Review

Enzymes as Working or Inspirational Electrocatalysts for Fuel Cells and Electrolysis

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Enzymes as Working or Inspirational Electrocatalysts for Fuel Cells and Electrolysis

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1. Introduction

The concept of a fuel cell dates back to 1839, from independent studies by Grove and Schoenbein.[1,2] Like a battery, a fuel cell is a device for obtaining electrical energy directly from a chemical reaction, but unlike a battery, electrical power is sustained as long as the reacting chemicals are supplied to each electrode with the cathode receiving oxidant and the anode receiving reductant or "fuel", hence "fuel cell".[3,4] There are environmental advantages over combustion because fuel cells avoid the high temperatures that cause NOx production, and they are usually reported as operating at a higher efficiency (typically 50−60%) than internal combustion engines (20−25%).[5,6] Applications of fuel cells were largely neglected until the "space age" (1960s) when there became a need for reliable electrical power in challenging, niche situations. In addition to environmental driving forces, energy demands for niche applications continue to drive fuel cell development today. Fuel cells vary greatly in their power output, ranging from large-scale (kW) building-integrated systems, known as "combined heat and power" systems,[4] to those that provide just enough power to operate electronics in special circumstances,[7] such as an implantable device for sensing and controlling glucose levels in the body.[8]

As we outline below, the power output of a fuel cell can be limited by the electrochemical reactions occurring at either of the two electrodes, the anode for oxidizing fuel and the cathode for reducing oxidant, and so the electrodes are usually coated with electrocatalysts. An enzyme fuel cell uses an enzyme as the electrocatalyst, either at both cathode and anode, or at just one of the electrodes. The catalytic properties of redox enzymes offer some interesting advantages in fuel cell applications, although examples of devices exploiting enzyme electrocatalysis are almost exclusively at a "proof of concept" stage.

Conventional low-temperature fuel cells are generally limited to H_2 or primary alcohols as fuels; however, the use of an enzyme as the anode electrocatalyst means that any substance that is oxidized by an organism can become a useful fuel because it is obvious that enzymes for carrying out that specific task must exist. Not only are enzymes capable of very high activity (on a per mole basis), but they are usually highly selective for their substrates. This simplifies the design of a fuel cell because fuel and oxidant need not be separated by an ionically conducting membrane, and they can be introduced as a mixture, that is, mixed reactant fuel cells are possible. This also makes it possible to miniaturize the fuel cell to an extremely small scale. Enzymes are also renewable, meaning that their components are fully recycled using sunlight as an energy source.

The main disadvantages of enzymes as electrocatalysts are as follows. First, they are usually very large molecules, so that although the active sites may be extremely active in comparison to the catalytic site of a conventional metal electrode, the catalytic (volume) density is low, and hence multilayers of enzyme are likely to be needed to provide sufficient current. Second, the catalytically active sites are usually buried, so that fast electron transfer to or from the electrode requires either use of an intrinsic electron relay system in the protein (such as a series of FeS clusters) or an extrinsic mediator that can penetrate sufficiently close to the active site. Third, enzymes are often unstable outside ambient

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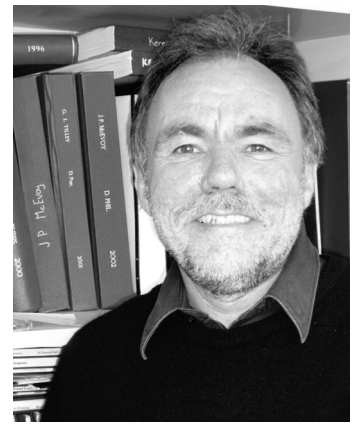
conditions of temperature and pH, and long-term durability is difficult to achieve.

Apart from application of enzymes in functional devices, enzyme electrocatalysis also provides inspiration for development of better synthetic catalysts. In this Review, we illustrate examples of fuel cells that are only possible because of the specific properties of the enzyme electrocatalysts. As we will discuss, a very significant impact may stem from the eventual replacement of platinum catalysts by ones based on abundant resources. For example, a strong case has been made for the active sites of "blue" copper oxidases being more efficient than Pt in catalyzing clean, four-electron reduction of O_2;[9,10] likewise, hydrogenases (the active sites of which are composed of the abundant metals Fe or Fe and Ni) have been likened to Pt in their ability to oxidize or produce H_2.[11–13] Studies of enzymes may yet inspire viable solutions to the poor electroactivity for methanol oxidation that limits the usefulness of this otherwise convenient and energy-dense fuel in small Pt-based fuel cells.[3]



Fraser Armstrong is a Professor of Chemistry at Oxford University and a Fellow of St. John's College. He obtained his Ph.D. at the University of Leeds with Geoff Sykes and then carried out postdoctoral research with Peter Kroneck (Konstanz), Ralph Wilkins (New Mexico), Helmut Beinert (Madison), and Allen Hill (Oxford). In 1983, he was awarded a Royal Society University Research Fellowship which he held in Oxford until 1989, when he joined the Chemistry Faculty at the University of California, Irvine. He moved to his present position in 1993. His interests are in biological redox chemistry, in particular the application of dynamic electrochemical techniques in studies of complex electron-transfer and catalytic reactions in proteins, and most recently the mechanisms and exploitation of biological hydrogen cycling. He was awarded the 1998 European Medal for Biological Inorganic Chemistry, the 2000 Royal Society of Chemistry award for Inorganic Biochemistry, the 2003 Carbon Trust Academic Innovation Award (with Kylie Vincent), the 2004 Max-Planck "Frontiers in Biological Chemistry" Award, and the 2006 Royal Society of Chemistry Medal for Interdisciplinary Chemistry. In 2008 he was elected a Fellow of the Royal Society.

1.1. Types of Enzyme Fuel Cells and Scope of This Review

This Review draws upon recent developments of fuel cells in which the catalysts are isolated enzymes, as opposed to "microbial" fuel cells in which whole organisms are exploited.[14,15] However, we will include cells in which the reaction at only one electrode is catalyzed by an enzyme, especially because it is still common to use a simple Pt catalyst for O_2 reduction, at least in initial tests on an enzyme anode. Enzyme electrodes are further classified into those in which the transfer of electrons between the enzyme and the electrode is direct and those in which a mediator is used to carry electrons between the two. We will focus on examples where both the enzyme and mediator (if used) are attached to the electrode. Several detailed reviews on enzyme fuel cells have appeared in the past few years,[8,16–18] and our aim is to complement rather than reiterate their views.

We expect that the majority of scientists engaged in research on enzyme-based fuel cells are physical chemists specializing in electrochemistry. However, despite the fact that it is essential "to get the electrochemical principles right", the complexity of enzymes should not be underestimated, and it is crucial that their properties and idiosyncrasies are fully understood and taken into consideration. We will therefore seek to highlight enzyme fuel cells from the point of view of the attributes of the enzymes themselves, starting with the notion that energy in biology is intimately associated with redox reactions and enzymes are biology's electrocatalysts.

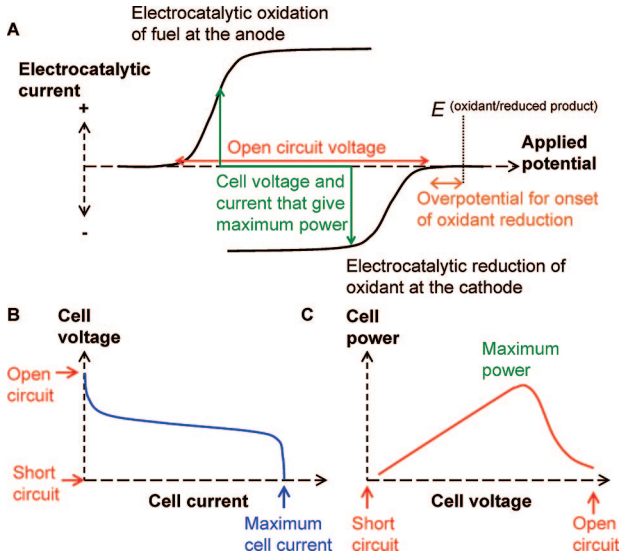


Figure 1. Voltage and current response for a pair of fuel cell electrodes tested separately (Panel A) or operating together in a fuel cell (Panels B and C). Features that determine fuel cell performance are highlighted.

1.2. Determinants of Cell Voltage, Current, and Power

Fuel cell performance is characterized in terms of power output, which depends upon the current achieved at different cell voltages (Figure 1). At one extreme, the open circuit voltage (OCV) provides a measure of the maximum voltage associated with a fuel cell. It is also termed the *resting potential* or *zero-current potential*, because it defines a potential at which there is no net current flow and, consequently, at which no work is done. In an ideal case, the OCV is determined by the difference between the thermodynamic potentials of the fuel/oxidized product redox couple and the oxidant/reduced product redox couple, adjusted for all the nonstandard conditions of fuel cell operation.

The measured OCV for a given fuel cell assembly is determined by the difference between the onset potential for catalysis at the respective electrodes (indicated in red in Figure 1A). Many redox enzymes operate very close to the thermodynamic potential of their substrate/product couple (i.e., interconversion is activated at minimal overpotential) and many are extremely good electrocatalysts in both directions. Examples include CO_2/CO interconversion by carbon monoxide dehydrogenase,[19] H_2 production and oxidation by many hydrogenases,[20] fumarate/succinate interconversion by fumarate reductase,[21] and NAD^+/NADH interconversion by soluble domains of complex I.[22] On the other hand, some redox enzymes require a larger overpotential to drive their reactions, and use of these catalysts in a fuel cell lowers the OCV from the value expected on the basis of substrate thermodynamics (indicated in Figure 1A for the cathode reaction). Examples include the reduction of O_2 by fungal laccases, which requires a small overpotential,[10] or by plant laccases[23] and heme copper oxidases,[24] which requires a much larger overpotential (several hundred millivolts). (We note here that Pt also requires an overpotential for O_2 reduction, as discussed later.)[25] The cell voltage may be diminished further by slow interfacial electron transfer because of poor electronic coupling of enzymes to the electrode or by use of electron-transfer mediators with potentials that are significantly more negative than the oxidant couple or more positive than the fuel couple. At the other extreme, sometimes termed *short circuit* because it arises when the anode and cathode are electrically connected without an applied load, no useful electrical work is done.

Useful power is achieved at current and voltage values that are a compromise between the limiting cases of open circuit and short circuit (Figure 1B and C). The highest fuel cell currents are usually delivered at low cell voltages; although in section 3.3, we note an interesting exception to this that arises from the reversible inactivation of hydrogenases at high potential. (We note that in high-power fuel cells it is important to minimize ohmic losses by operating at high cell voltage.) The magnitude of the current is determined by the rate of electrocatalysis at the anode and cathode and will be limited by the electrode with the lowest electrocatalytic rate (in Figure 1A this is the reduction of oxidant at the cathode).

In contrast with conventional, precious-metal catalyzed fuel cells, enzyme fuel cells are generally aimed at the low-power end of the power spectrum. Typical power outputs are in the range of micro to milliwatts, compared with the kilowatt outputs from conventional fuel cells, although it is observed that, unlike conventional fuel cells, enzyme fuel cells operate close to ambient temperature and pressure.[3] In common with conventional fuel cells, the power output can be increased, for example, by "stacking" the individual cells.

The maximum electrocatalytic current achievable at either electrode depends on the density of catalytic active sites (this is the electroactive coverage, which for enzymes tends to be low, because of their large size) and the rate of catalysis per active site (which, conversely, can be very high for enzymes).[8] A monolayer of enzyme usually only contains at most a few picomoles of catalyst per square centimeter, which is too low to provide useful currents for most purposes.[8] The cell current can be improved greatly by employing multilayers of enzyme on an electrode; this depends upon the resulting "3D electrode" being structured to ensure that mass transport of reactants and products is not impeded and all enzyme molecules are electronically well coupled to the electrode. Even so, the overall efficiency (electroactivity per mole of enzyme applied to the electrode) may be low.

