[6,55] In this system, an electron is photo-excited in the dye and transfers to the conduction band of the semiconductor particles. The dye is regenerated by a sacrificial electron donor while the electron transfers to hydrogenase where reduction of H^+ to H_2 occurs. Under illumination of 45 mW cm^{-2} a level of 4.6% H_2 was reached in the headspace, corresponding to a turnover number of 1.9×10^5 for the hydrogenase.[55] The limiting part of the system appeared to be the ruthenium dye, rather than the enzyme.

Avoiding the need for a separate dye-sensitiser, H_2 production has been reported for *T. roseopercina* [NiFe]-hydrogenase on CdTe quantum dots,[8] and *C. acetobutylicum* [FeFe]-hydrogenase I on CdS nanorods.[7] Similarly, reduction of CO_2 to CO by carbon monoxide dehydrogenase has been shown on CdS quantum dots.[56] These demonstrations show that enzyme adsorption on nanoscale semiconducting particles is a versatile approach for a range of bio-electrocatalytic transformations.

The field of light-driven biocatalytic devices still has many opportunities for creative combination of components and reactions and we can expect many exciting new developments in this area in the future. These need to be accompanied by detailed studies to improve our fundamental understanding of electron transfer between enzymes and semiconductor materials and analysis of the limiting factors in stability of these systems.

4.3 H_2-driven chemical synthesis or H_2 production via coupled catalysis

The observation that a range of different enzymes will exchange electrons with graphitic surfaces has led to coupling or wiring of enzymes together on graphite particles. In one proof-of-concept demonstration, hydrogenase was co-immobilised onto pyrolytic graphite particles or platelets with nitrate reductase, such that electrons from H_2 oxidation passed from the hydrogenase into the particle, and were then taken up by the nitrate reductase for reduction of nitrate to nitrite.[57] A more interesting manifestation involved demonstration of the water gas shift reaction, interconversion of CO and H_2O with CO_2 and H_2, on graphite platelets modified with hydrogenase and carbon monoxide dehydrogenase, as represented in Figure 2f.[14] We recently used a similar particle concept for recycling of the important biological cofactor NADH, Figure 2g. Under 1 bar H_2 and with NAD^+ in solution, reduction of NAD^+ by H_2 is thermodynamically favourable. The NAD^+-reducing catalytic moiety of *R. eutropha* soluble hydrogenase was combined with *E. coli* Hyd-I on pyrolytic graphite particles to couple electrocatalytic reduction of NAD^+ to NADH to the oxidation of H_2, providing a modular system for recycling these important biological cofactors.[15] Most NADH-dependent dehydrogenases will not exchange electrons directly with an electrode because they require electron transfer in the form of hydride ($H^- = 2e^- + H^+$) from NADH. By co-immobilisation of lactate dehydrogenase on the particles, it was possible to demonstrate H_2-driven reduction of the model substrate pyruvate, to lactate, via the recycled NADH, as shown in Figure 9.[15] This opens up new opportunities for driving electron transfer to NADH-dependent enzymes from H_2 or an electrode via NADH.

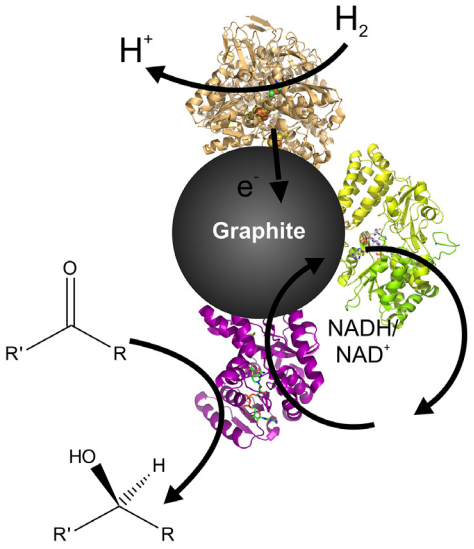

Figure 9. NADH-dependent catalysis by a dehydrogenase can be coupled to H_2 driven NADH recycling by co-immobilising enzymes on graphite beads.[15]

In each case, the critical features are the conductivity of the graphite to transfer electrons between the two enzymes, and adsorption of the two enzymes in correct orientations for fast electron transfer, and in an appropriate ratio that their catalytic rates are matched fairly evenly.

5. Future Scope

One of the historical barriers to the development of enzyme-based devices has been the research specialisation necessary for culturing the variety of specialised host organisms under suitable conditions for enzyme expression. Increased interest in hydrogenase chemistry has led to isolation and characterisation of

new hydrogenases, including several temperature-stable enzymes from hyperthermophilic organisms.[29,58,59] Isolation of two [NiFe]-hydrogenases from *E. coli* (including crystallographic characterisation of hydrogenase-1) and genetic engineering of these enzymes,[4,27] and successful heterologous expression of several other hydrogenases in *E. coli,* are helping to broaden the availability of hydrogenases for study and application. The feasibility and economics of scale-up of hydrogenase preparations, for example to gram and kilogram scales, still need to be established if they are to be used in commercial devices. The demonstration that [FeFe]-hydrogenase precursors lacking the 2Fe2S unit of the active site can be activated by incorporation of a synthetic 2Fe2S cluster provides a tantalising indication that it might be possible to produce part-synthetic enzymes on a larger scale.[19,60]

Two main areas emerge where further work is needed. The first is in electroactive attachment of enzymes at high coverage, on high surface area electrodes or particles. Detailed insight into the range of interactions involved in stabilising protein-protein complexes is available from crystallographic structures, studies involving mutagenesis of surface residues and coupling between spin-labels observed through advanced electron paramagnetic resonance (EPR) methods. In comparison, we know relatively little about the specific interactions that support protein-electrode interactions, and efforts to expand the range of useful materials often involve trial and error rather than rational design.

A second area that still requires further attention is in development of O_2 tolerance. We now understand much about the competing reactions of H_2 and O_2 at hydrogenase active sites, particularly for [NiFe]-hydrogenases, but more needs to be done to control the relative rates of these reactions to avoid short circuiting of H_2 oxidation currents to un-productive O_2-reduction. Nevertheless, a number of hydrogenases are available with sufficient O_2 tolerance that all types of devices discussed in this review have been demonstrated operating under air, or at least with low level O_2.

Arising from the wealth of possibilities demonstrated with enzyme electrocatalysts, we can expect to see new generations of bio-inspired catalysts that incorporate synthetic metal centres alongside polymer, oligo-peptide or even protein shells that provide the functionality of proteins (eg., proton transfer, electron transfer, substrate selectivity, stabilisation and protection) but with higher active site density and improved robustness. This is a fast evolving field, and the most exciting developments are emerging at the interfaces between biocatalysis, enzyme engineering, materials science, chemical synthesis and device electronics.

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