1.3. Comparison with Biology

There is an instructive connection between enzyme fuel cells and energy-transducing electron-transfer chains in biology. As an example, a variety of oxidation and reduction reactions are coupled in the inner-membrane respiratory chain of *Escherichia coli* via lipid-soluble quinone (Q)/quinol (QH_2) redox mediators, Figure 2A. An even wider range of other fuels and oxidants is used by microorganisms from specialized environments.[26,27] The redox-derived energy is used to sustain a proton gradient across the cytoplasmic membrane, as shown schematically (Figure 2B) for formate oxidation coupled to nitrate reduction. This pair of enzymes can be considered to be, in structural terms, the best characterized redox (Mitchell) loop.[26,28] Two electrons released from formate oxidation in the periplasm are transferred, together with H^+ from the cytoplasm, to the electron-acceptor menaquinone (MQ). The reduced mediator, menaquinol (MH_2) passes electrons to nitrate reductase for nitrate reduction in the cytoplasm and releases two protons into the periplasm. The redox loop is equivalent to using a fuel cell to charge a capacitor, Figure 2C and D.[26] Transport of protons back into the cytoplasm through the ATP-synthase

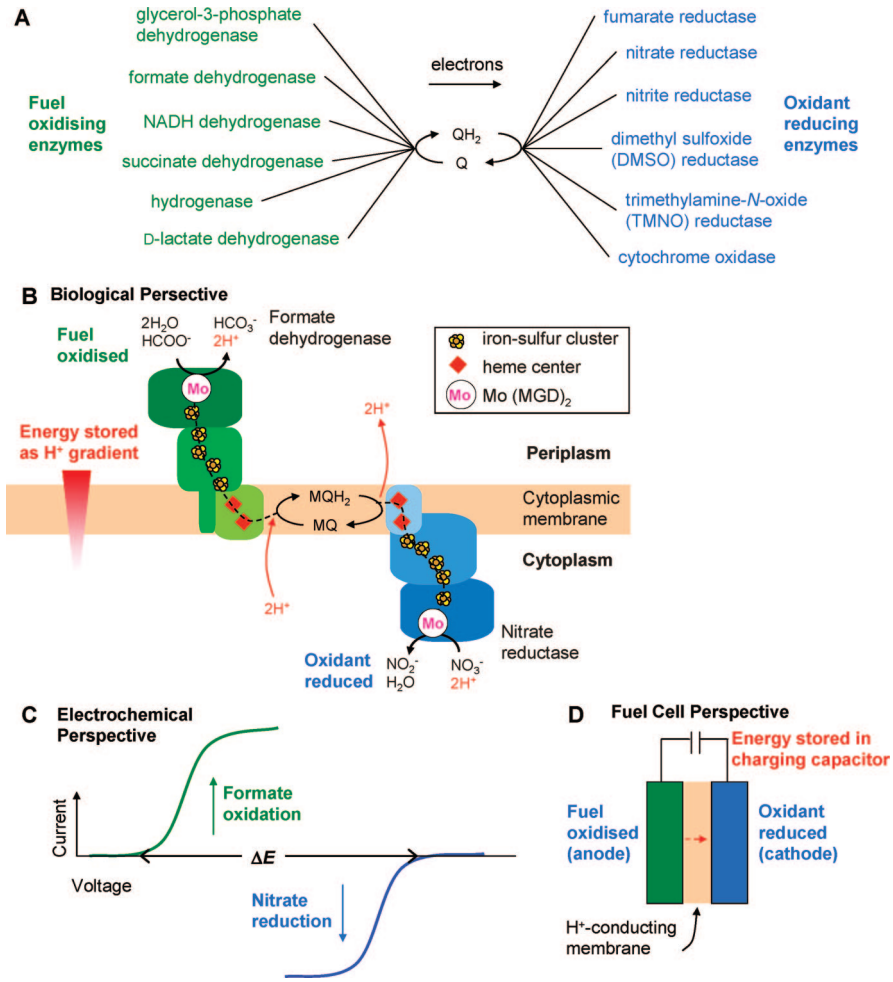


Figure 2. Panel A: Selected respiratory enzymes found in the bacterium *E. coli*.[26] Organisms ranging from the simplest microbes to the higher mammals express specific redox enzymes to extract energy from a variety of fuels and oxidants. The enzymes couple fuel oxidation to oxidant reduction. Panel B: An anaerobic respiratory "Mitchell" redox loop involving a pair of respiratory enzymes, formate dehydrogenase, and nitrate reductase, coupled across the inner membrane of *E. coli* via menaquinone (MQ) and menaquinol (MQH_2).[26,31] Energy from the redox reactions is stored as a transmembrane H^+ gradient, generated by H^+ uptake from the cytoplasm, and released into the periplasm. Panel C: Monitoring catalytic current versus potential for isolated redox enzymes on an electrode surface in a voltammetric experiment provides an electrochemical perspective on the redox loop. Panel D: The redox loop is analogous to a situation in which a fuel cell is used to charge a capacitor as noted in ref 26.

complex harnesses the energy stored in the H^+ gradient for synthesis of ATP. The membrane-spanning "proton pump,' cytochrome *c* oxidase, functions as a single-molecule redox loop, reducing O_2 to water at one site and oxidizing cytochrome *c* (the fuel) at another; the energy released is channeled internally to "pump" protons across the membrane.[24] This enzyme, or a pair of enzymes coupled by the quinol pool, can be considered the smallest examples of working fuel cells.

Dihydrogen is not used as a fuel by *E. coli* during oxygenic growth conditions.[26] However, certain aerobic bacteria such as *Ralstonia eutropha* couple periplasmic H_2 oxidation to cytoplasmic O_2 reduction via the quinone pool,[29] analogous to the situation in a H_2/O_2 fuel cell. Similarly, acetic acid bacteria couple periplasmic oxidation of alcohols, such as glucose or ethanol, to cytoplasmic reduction of O_2.[30] Unlike a proton-exchange membrane in a conventional low-temperature Pt fuel cell, a biological membrane is permeable to small neutral molecules,[26] so the fuel and oxidant can mix. The selectivity of enzymes as catalysts is therefore critical in allowing bacteria to derive energy from these processes.

The general catalytic behavior of an isolated enzyme is considered in terms of two common quantifiable properties: its substrate affinity (defined by the Michaelis constant, K_M) and the turnover frequency, k_{cat}. When two redox enzymes work together, another property appears: this is the potential difference between the redox couples reacting at each enzyme, which translates into the maximum voltage available to the organism from respiration. When the k_{cat} values are bought into the equation we obtain the concept of *microscopic power* because of the proportional relationship between current and k_{cat}. Figure 2C provides an electrochemical perspective on the coupling of two redox enzymes in a Mitchell loop, and we note the similarity with the voltage-current relationship for a fuel cell as shown in Figure 1. The reduction potentials for a variety of biologically relevant half-reactions are given in Table 1. Methanogenic CO-oxidizing bacteria, such as *Carboxydothermus hydrogenoformans*, couple CO oxidation to H^+ reduction generating just 0.1 V, whereas bacteria coupling H_2 oxidation to the two-electron reduction of NO_3^- to NO_2^- access more than 0.7 V. The thermodynamics of H_2 oxidation and O_2 reduction suggest that aerobic H_2 oxidizers should have access to over 1 V.

For *E. coli* respiring on H_2 (the fuel) and fumarate (the oxidant), the maximum voltage available is around 0.45 V.

Table 1. Approximate Reduction Potentials ($E^{\circ\prime}$) under Non-Standard Conditions (pH 7, 25 °C) of Redox Couples That Are Relevant to Microbial Energy Cycling

redox couple	$E^{\circ\prime}$ (V)	ref
H_2O_2/H_2O	1.35	32
O_2/H_2O	0.82	32
NO_3^-/NO_2^-	0.42	33
ubiquinone/ubiquinol	0.10	34
fumarate/succinate	0.03	33
menaquinone/menaquinol	−0.07	34
CH_2O/CH_3OH	−0.18	33
$NAD^+/NADH$	−0.33	27
H^+/H_2	−0.41	32
glucose/gluconolactone	−0.45	calculated from ref 35
CO_2/CO	−0.51	19
acetate/acetaldehyde	−0.59	calculated from ref 35

Both hydrogenase and fumarate reductase have high catalytic rates at substrate levels above K_M,[21] but taking 100 s^{-1} as a conservative estimate for the limiting activity, the current flow through a redox loop involving a pair of these enzymes would be just 3.2×10^{-17} A, representing a microscopic power output of 1.4×10^{-17} W. This represents the "rating" of one of the smallest fuel cells possible. Note that the potentials given in Table 1 are valid for high substrate concentrations that are unlikely to be encountered in vivo; thus, for example, $E^{\circ\prime}(H^+/H_2)$ shifts to −0.27 V at 10 ppm H_2 (equivalent to about 10 nM dissolved H_2). Some hydrogenases have very low K_M values for H_2 oxidation (equivalent to <100 ppm), and this reflects the environmental H_2 levels that are experienced by many H_2-uptake microorganisms.[36,37] In the lower atmosphere, H_2 is present at a level of about 0.5 ppm. The ability to scavenge low levels of H_2 from the environment means that hydrogenases effectively concentrate the energy available in the dilute fuel by contributing to generation of a transmembrane proton gradient. Aside from H_2 oxidation, some organisms produce H_2 either by fermentation or, in special cases, by photosynthesis because the proton is used as the oxidant.[38]

1.4. Historic Aspects

Since the 1970s, there have been numerous papers describing electrocatalysis by enzymes attached to electrodes, in which authors have focused either on fundamental studies of enzyme electron transfer and catalytic mechanism or on applications to biosensors. It is only comparatively recently that papers have appeared dealing specifically with enzymes as fuel cell catalysts. Because any one development depends on others, it is instructive to view how the use of enzymes as fuel cell catalysts relates chronologically to breakthroughs in technology or understanding. Some perspective on this is provided by the chronogram shown in Figure 3. The main points, apart from the seminal work of the 1830s,[1,2] are the invention of dynamic electrochemical methods,[39,40] dawn of the space age, understanding of enzyme kinetics,[41,42] development of protein technologies, particularly enzyme purification,[43] X-ray crystallography,[44,45] genetic engineering,[46] development of the amperometric glucose sensor,[47,48] and the advent of nanomaterials and new carbon materials.[49,50] It is these revolutions and breakthroughs that have led to the ideas behind enzyme fuel cells.

To our knowledge, the first enzyme fuel cell was reported by Yahiro et al. in 1964.[51] The cell featured Pt foil electrodes immersed in phosphate buffer solutions in both the anode and cathode compartments, which were separated by a membrane. Glucose and glucose oxidase were placed in the anode solution and the cathode solution was either open to air or sparged with O_2. The cell gave OCV values in the range 625−750 mV and a tiny current density of 30 nA cm^{-2} at 330 mV (in this Review, all current densities refer to electrode area). The poor performance was probably the result of the absence of any mediator to transfer electrons between glucose oxidase and the electrode, and given that glucose oxidation at Pt occurs at high potential, it is unclear whether the enzymes were functioning at all. In 1981, Hill and co-workers reported a methanol-oxidizing fuel cell that used a bacterial methanol dehydrogenase in solution as the anode catalyst with phenazine ethosulfate mediating electron transfer to a Pt electrode. This enzyme contains a pyrroloquinoline quinone (PQQ) cofactor and is able to catalyze two-electron oxidations of methanol to formate and further to formaldehyde. An OCV of 0.3 V was recorded, with a maximum (short-circuit) current density of about 0.5 mA cm^{-2} (based on anode dimensions).[52] A later device produced 0.03 mA cm^{-2} at an operating voltage of 50 mV.[53] Throughout the 1980s and 1990s, there were simultaneous and more intense activities devoted to enzyme-based biosensors; here, rapid analysis of critical analytes such as glucose had a more obvious market, and low power was not an issue. Significantly, Hill and co-workers went on to develop a very successful amperometric glucose sensor, the "Exactech", that used glucose oxidase with functionalized ferrocenes as electron mediator.[54,55] A more ambitious cell was reported by Yue and Lowther in 1986: this used two immobilized enzymes, methanol dehydrogenase and formate dehydrogenase, to catalyze the complete oxidation of methanol to CO_2 in the anode compartment.[56] The device gave a current density of about 0.02 mA cm^{-2} but a cell voltage of just 65 mV.

In 1999, Willner and co-workers reported a membraneless fuel cell with immobilized enzymes as catalysts at both anode and cathode. The device was based upon anodic oxidation of glucose catalyzed by flavin-containing glucose oxidase coupled to cathodic reduction of peroxide or O_2 by microperoxidase or cytochrome *c* oxidase, respectively.[57,58] Since then, the idea that enzymes allow a fuel cell to be extremely simple and tiny, free from the need to separate fuel and oxidant with a membrane, has gathered support. With such a specification, enzyme-based fuel cells could occupy a technological niche, for example as implantable power sources (operating in biological fluids) or ambient, self-powered sensors. Heller and co-workers have since focused on gaining large improvements in the performance of cathodes catalyzing O_2 reduction. A major development has been that the enzymes (laccase or bilirubin oxidase) are embedded into a conducting hydrogel polymer to which Os-complexes are attached as electron mediators.[59] This produces a "3D electrode" equivalent to having a large number of monolayers of electronically coupled enzyme. Heller and co-workers have developed similar modification strategies for anodes catalyzing the oxidation of sugars including glucose and fructose. A working fuel cell using this principle and operating on fructose was constructed and studied either in aerated fructose solution or as an implanted device in a grape.[60] Willner and co-workers have developed elaborate strategies for attaching flavin or PQQ-dependent enzymes to electrodes, employing covalent attachment of the cofactor and allowing it to reinsert into the apoenzyme.[61,62] A similar concept was successfully demonstrated for laccase. In this case, substrate-like electron-conducting functionalities at-

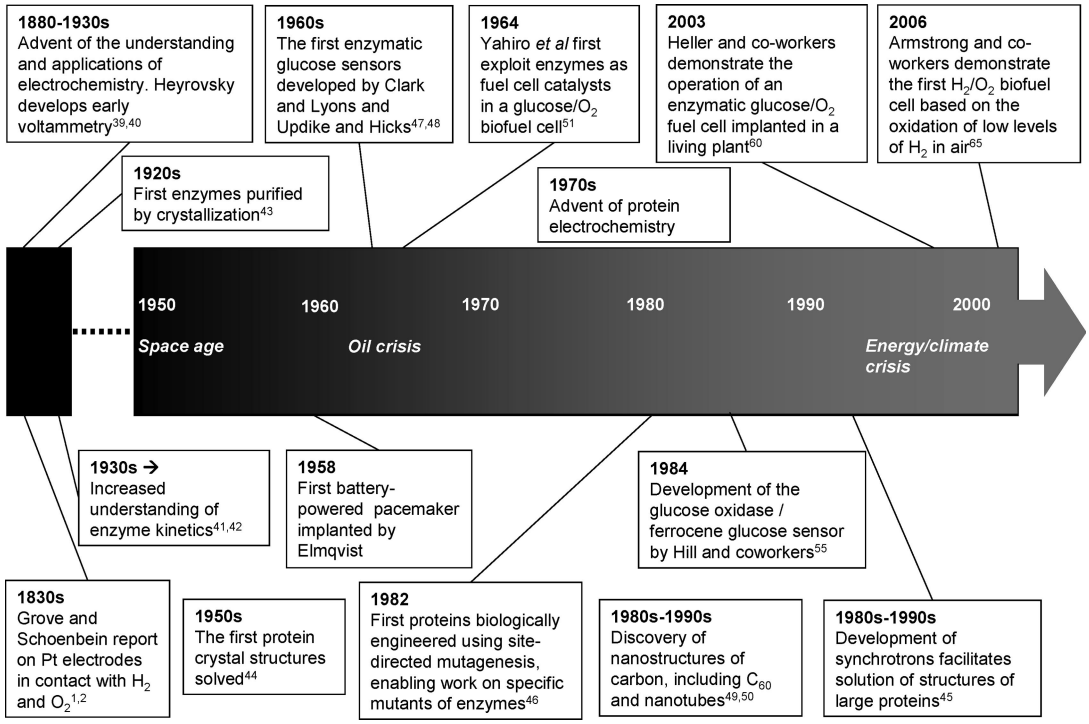


Figure 3. Some key chronological landmarks in the development of enzyme fuel cells.

tached to the electrode are thought to penetrate the enzyme close to an electron-relay center and provide direct electronic coupling.[63]

So far, we have mentioned only fuel cells that act on alcohol/sugar fuels, but hydrogen (H_2) has long been the "standard" because of its low reduction potential, clean oxidation to water, and the ease of supplying it as a gas. There have recently been some interesting reports dealing with the application of hydrogenases to replace Pt. It has been proposed that the active sites of hydrogenases are comparable in activity to Pt;[12,13] however, most hydrogenases are inactivated even by traces of O_2, and this makes them very difficult to use in real practical situations. The situation has become more favorable with the discovery and characterization of hydrogenases from aerobes that are tolerant to O_2 and can catalyze the oxidation even of trace H_2 in air.[36,64] This has led to the demonstration of a membraneless fuel cell that can power a wristwatch from a mixture of 3% H_2 in air, not a high power demand, but proof of the ability of highly active enzymes to extract energy from an otherwise benign gas mixture (4% H_2 in air is the lower combustion limit).[65]

Further recent developments in enzyme-based fuel cells have exploited high-surface area electrode materials such as porous carbon, carbon cloth, and nanotubes to increase catalytic current.[66–68] In addition, enzymes have continued to inspire development of new synthetic electrocatalysts, with particular examples being O_2 reduction (porphyrins)[69,70] and H_2 oxidation.[71–78] Finally, by 2007, Sony is advertising a glucose-fueled enzyme fuel cell for powering small consumer electronics. Glucose dehydrogenase and bilirubin oxidase, both in solution with electron mediators and separated by a membrane, are used as the anode and cathode catalysts respectively.[79]

1.5. Feasibility of Enzymes As Catalysts

Some of the benefits and disadvantages of employing enzymes in fuel cell catalysis are summarized in Table 2.

Table 2. Advantages and Disadvantages of Enzymes in Fuel Cell Electrocatalysis

advantages	disadvantages
overpotential is often close to zero	low current density per catalyst volume
enzymes are infinitely renewable	time and cost of isolation and purification
excellent specificity, able to scavenge fuel and oxidant from ambient environment	poor stability and restricted temperature range
oxidation of unusual (biological) fuels	difficulty in achieving good electronic coupling to the electrode in some cases
can be used in disposable and completely biodegradable devices	

Conventional low-temperature fuel cells are essentially limited to pure methanol or H_2 as fuels and O_2 as the oxidant. With enzymes, many more fuels are possible and more oxidants are possible, although O_2 is an excellent oxidant because it has a high potential and is widely available.

The two most important *intrinsic* properties of any enzyme suiting it for fuel cell applications are its turnover frequency (k_{cat}), which is the ultimate rate at which the catalyst can cycle its substrate (either a fuel or an oxidant), and the driving force (potential) necessary to achieve catalysis. Good values of k_{cat} are upward to 100 s^{-1}, for example with blue copper oxidases,[80] with values exceeding 10^4 s^{-1} likely for some hydrogenases.[20,81] (For comparison, on a per Pt atom basis ($\sim$10 Å^2 footprint), assuming a chemical rate-determining step and a current density of 10 mA cm^{-2}, we estimate the equivalent turnover frequency of Pt to be approximately 15 s^{-1}.) Without a high k_{cat} an enzyme cannot function as an effective catalyst, and it is important to achieve this rate efficiently, that is, with the minimum voltage loss possible. As mentioned above, many enzymes operate at potentials very close to that of their substrates, and these are usually the most suited to fuel cell applications.

Next in importance come substrate specificity, the K_M values for the substrates, both fuel and oxidant, and the potential difference between fuel oxidation and oxidant reduction. Ideally, for a fuel cell, the substrates should be available at concentrations well above K_M. In contrast, for biosensors, the catalytic activity should be strongly dependent on the substrate concentration in the range of interest, i.e., around K_M or below. For example, sugar oxidizing enzymes tend to have fairly high values of K_M for their sugar substrates (often greater than 10 mM, section 2.2.1). This means that the enzyme active site will not be substrate saturated at the levels of glucose normally found in blood (4−8 mM).[82] Consequently, whereas these enzymes are well-suited to sensing glucose levels in blood, optimal activity would not be achieved in an implanted fuel cell under these conditions. Furthermore, the tricks available to electrochemists, such as using a rotating disk electrode (RDE) to control substrate mass transport, are not applicable in fuel cells. Fuel cell electrodes usually operate in quiescent solution and substrate is depleted close to the electrodes so that the current can become limited by diffusion from the bulk solution. A cathode catalyst having a relatively high K_M value for O_2 is less of a problem because, in a solution exposed to air under ambient conditions, the concentration of O_2 is already about 0.27 mM,[83] and O_2 diffuses much more rapidly than glucose.

Because enzymes are so large, they yield only a low current density per unit electrode area. However, the protein material surrounding an active site is not just a bulky dressing; it produces the substrate selectivity and provides acidic and basic functionalities, internal electron coupling (a relay system), substrate access, proton access, and surface features that allow it to bind to other proteins (or an electrode). Barton and co-workers have calculated the scale of the challenge arising from the poor catalytic density of enzymes. A monolayer of a typical enzyme, having a 100 nm^2 "footprint" and a turnover frequency of 500 s^{-1}, will produce a current density of just 80 $\mu A\ cm^{-1}$.[8] On this basis, 6000 monolayers would be needed to achieve a current of 0.5 A cm^{-1}. Therefore, even if laccases (O_2 reduction) or hydrogenases (H_2 oxidation) have active-site activities comparable to Pt, as has been argued (see below), their practical activity will be far lower. Hence it is desirable to construct "3D-electrodes" like ones based on enzymes entrapped in a redox hydrogel[59,67] or immobilized on high-surface area conducting carbon supports.[66–68,84]

Another factor which can be regarded as intrinsic is enzyme stability, and this is obviously important because a fuel cell is generally expected to have a long lifetime (particularly for implantable devices). Enzymes can be quite stable under ambient conditions in the absence of proteases, and enzymes from thermophilic organisms may offer further improvements in stability. Attachment to a surface can also extend enzyme lifetime. For example, De Lacey and co-workers report that *Desulfovibrio gigas* hydrogenase covalently attached to a nanotube-coated electrode gives a fairly stable catalytic response over >30 days (see section 2.2.2),[85] and it is unlikely that solution samples of this enzyme would retain substantial activity over this time frame. In another study, Blanford et al. showed that laccase on an anthracene-modified pyrolytic graphite electrode (see section 2.1) is stable over at least 60 days.[63] Factors that lie beyond the scope of natural enzymes are those structural properties that allow it to be incorporated at an electrode or into a redox polymer. Long-range electron-transport capability is a problem for some enzymes that possess no relay system, but "wiring" can be artificially introduced, for example by incorporating ferrocene units into glucose oxidase.[86]

Enzymes exploited in fuel cells are derived from a wide range of organisms and it is difficult to generalize on issues relating to their isolation and purification. Heterologous expression of complex metalloenzymes in readily grown host organisms such as *Escherichia coli* or yeast is often challenging because of the number of accessory proteins required for metal insertion and assembly of the active enzyme.[29] If corresponding assembly proteins are not expressed by the host, the relevant genes must be transferred along with the gene encoding the protein of interest.[29] The enzymes that are widely used in fuel cell research are often chosen because they are easily obtained, but in many cases it is possible to isolate alternatives that have more desirable electrocatalytic properties. Furthermore, commercially available enzyme samples are often of low purity and contaminants may block sites on an electrode or impair activity.

1.6. Design Considerations

Biological fuel cells vary considerably in design, but retain several common features. The two electrodes are separated by an electrolyte (ionically conductive but electronically resistive) and are connected via external circuitry that includes an electronic device.

Platinum-based catalysts are largely nonspecific (catalyzing both fuel oxidation and oxidant reduction). If the fuel and oxidant are present together, no net reaction can take place, so the anode and cathode must be separated, most commonly by Nafion (an expensive polyfluorinated proton-permeable polymer that has low permeability to O_2).[87] Other attempts to keep the fuel and oxidant apart have been based on laminar flow.[88,89] In contrast, enzymes are usually highly selective for their substrates, and provided that the cathode catalyst is insensitive to the fuel and, more challengingly, the anode catalyst is not inhibited by the oxidant (usually O_2 in air), a membrane should not be necessary. This greatly simplifies design,[65] and enzymatic fuel cells can therefore be very small.[60,90]

Proteins are often unstable above ambient temperatures and at extremes of pH. Usual operating conditions for enzyme fuel cells are in the temperature range 20−50 °C and between pH 4 and 8. By contrast, conventional Pt-catalyzed PEM fuel cells are typically operated at 80−100 °C in strongly acidic or alkaline electrolytes. Enzymes often operate only in a narrow pH range, and it is necessary to choose conditions that suit both the anode and cathode catalyst. For example, it would not be possible to couple fungal laccase (pH optimum 3−5)[91] with a PQQ-dependent methanol dehydrogenase (pH optimum generally above 9),[92] but it is possible to couple laccase to an O_2-tolerant hydrogenase from *Ralstonia* that also has an acidic pH optimum.[64,65]

Some reported enzyme fuel cells incorporate a magnetic stirrer or use a peristaltic pump both to supply fuel or oxidant and agitate the solution. However the power requirements of these processes invariably outweigh power produced by the fuel cells, and these systems cannot be considered to be useful working devices.

In our view, the most attractive electrode materials are based on carbon, which is abundant, renewable, relatively cheap, comes in many forms and can be chemically modified in a variety of ways. Many enzymes can be directly adsorbed

onto the "edge" surface of pyrolytic graphite (PGE) or carbon materials such as Ketjen Black. Examples include hydrogenases,[20] laccases,[63] fructose dehydrogenase,[84] and cytochrome *c* peroxidase.[93]

An electrode can be "extended into 3D space" with an electrically conducting network, greatly increasing the loading of coupled enzyme.[66,67,84] Not only can this increase the current by engaging more enzyme molecules, but it can also increase the *apparent* stability. This arises if the net current becomes limited by substrate diffusion rather than the loading of enzyme so that enzyme molecules can be lost or inactivated without altering the current magnitude. The same principle applies when optimizing the loading of Pt at PEM fuel cell electrodes.

Single carbon fibers (approximately 7 μm diameter) have been used as the electrodes in miniature enzyme fuel cells[16] and form a high surface-area conducting material when bundled or woven into cloth.[94] Enzyme electrocatalysis for fuel cells has also been described at gold electrodes that have the advantage of being readily modified by thiol-containing functionalities.[57] Electrodes have also been produced using low-temperature sol−gel technology, where enzyme molecules and mediators are encapsulated in a porous network of hydrated silica, $SiO_2 \cdot nH_2O$.[95–97] This has been applied to glucose oxidase[98] or horseradish peroxidase.[99]

1.7. The Electronic Coupling Problem

For an enzyme to catalyze fuel oxidation or oxidant reduction, there must be fast and efficient electron transfer to or from the electrode surface. Some rules for electron tunneling between redox sites within enzyme molecules have been described by Dutton and co-workers.[100] Efficient electron transfer (without requirement for a large driving force) can occur between two sites having low reorganization energies and separated only by a short distance. The same is true for intermolecular electron transfer (between two protein molecules) and for electron transfer across the enzyme−electrode interface. To provide a sufficiently high rate of electron transfer for enzyme catalysis, where turnover frequencies lie in the range $10-10^4$ s^{-1}, a good rule is that redox-sites can be separated by up to 15 Å but no more.[100] Achieving a good electrical connection between the electrode and the enzyme active site is *the coupling problem*. One answer is to immobilize the enzyme on the electrode in such a way that a direct electron transfer pathway is engaged, and no electron mediator is required. Direct electron transfer is possible if the active site itself lies close to the protein surface or, more commonly, if an electron relay center lies close to the protein surface. The direct transfer pathway can be established by linking the enzyme strongly to the electrode in the correct orientation, either by directed covalent or strong, noncovalent bonding, such as multisite electrostatic interactions (Figure 4). Strong attachment can lead to enhanced stability of the enzyme electrode and may also increase the electroactive surface coverage by directing only the most favorable orientation.

The alternative way of overcoming the coupling problem is to employ mobile mediators to shuttle electrons between the electrode surface and the enzyme.[101,102] The mediator is a small organic molecule or metal complex: it may be soluble and free in solution or tethered to a long linker that allows free movement but no escape. If mediators are present in solution, it is necessary to incorporate a membrane to prevent relay of electrons, through solution, directly from the anode to the cathode. Importantly, the mediator must also be capable of efficient electron transfer at a particular potential, that is, it must be able to transfer electrons rapidly when a small driving force is applied.

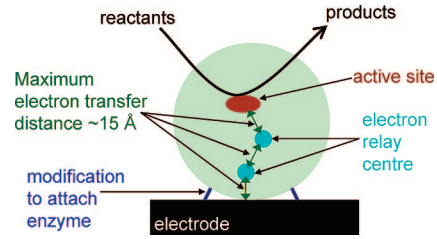


Figure 4. Cartoon diagram of an enzyme molecule immobilized on an electrode surface. Covalent or other strong attachments can be used to promote stable binding in the correct orientation for efficient electron transfer from the electrode to redox sites in the protein, such as electron relay centers or the active site.

There are both advantages and disadvantages to using electron mediators. In their favor, they are essential if the active site of the enzyme is occluded and consequently is unable to exchange electrons directly with the electrode surface. Analogously, they are also essential if the enzyme preferentially attaches in a nonelectroactive orientation on the electrode surface, however stable this attachment may be. Mediators are necessary for coupling to multilayers of enzyme on the electrode surface (raising the catalytic density) because, otherwise, only the lower layer will lie within the electron's tunnelling distance from the electrode surface.[8] In these cases, redox polymers can be used to create a conductive matrix extending from the electrode surface, hence 3D-electrode. An alternative approach is coentrapment of mediators with the enzyme in a polymer or sol−gel.[103]

The reduction potential of the mediator is important as it usually dictates the potential at which the electrode will operate. For mediators that rely upon diffusion, the K_M of the enzyme for the mediator is also relevant. A mediator adds an extra step to the electron transfer chain and it can actually lower the catalytic rate. This unusual result is the case with hydrogenases, where direct interfacial electron transfer is very fast, and a mediator is not required. Indeed, the electron-transfer reaction of hydrogenases with methyl viologen is slower than that observed for adsorbed hydrogenase reacting directly at an electrode.[104] Mediators may not be biocompatible: viologens are extremely poisonous and the same may be true for Os complexes.

2. Enzyme Structures and Properties Suited for Electrocatalysis

2.1. Enzymes for Cathodes

2.1.1. Reduction of O_2

For most enzyme fuel cells, O_2 is the oxidant of choice because it is freely available and has a high reduction potential, thus maximizing the voltage output by the cell. Catalysis of the four-electron reduction of O_2 to H_2O is complex and sluggish at mild overpotentials, and the driving force required is a key determining factor in the cell voltage available in Pt-based fuel cells.[25] By analogy, the difficulty of H_2O *oxidation* limits the efficiency of electrolytic and photolytic H_2 production. The overpotential for oxygen cycling (cf., hydrogen cycling, see below) is also a problem in biology: the only enzyme active site able to catalyze the formation of O_2 from water, the Mn_4CaO_x cluster of

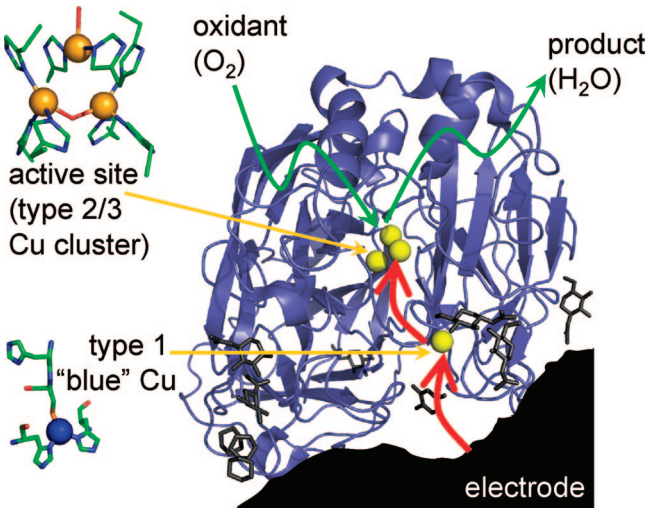


Figure 5. Ribbon representation of the crystal structure of *T. versicolor* laccase (PDB code 1KYA; structure solved by Bertrand et al.[109]) showing the protein superstructure in blue and the copper atoms as yellow spheres.

photosystem II, requires an overpotential of at least 0.1 V;[105] likewise four-electron O_2 reduction by enzymes always costs hundreds of millvolts.[9,10,23,63] An extensively studied enzyme for O_2 reduction is cytochrome oxidase, which in vivo couples the reaction to proton pumping and is less useful in terms of electrochemical efficiency because of its high overpotential.[24] Electrocatalysis of O_2 reduction by cytochrome oxidase at an electrode modified with its physiological electron donor, cytochrome *c*, is only observed at potentials below about 0.1 V,[57] meaning that this enzyme is a poor fuel cell catalyst.

By contrast, the blue Cu oxidases have been widely studied as fuel cell catalysts, and attention has focused on laccase and bilirubin oxidase, both of which display electrocatalytic O_2 reduction characteristics that compare favorably with Pt.[9,10] These enzymes have similar structures. The active site for O_2 reduction comprises three Cu atoms (the type 2/3 cluster) coordinated by histidine ligands. Electrons are transferred to the active site, one at a time, from a fourth Cu site known as a type 1 or blue Cu center, located at the top of a hydrophobic pocket at which organic substrates are bound and oxidized. The X-ray crystallographic structure of laccase from *Trametes versicolor* is shown in Figure 5 in the orientation (with respect to the electrode) that it must adopt for efficient electron transfer in an electrochemical experiment. (This structure and all subsequent protein structure representations in this Review were prepared in the same color scheme using PYMOL.)[106] The protein backbone is shown in a blue "ribbon" representation, while the electron-relay Cu center and active site Cu atoms are shown as yellow spheres. Surface sugars, shown in gray, probably contribute to the stability of the protein[107] and may present useful sites for chemical attachment of the enzyme to an electrode.[108]

Laccases that are useful for fuel cell applications are those produced by fungi because these are able to reduce O_2 to water at a higher potential than plant laccases,[23] of which *Rhus vernicifera* is the best studied example. Laccases are secreted by fungi, such as white rot, to catalyze the oxidation of various organic molecules (particularly products of lignin degradation).[91]

Laccases from fungi have pH optima in the region of 3−5,[91] which makes them unsuitable for certain novel fuel

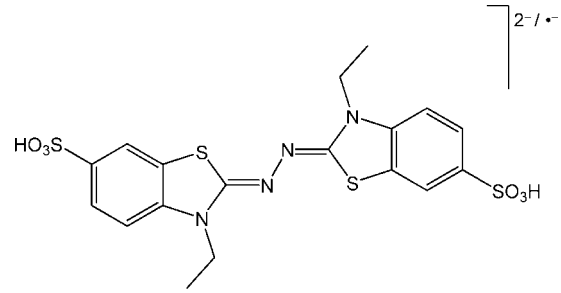


Figure 6. ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)).

cells designed to perform in human tissue and fluids for which the pH is closer to neutrality. Laccases are also inhibited by halide ions, particularly F^-, and the more ubiquitous Cl^- ion.[23,110–112] To overcome these problems, bilirubin oxidase has been studied as an alternative to laccase.[113,114] The operating potential of bilirubin oxidase is similar to that of most high-potential laccases, but it is active at higher pH (around 7) and tolerant to Cl^-.[9] The application of bilirubin oxidase as an O_2 cathode catalyst in a fuel cell was first suggested by Ikeda and co-workers in 2001, who showed that it reduces O_2 to H_2O at potentials higher than 0.7 V at pH 7, in the presence of ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate, Figure 6) as a mediator.[113] (Prior to this, bilirubin oxidase had been used in an enzymatic amperometric bilirubin biosensor.)[115] Bilirubin oxidase has been cloned and expressed in *Pichia pastoris*, and efforts have been made to change its activity by altering the potential of the blue Cu site (which is the electrochemical control center of the enzyme).[80,116]

Although earlier studies on laccase showed it could interact directly with an electrode, most experiments aimed at fuel cell applications have used mediators to improve the current density. With a reduction potential of 0.62 V for the dianion/radical (2−/•−) redox couple, ABTS is able to provide a reasonably efficient driving force for O_2 reduction at different types of electrode.[102,113] However, for membraneless fuel cells, soluble mediators are unsuitable, and dedicated wiring to each enzyme is required. Consequently Heller and co-workers have undertaken extensive studies aimed at wiring laccase or bilirubin oxidase to electrode surfaces using water-soluble polymers containing redox active Os complexes to serve as electron relays, as shown in Figure 7A.[9,10,117] Osmium forms stable complexes with pyridine ligands, and the reduction potentials can be tuned by appropriate choice of substituents on pyridine, bipyridine, and terpyridine ligands.[59] The redox polymer confines a large quantity of enzyme in good electronic contact with the electrode and allows construction of membraneless fuel cells giving high current densities. The length of the linker, which affects the mobility of the redox group, was found to be important, Figure 7B.[10]

Blanford et al. recently described a rational strategy for direct attachment of laccase to graphite.[63] As described above, the crystallographic structure of fungal laccase reveals a hydrophobic pocket at the surface of the protein in which organic substrates bind, close to the mononuclear type 1 blue Cu electron-transfer center. Specific electrode attachment at this region of laccase should provide the optimal electron-transfer pathway through to the trinuclear catalytic site where O_2 is reduced to water. A PGE electrode modified with 2-aminoanthracene (by electrochemical reduction of the anthracene-2-diazonium ion) was found to give stable

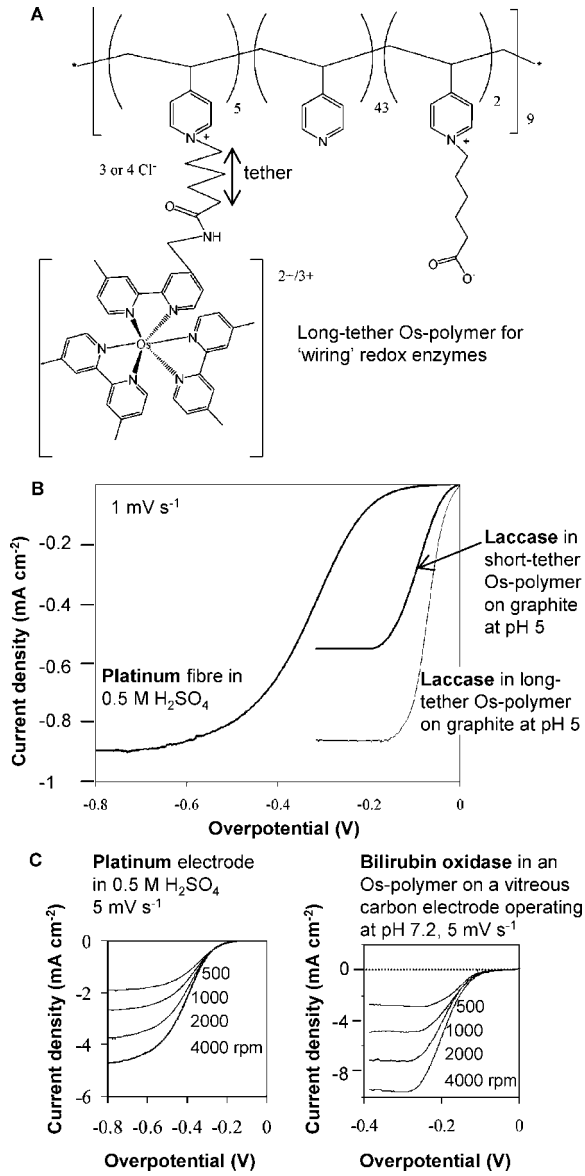


Figure 7. Panel A: An Os-containing polymer developed by Heller and co-workers for the attachment and electronic wiring of redox enzymes. Panel B: Comparison of electrocatalytic reduction of O_2 from air in quiescent solution at 37 °C, (i) by laccase in Os-polymers on a graphite electrode operating in pH 5 citrate and (ii) at a Pt electrode operating in 0.5 M H_2SO_4. Panel C: Comparison of electrocatalytic O_2 reduction at 1 atm O_2, 37 °C at a platinum fiber and by bilirubin oxidase in an Os-polymer on a vitreous carbon electrode operating in pH 7.2 phosphate containing 0.15 M NaCl. Panels A and B are reprinted with permission from ref 10 (Copyright 2006 American Chemical Society), and panel C is reprinted with permission from ref 9 (Copyright 2003 American Chemical Society).

adsorption of laccase and high electrochemical activity, probably because anthracene mimics physiological organic substrates, Figure 8A.[63] The laccase-modified electrode shows a well-defined electrocatalytic wave in the presence of O_2, in which reduction commences above 850 mV vs SHE at pH 4, Figure 8B. The magnitude and the long-term stability of the current response are far superior to those observed for laccase adsorbed at an unmodified electrode, indicating that the modified electrode both stabilizes and orientates laccase molecules for effective electron transfer (Figure 8B inset). This approach would also be suitable for extension to high surface-area forms of carbon.

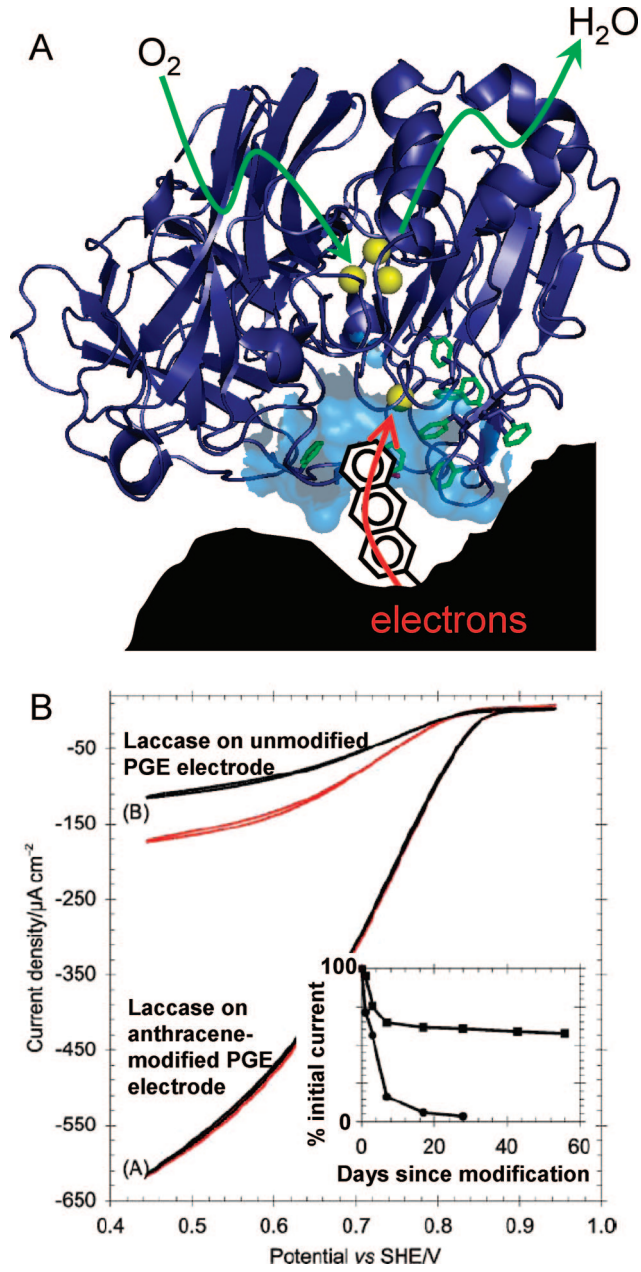


Figure 8. Panel A: Schematic representation of electron transfer via the 2-aminoanthracene linker to the type 1 (blue copper) center of laccase. The "hydrophobic pocket" is highlighted as a textured surface. Panel B: Electrocatalysis of O_2 reduction by *Pycnoporus cinnabarinus* laccase on a 2-aminoanthracene modified pyrolytic graphite edge (PGE) electrode and an unmodified PGE electrode at 25 °C in pH 4 citrate. Red curves were recorded immediately after spotting laccase solution onto the electrode, while black curves were recorded after exchanging the electrochemical cell solution for enzyme-free buffer solution. The inset shows the long-term percentage change in limiting current (at 0.44 V vs SHE) for electrocatalytic O_2 reduction by laccase on an unmodified PGE electrode (●) or a 2-aminoanthracene modified electrode (■) after storage for different periods of time at 4 °C. Panel B is from ref 63. Reproduced by permission of the Royal Society of Chemistry.

2.1.2. Reduction of Peroxide

Hydrogen peroxide is a stronger oxidant than O_2 and fuel cells using highly active peroxidases as the cathodic electrocatalyst have been reported.[118,119] Peroxidases contain an Fe-porphyrin group as the redox cofactor, and by far, the most applications have been described for the commercially

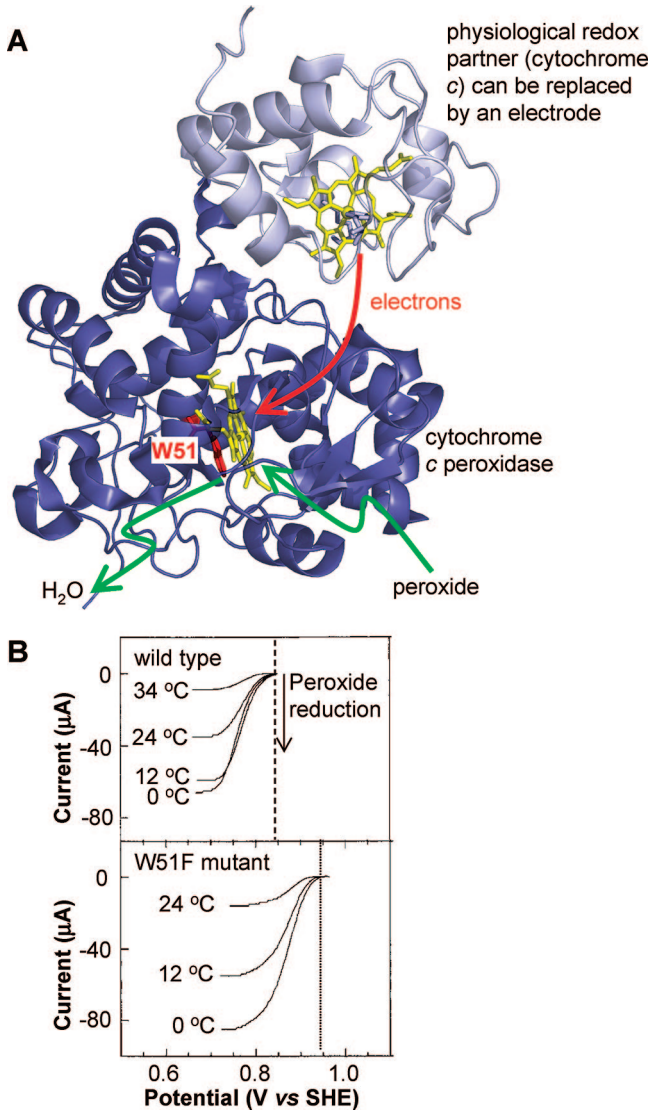
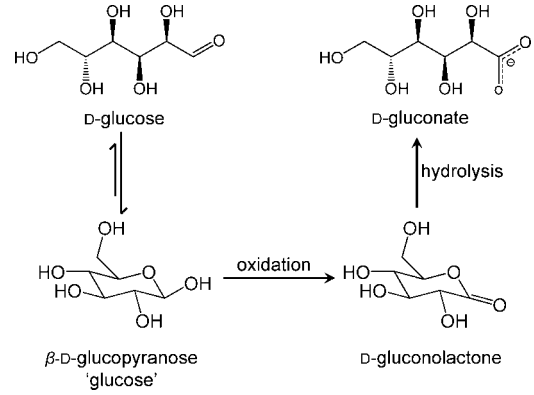


Figure 9. Panel A: How cytochrome *c* peroxidase interacts with its natural electron-transfer partner cytochrome *c*. The structure shows the enzyme from bakers' yeast (*Saccharomyces cerevisiae*) cocrystallized with cytochrome *c* (PDB code 1U74; structure solved by Kang et al.).[120] The protein backbone is shown in ribbon representation, and the porphyrin groups are shown in yellow. Tryptophan (W) 51 is highlighted in red. Panel B: The electrocatalytic responses obtained when the wild type or W51F mutant interact directly with a PGE electrode (N.B., cytochrome *c* is not present). The voltammograms for H_2O_2 reduction show that the W51F mutant has an increased electrocatalytic potential but a lower thermal stability. Adapted from ref 93. Coyright 1998 American Chemical Society.

available enzyme preparations from horseradish that are in widespread use in biotechnology. Horseradish peroxidase catalyzes the oxidation by H_2O_2 of small organic molecules that are able to approach the Fe-porphyrin, and a mediator is normally required to achieve electron transfer with an electrode. However, fast and efficient electron exchange with a PGE electrode has been demonstrated for cytochrome *c* peroxidase for which the physiological electron donor is cytochrome *c* (Figure 9), and some aspects of these studies are instructive.[93] First it should be stressed that cytochrome *c* peroxidase has not so far yielded stable electrochemistry, a serious disadvantage for fuel cells! (This is shown clearly in Figure 9B where raising the temperature actually *decreases* the catalytic current, because it destabilizes the enzyme film.) However, the electrocatalytic potential is high, greater than

Scheme 1. Biologically Relevant Forms Of D-Glucose and Its Oxidized Product, D-Gluconolactone



0.75 V vs SHE at pH 5.4, and this increases to 0.88 V when a tryptophan residue in the active site is replaced by phenylalanine (W51F mutation, see Figure 9).[93] This illustrates the possibility of genetically modifying enzymes to obtain large improvements in potential and hence the cell voltage.

Highly truncated forms of mitochondrial cytochrome *c* in which the heme group and some surrounding amino acids are retained after enzymic digestion are known as "microperoxidases". These tiny "enzymes" (commonly MP-8 or MP-11, indicating the number of amino acids retained, have some peroxidase activity). MP-8 has been used as the cathodic catalyst in an elaborate enzyme fuel cell in which H_2O_2, generated by the glucose oxidase-catalyzed reaction of glucose with O_2 is the oxidant.[121] At the anode, ethanol is converted to acetaldehyde by quinoheme alcohol dehydrogenase.

2.2. Enzymes for Anodes

2.2.1. Oxidation of Sugars and Other Alcohols

Among common sugars that can be used as fuels, glucose has received the greatest attention. Most glucose-oxidizing enzymes target the C1 hydroxyl group of β-D-glucopyranose (hereafter referred to simply as 'glucose', see Scheme 1), forming gluconolactone which spontaneously hydrolyses to gluconate. This reaction is catalyzed by a range of oxidase and dehydrogenase enzymes that differ in their cofactors and physiological electron acceptors. Enzymes using O_2 as the electron acceptor are classified as oxidases, whereas those using another small acceptor, such as a heme protein (cytochrome), are classified as dehydrogenases. In all cases, the catalytic centers are buried fairly deeply in the protein, and achieving efficient electron transfer at an electrode is a key challenge.

Some classes of enzymes that catalyze oxidation of alcohol functionalities in sugars also oxidize simple primary alcohols including methanol and ethanol. Table 3 lists selected alcohol oxidizing enzymes and their substrate affinities as represented by the Michaelis constants, K_M. It is notable that these affinities are fairly weak, and this can limit catalysis at the anode under low fuel conditions as discussed below. Alcohol-oxidizing enzymes are not damaged by O_2, so they are usually suitable as anode catalysts in membraneless fuel cells, although direct reaction of glucose oxidase with O_2 can cause a short circuit at the anode.

Fungal glucose oxidase couples the oxidation of glucose at a flavin center to the reduction of O_2 to H_2O_2. The structure

Table 3. The substrate affinity of selected enzymes that oxidize the C-OH functionality, which are relevant in fuel cell electrocatalysis.

organism	enzyme	cofactor	substrate	in vitro electron acceptor	K_M (mM)	ref
Aspergillus niger	glucose oxidase	flavin	glucose	O_2 or benzoquinone	42	122
Acinetobacter calcoaceticus	glucose dehydrogenase	PQQ	glucose	2,6-dichlorophenol-indophenol (DCIP)	25	123
Thermoplasma acidophilum	glucose dehydrogenase	$NAD(P)^+$	glucose	$NADP^+$	10	124
Acetobacter aceti	alcohol dehydrogenase	PQQ	ethanol	ferricyanide	1.6	125
Hyphomicrobium X	methanol dehydrogenase	PQQ	methanol	phenazine methosulfate	0.3	126
Gluconobacter industrius	fructose dehydrogenase	flavin	fructose	ferricyanide	10	127

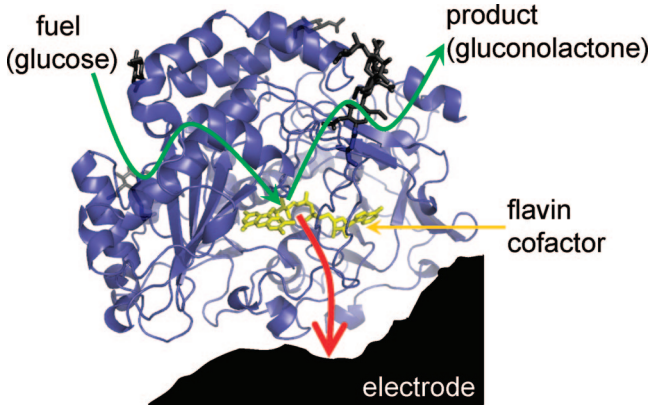


Figure 10. Representation of the structure of glucose oxidase from *Aspergillus niger* (PDB code 1CF3; structure solved by Wohlfahrt et al.)[128] showing the protein as blue ribbons, the flavin cofactor as yellow sticks, and sugars on the surface of the protein as gray sticks.

of the enzyme from *Aspergillus niger* is shown in Figure 10. The flavin adenine dinucleotide (FAD) cofactor is buried in a cleft in the protein so that direct electron exchange with an electrode is very sluggish. Nevertheless, using a range of strategies for mediating electron transfer, glucose oxidase has been exploited extensively for glucose sensing and fuel cells.

An immobilization and mediation strategy based on reconstitution of cofactor-deficient glucose oxidase using a linker capped with the FAD group (Figure 11) has been developed by Willner, Katz, and co-workers.[129,130] Ferrocene, PQQ, gold nanoparticles, and carbon nanotubes have been used as electron relays in the linkers, and subsequent chemical cross-linking of the attached enzyme molecules with glutaraldehyde has been used to stabilize the layer.[62,129,131] Very high rates of electron transfer through these wires have been reported (up to 5000 s^{-1}), but often a high overpotential is required for glucose oxidation (see for example Figure 11C). It is therefore unsurprising that a glucose oxidase electrode assembled in this way provided an OCV less than 120 mV in a fuel cell when coupled with a cytochrome oxidase cathode that also requires a high overpotential for O_2 reduction.[57] A great improvement in terms of efficiency was achieved using supramolecular architectures, in which Willner and colleagues used, as a mediator, a rotaxane threaded onto a molecular wire connecting the FAD cofactor of glucose oxidase to a gold electrode, Figure 11D.[130] The

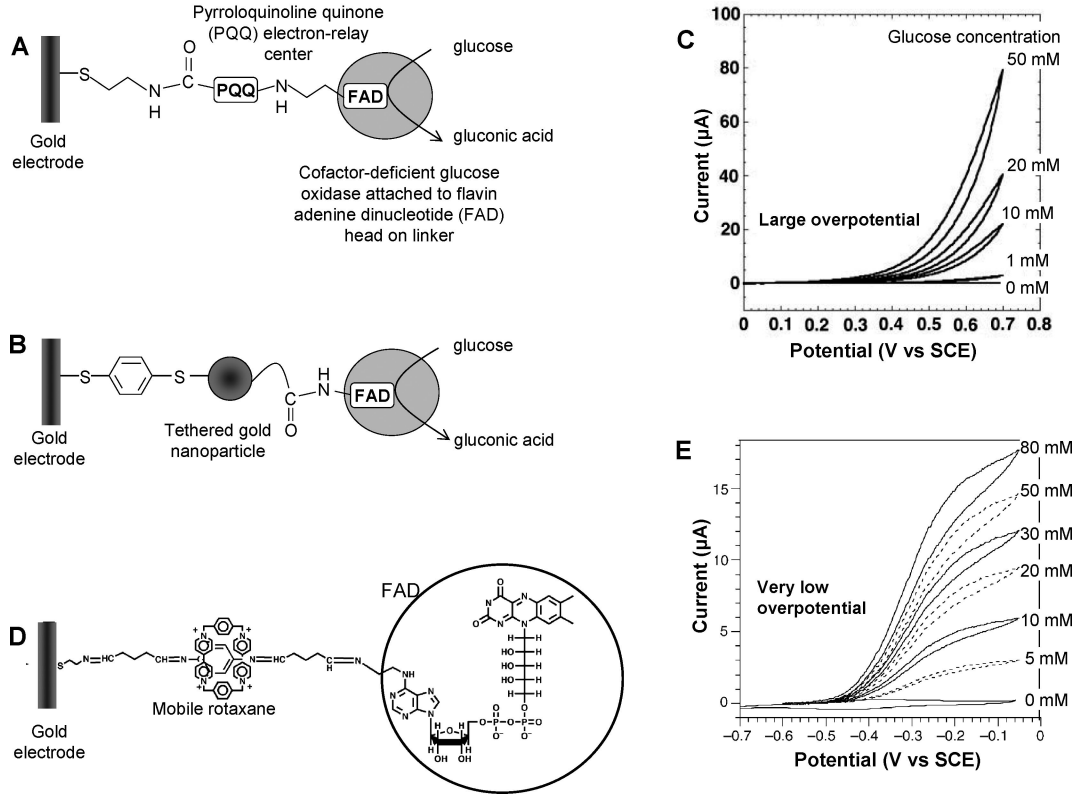


Figure 11. Strategies developed by Katz, Willner, and co-workers for tethering glucose oxidase using either a PQQ-incorporating wire (Panel A), gold nanoparticles (Panel B), or a threaded redox-active rotaxane (Panel D). Cyclic voltammograms for the systems shown in panels B and D are recorded in Panels C and E, respectively, in pH 7 phosphate buffer (0.1 M) under argon at a scan rate of 5 mV s^{-1}. Panel C is from ref 129 (www.sciencemag.org) (Reprinted with permission from AAAS), and panels D and E are reproduced from ref 130 (Copyright 2004 Wiley-VCH).

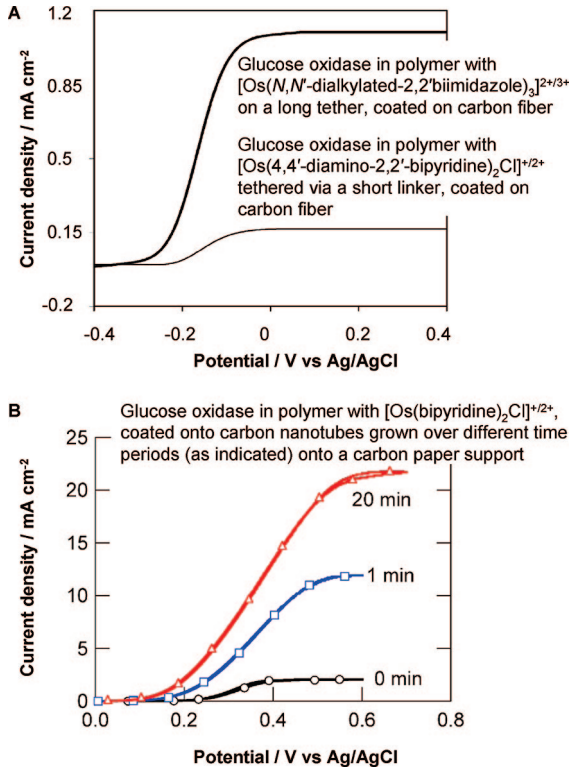


Figure 12. Os-containing redox polymers have been used to mediate electron transfer to the buried flavin center of *A. niger* flavin-containing glucose oxidase. The electrocatalytic current density (per geometric area) for glucose oxidation at electrodes prepared in this way is shown for (A) carbon fiber or (B) a multiscale carbon RDE. The polarization curve in Panel A was recorded at 1 mV s^{-1} in quiescent buffer solution (pH 7.4) containing 15 mM glucose and exposed to air at 38 °C. The voltammogram in panel B was recorded at 1 mV s^{-1} in N_2-saturated buffer (pH 7.1) containing 50 mM glucose at 37.5 °C with the electrode rotated at 4000 rpm. Panel A is reproduced from ref 133 (Copyright 2003 American Chemical Society), and panel B is reproduced from ref 67 (Reproduced by permission of ECS—The Electrochemical Society).

resulting electrocatalytic behavior observed by cyclic voltammetry is excellent, with glucose oxidation commencing at about −0.2 V vs SHE, Figure 11E.

A different approach to mediation of electron transfer to glucose oxidase has been adopted by Heller and co-workers. In early work, ferrocene groups were attached to the enzyme as electron relays,[132] but more recently, Os-containing redox hydrogels, similar to those described for laccase and bilirubin oxidase (section 2.1), were used to entrap glucose oxidase or lactate oxidase and facilitate electron transfer to the flavin active site.[60] Electrocatalysis at a low overpotential was achieved by varying the ligands to the Os center to tune the redox potential of the metal to match that of the flavin center in the enzyme, Figure 12A. The rate of catalysis was also improved by increasing the length of the tether between the Os center and the polymer backbone, presumably because this increases the mobility of the mediator.[59] Barton et al. recently reported high electrocatalytic currents for glucose oxidation (<20 mA cm^{-2} geometric area) when similar Os-polymers were used to attach glucose oxidase to high surface-area multiscale carbon supports (multiwall carbon nanotubes grown on carbon fiber paper), but a much higher overpotential was required for electrocatalysis, Figure 12B.[67]

It has been suggested that carbon nanotubes may facilitate direct electron transfer to glucose oxidase by protruding into the enzyme and shortening the electron-transfer distance to the buried flavin, but direct electrocatalysis has not been achieved at mild overpotentials.[134]

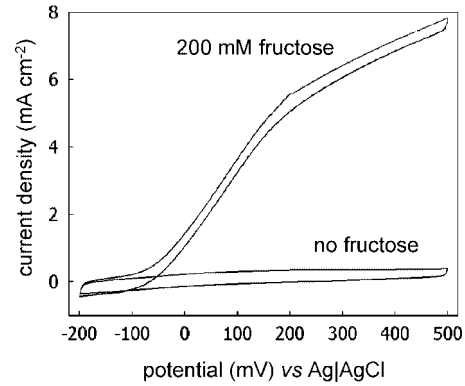


Figure 13. Cyclic voltammogram showing direct electrocatalytic oxidation of fructose by the heme-containing flavin fructose dehydrogenase from *Gluconobacter* species at a Ketjen Black modified glassy carbon electrode, pH 5.0, 20 mV s^{-1}. Reproduced with permission from ref 84. Copyright 2007 Chemistry Society of Japan.

Acetogenic bacteria express a range of membrane-bound dehydrogenases that selectively oxidize substrates such as glucose, fructose, and primary alcohols at a flavin center. Although mediators have been used in most reports of electrocatalysis,[57,135,136] fructose dehydrogenase from *Gluconobacter* species has been isolated with a *c*-type heme-containing subunit or partner protein tightly bound to the dehydrogenase moiety, and this system undergoes efficient, direct electrocatalysis of fructose oxidation at an electrode, Figure 13.[84] Sode and co-workers also report that the thermostable heme glucose dehydrogenase from *Burkholderia cepacia* undergoes direct electrocatalysis when adsorbed at a graphite powder-packed bed electrode, but currents were very low in this case, suggesting that few enzyme molecules are engaged in electrocatalysis.[137]

In other microbial alcohol and sugar dehydrogenases, the catalytic center is the hydride carrier pyrroloquinoline quinone (PQQ) coordinated to a Ca^{2+} ion, and electrons are transferred to acceptors such as cytochrome *c* or ubiquinone.[92] Several early examples of fuel cells made use of PQQ-methanol dehydrogenases from different strains of *Pseudomonas* for oxidation of methanol with the aid of soluble mediators, phenazine ethosulfate (PES),[52] or tetramethyl-4-phenylenediamine (TMPD).[53] While these enzymes were suitable for fuel cells that use Pt as the cathode catalyst, the high pH optimum of PQQ-methanol dehydrogenase (pH 9 or above),[92] means that they are not suitable for coupling with an O_2-reducing enzyme cathode modified with laccase or bilirubin oxidase.

Willner and co-workers tested a strategy for mediation of electron transfer to a buried PQQ-cofactor of glucose dehydrogenase based on attachment of cofactor-deficient enzyme to PQQ-capped linkers incorporating gold nanoparticles. This is similar to the approach described above for flavin-dependent dehydrogenases, and the overpotential for glucose oxidation is also quite substantial.[62] There are some examples of PQQ-dependent alcohol dehydrogenases that incorporate one or more heme centers able to relay electrons between the catalytic center and the surface of the protein,[92] and the structure of one such enzyme is shown in Figure 14. Dehydrogenases of this type have been shown to undergo direct electron transfer and electrocatalysis.[138] Sode and co-

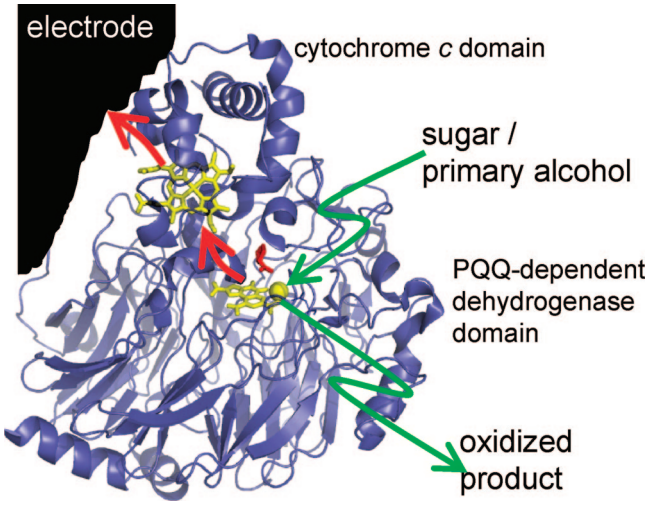


Figure 14. Representation of a PQQ-dependent alcohol dehydrogenase from *Comamonas testosteroni* (PDB code 1KB0; structure solved by Oubrie et al.).[139] The protein backbone is shown as a blue ribbon, the heme and PQQ centers are shown as yellow sticks, and a Ca atom is shown as a yellow sphere. Electron density close to the PQQ center has been assigned to tetrahydrofuran-2-carboxylic acid (red sticks) and presumably arises from enzyme-catalyzed oxidation of the corresponding alcohol.

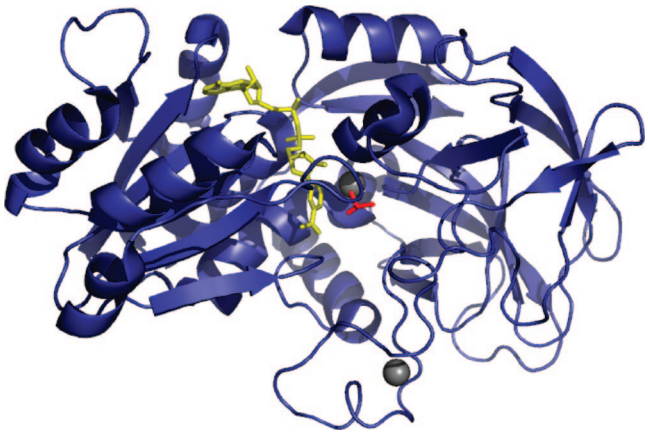


Figure 15. Structure of horse liver alcohol dehydrogenase (PDB code 2OHX; structure solved by Al-Karadaghi et al.),[140] with NAD shown in yellow, Zn atoms in gray, and co-crystallized dimethylsulfoxide in red.

workers have developed a genetic construct in which the PQQ glucose dehydrogenase from *Acinetobacter calcoaceticus* is fused to the cytochrome *c* domain of ethanol dehydrogenase from the soil bacterium *Comamonas testosteroni* (see Figure 14). This system undergoes direct electrocatalysis at a carbon paste electrode and has been demonstrated in a glucose sensor; however, currents were very low when the electrode was used in a fuel cell.[163]

Other dehydrogenases for alcohol oxidation depend upon the more ubiquitous hydride carrier, $NAD(P)^+$ (the oxidized form of nicotinamide adenine dinucleotide, NADH, or its phosphorylated derivative, NADPH). A well-characterized example is the zinc-containing liver alcohol dehydrogenase, Figure 15. Use of the $NAD(P)^+$-dependent dehydrogenases at an electrode requires rapid, continuous regeneration of the oxidized cofactor, and the high overpotential and slow kinetics for this reaction at unmodified electrodes mean that direct oxidation is inefficient. Multicomponent NAD^+ regeneration systems using mediators and diaphorase enzymes have been employed in a number of early reports[101] but are probably too complex to be useful in fuel cell catalysis.

A strategy that leads to moderately low overpotentials for electrocatalysis by $NAD(P)^+$-dependent dehydrogenases involves electropolymerization of a redox dye at the electrode which acts as a catalyst for regeneration of soluble $NAD(P)^+$. Examples include polymethylene green on carbon felt for alcohol dehydrogenase or aldehyde dehydrogenase[141] or polymethylene blue on single-walled carbon nanotubes for glucose dehydrogenase cross-linked with the readily available protein bovine serum albumin.[142] Yan et al. recently elaborated on this approach by coupling NAD^+ to a Nile Blue-modified single-walled carbon nanotube electrode via a boronate linkage and then attaching glucose dehydrogenase or alcohol dehydrogenase.[131] A promising alternative for cofactor regeneration was described recently by Barker et al. in which a two-subunit subcomplex of mitochrondrial respiratory complex I (NADH dehydrogenase) exhibits efficient NAD^+/NADH interconversion at a graphite electrode.[22]

2.2.2. Oxidation of H_2

Fast interconversion of H_2 and H^+ is critical in microbial energy-cycling, where it is catalyzed by enzymes known as hydrogenases.[143] Oxidation or production of H_2 is catalyzed at a bimetallic active site consisting of commonly available metals: iron ([FeFe]) or nickel and iron ([NiFe]), with the Fe atom coordinated by the biologically unusual ligands CO and CN^-. For fuel cell applications the [NiFe]-hydrogenases, Figure 16, are generally favored over [FeFe]-enzymes because they tend to be less sensitive to damage by O_2 and are often extremely active catalysts for H_2 oxidation. As we discuss later, however, the [FeFe] hydrogenases can be extremely active electrocatalysts for H^+ reduction and have been used in photohydrogen production (section 4).[11,20] In both cases, a chain of iron−sulfur clusters facilitates fast electron transfer between the buried active site and the surface of the protein (Figure 16) and makes direct electron transfer with an electrode possible in many cases.[20]

The H_2-oxidation activity of carbon electrodes modified with [NiFe]-hydrogenases has been compared with that of platinized electrodes operating in aqueous solution.[12,13,20] A PGE RDE modified with *A. vinosum* [NiFe]-hydrogenase (operating at +0.242 V vs SHE, 45 °C, 1 bar H_2, pH 7.0), was compared with a platinized carbon or gold RDE operating under the same conditions. In both cases, the catalytic current was diffusion-controlled at 2500 rpm; catalysis at the hydrogenase active site is clearly very fast (Figure 17A).[12] At less positive potentials, however, the catalytic current at the hydrogenase-modified electrode was lower than at the platinized electrode.[145] This is probably the result of poor electronic contact between some of the enzyme molecules and the electrode, giving rise to slow interfacial electron-transfer rates. Karyakin and co-workers reported similar findings based on comparison of a carbon filament electrode modified with *Desulfomicrobium baculatum* [NiFeSe]-hydrogenase operating in aqueous solution (pH 7.0, 60 °C, agitated by H_2 bubbling), with a Pt/Vulcan RDE operating in 0.5 M H_2SO_4 (Figure 17B).[13]

Catalytic H_2 oxidation currents higher than 3 mA cm^{-2} have been reported for electrodes modified by adsorption of [NiFe]-hydrogenase (and relying on direct electron transfer, as in Figure 17).[12] However, improvement of the stability

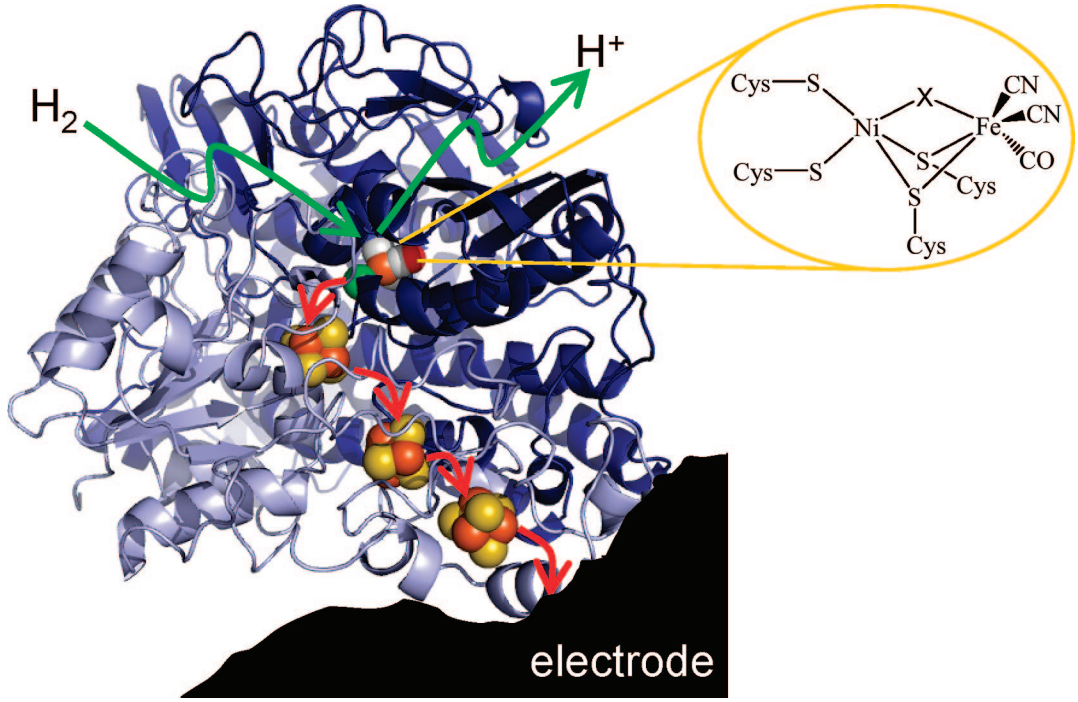


Figure 16. Representation of the crystal structure of *Desulfovibrio fructosovorans* [NiFe]-hydrogenase (PDB code 1YRQ; structure solved by Volbeda et al.),[144] highlighting the structure of the [NiFe]-active site, where X is a bridging ligand, the identity of which varies according to the state of the enzyme.

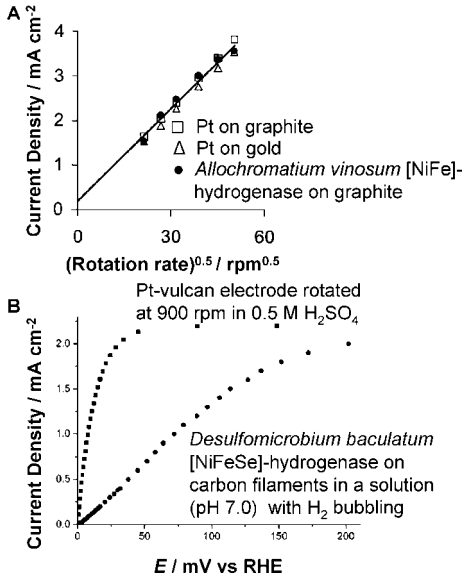


Figure 17. Two attempts to compare the electrocatalysis of H_2 oxidation by hydrogenases and platinum-modified electrodes. Panel A: Levich plot (limiting current density vs square root of the rotation rate) achieved at 0.242 V vs SHE, 1 bar H_2, 45 °C and pH 7, at platinized PGE, platinized gold, or a PGE electrode modified with a [NiFe]-hydrogenase from *Allochromatium vinosum*. Panel B: Quantifying the maximal current density for Pt/Vulcan (in 0.5 M H_2SO_4, electrode rotation rate: 900 rpm) vs a carbon filament electrode modified with a [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum* (stationary electrode, but with H_2 bubbling to assist mass transport, pH 7.0) showing the electrode potential (driving force) required to attain maximum activity. Panel A from ref 12. Reproduced by permission of The Royal Society of Chemistry. Panel B from ref 13. Reproduced with permission from Portland Press Ltd.

of the adsorbed film remains a challenge for exploiting hydrogenase electrodes in fuel cells.[65]

Karyakin and co-workers achieved an improvement in stability and fairly high catalytic currents (0.5−1.5 mA per flat cm^2 electrode area) by electropolymerization of a pyrrole layer on carbon filament material, on which they immobilized *Thiocapsa roseopersicina* [NiFe]-hydrogenase.[94] The electrode was stored at 4 °C and tested periodically. Around 50% of the initial current remained after 6 months.

Site-specific covalent coupling strategies have been proposed to improve film stability and correctly orientate hydrogenase for optimum electron-transfer efficiency. DeLacey and co-workers used carbodiimide chemistry to couple *Desulfovibrio gigas* [NiFe]-hydrogenase to pyrolytic graphite or carbon nanotubes, taking advantage of a dipole moment in the protein arising from a high concentration of glutamate residues around the surface [4Fe4S] cluster that could be linked to an amine-functionalized surface.[66,85] Although the covalent modification did not provide a substantial improvement in H_2 oxidation current relative to directly adsorbed hydrogenase (Figure 18A), a stable current (greater than 1 mA cm^{-2}) persisted for over 1 month (Figure 18B).[66] However, the catalytic current showed evidence of being limited by mass transport of H_2 (the current was rotation-rate dependent at all rotation rates tested),[66] and this may disguise some loss of enzyme activity or enzyme dissociation from the electrode (see section 1.6).

Direct electrochemical methods have been used extensively to study the reactions of a range of hydrogenases with small molecules, such as O_2, CO, and sulfide, that can compete with H_2 at catalytic sites on precious metals, (reviewed in ref 20). In general, hydrogenases are highly O_2-sensitive; therefore, hydrogenase activity has usually been studied under anaerobic conditions. However, tolerance to O_2 is essential if hydrogenases are to have a future as anode catalysts in simple membraneless enzyme fuel cells. More generally, this is also important if organisms able to produce H_2 are to be "farmed". To this end, one recent significant

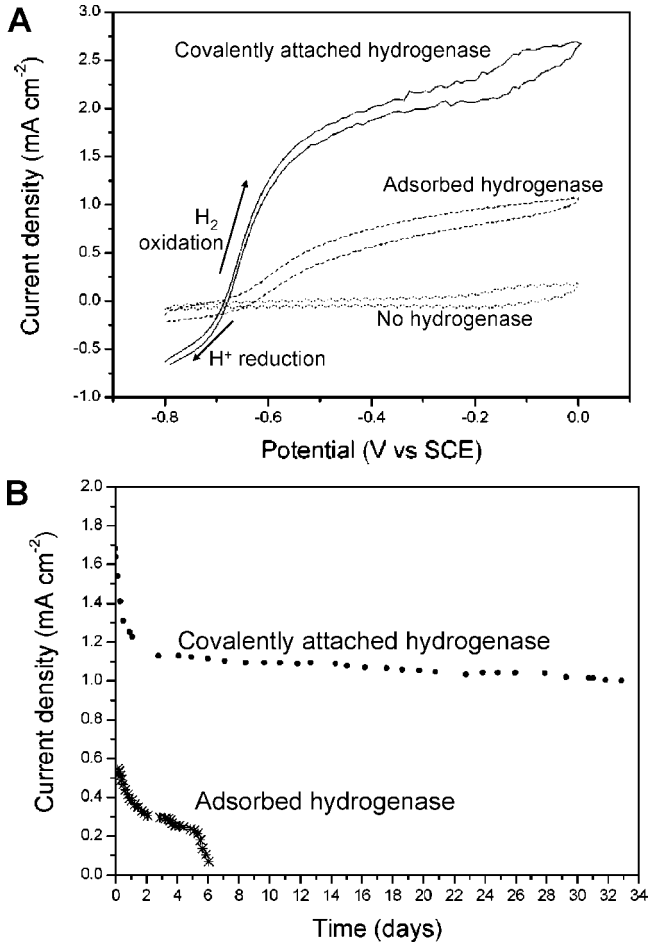


Figure 18. Cyclic voltammograms showing electrocatalysis of H_2 oxidation by a PGE electrode modified with multiwalled carbon nanotubes, with *D. gigas* hydrogenase covalently attached or directly adsorbed. Measurements were made at pH 7.0, 40 °C, 20 mV s^{-1} under 1 atm H_2, and at an electrode rotation rate of 2500 rpm. Reproduced with permission from ref 66. Copyright 2007 American Chemical Society.

finding was that the [NiFe]-membrane-bound hydrogenases from *Ralstonia* spp. of Knallgas bacteria retain substantial H_2 oxidation activity under atmospheric levels of O_2.[20,64] This is consistent with the strictly aerobic growth conditions of *Ralstonia*, in which periplasmic H_2 oxidation by hydrogenase is coupled to O_2 reduction. By contrast, the [NiFe]-hydrogenase from the purple photosynthetic bacterium *Allochromatium vinosum* is inactivated by just 0.5% O_2.

Most hydrogenases are inhibited reversibly by CO, but the *Ralstonia* membrane-bound hydrogenases were found to be essentially insensitive to high levels (9-fold excess) of CO.[20,64] Hydrogenases are also inhibited reversibly by sulfides but only at very high potentials that would not be accessed under fuel cell operation.[146] The chemical basis for discrimination between H_2 and other small molecules in hydrogenases still remains uncertain and is a subject of active investigation.[20] The protein environment around the active site in O_2 sensitive [NiFe]-hydrogenases is fairly well-conserved in the *Ralstonia* membrane-bound hydrogenases,[29] so it appears that residues further from the active site are somehow important in tuning their reactivity.

A common characteristic of [NiFe]-hydrogenases is the reversible formation of an inactive state at high potentials under anaerobic conditions, which is associated with a

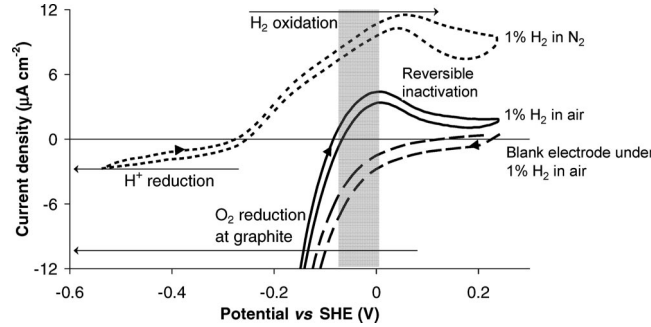


Figure 19. Cyclic voltammograms for an electrode modified with *Ralstonia metallidurans* CH34 membrane-bound hydrogenase operating under 1% H_2 in N_2 (dotted line) and 1% H_2 in air (solid line). Also shown is the response of a blank electrode under 1% H_2 in air (dashed line). Other conditions: pH 5.5, 30 °C, electrode rotation rate 4500 rpm, scan rate 2 mV s^{-1}. The gray bar indicates the potential window within which useful H_2 oxidation in air occurs under these conditions. Reproduced with permission from ref 36. Copyright 2008 American Chemical Society.

bridging hydroxide or peroxide ligand becoming trapped in the active site (X in Figure 16).[20] This inactivation is evident in the anaerobic voltammogram for *Ralstonia metallidurans* CH34 [NiFe]-membrane-bound hydrogenase directly adsorbed on a PGE electrode, Figure 19 (dotted line).[36] Reversible inactivation at high potentials is even more pronounced for this enzyme operating in air (solid line).

The similarity of hydrogenase active sites with established organometallic iron−sulfur clusters has led to much interest in the synthesis of inorganic mimics.[71–76] So far, however, no examples with significant catalytic activity or stability in the presence of air and moisture have been generated. It is important to note that aside from comparing Pt with enzymes, there have been important developments in the mainstream research on Pt catalysts. This includes alloying Pt with other metals to produce materials with different properties, such as PtRu which exhibits enhanced tolerance to CO relative to Pt alone,[147] and modifying the Pt surface with organometallic compounds to enhance the catalytic current.[148]

3. Examples of Enzyme-Based Fuel Cells

In this section, we discuss recent efforts to apply enzymes as electrocatalysts in functioning fuel cells. The fuel is generally the variable, so we will describe different types of enzyme fuel cell on this basis, focusing particularly on the inherent characteristics of the enzymes. We address questions of what niche is fulfilled and what improvements are necessary to develop reliable devices.

3.1. Enzyme Fuel Cells Using Sugars As Fuels

The relatively high K_M of sugar-oxidizing enzymes for their substrates (see Table 3) presents a challenge for operation under low-substrate conditions, section 1.5. A number of attempts at fuel cells have included a magnetic stirrer or relied upon agitation caused by pumping the fuel−oxidant mixture through the cell: as noted in section 1.6 the power requirement for these processes far outweighs the power generated. Electron transfer is another key challenge in exploitation of sugar oxidation by sugar/alcohol oxidases and dehydrogenases, and most fuel cell electrodes based on these enzymes have incorporated mediators as discussed in section 2.2.1. In early examples, the mediators and sometimes the enzymes themselves were free in the

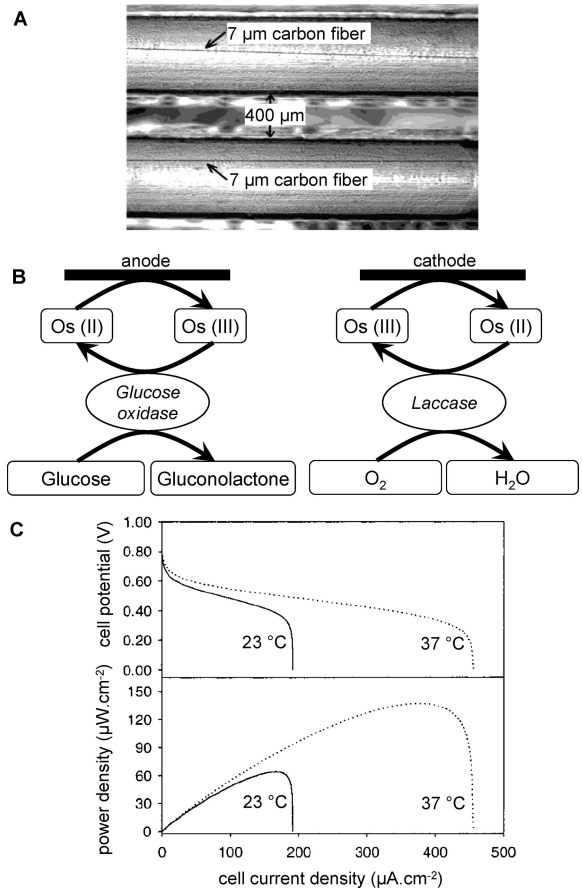


Figure 20. Glucose/O_2 enzyme fuel cell reported by Heller and co-workers in 2001, using carbon fibers modified with Os-polymers containing laccase and glucose oxidase in quiescent pH 5 citrate containing 15 mM glucose and equilibrated with air. Panel A: A portion of the cell. Panel B: Schematic representation of the mediated electron-transfer reactions at each electrode. Panel C: Cell potential and power density versus cell current density. Panels A and C are reproduced with permission from ref 90. Copyright 2001 American Chemical Society.

electrolyte solution, but more recent approaches have improved on this by co-immobilizing or entrapping the enzymes and mediators.

Heller and co-workers tackled the challenge of electron transfer by incorporating enzymes in Os-containing redox polymers as described in section 2.2.1. In an early fuel cell reported by this group, carbon fibers (7 μm diameter) were modified with Os-polymers incorporating glucose oxidase for the anode and a fungal laccase for the cathode, Figure 20A.[90] Insertion of the miniature anode and cathode into a still, aerated buffer solution (pH 5) containing 15 mM glucose gave an OCV close to 0.8 V, and a maximum power of 137 μW cm^{-2} (0.4 V, 343 μA cm^{-2}) at 37 °C, Figure 20B and C. The potentials of redox centers in the polymer were tuned to suit each enzyme by varying the ligands to the Os, and this was an important factor in achieving the impressive cell voltages. The starting glucose concentration in these experiments was close to the K_M value for the anode enzyme, *Aspergillus niger* glucose oxidase (around 20 mM glucose),[149] and efficient cylindrical diffusion of substrate at the fiber electrodes was necessary to maintain substrate concentrations at the electrodes close to the levels in the bulk electrolyte. The authors report a 10% loss of current after 24 h of continuous operation and a 25% loss after 72 h. Heller and co-workers have made further developments, replacing laccase at the cathode by bilirubin oxidase, which has a more neutral pH optimum and tolerates Cl^-,[114,150,151] and they also demonstrated fuel cell operation in a living plant (see below).[60]

Willner, Katz, and co-workers described a fuel cell using a glucose oxidase anode, assembled by reconstitution of the apoenzyme with a flavin-capped linker as described in section 2.2.1.[57] The cathode catalyst was cytochrome *c* oxidase immobilized on a cytochrome *c*-modified electrode, but the significant overpotential required for electrocatalytic O_2 reduction by this enzyme meant that the OCV for the fuel cell was only about 120 mV. The power output was also reliant upon pumping air-equilibrated glucose solution (80 mM) through the cell using a peristaltic pump.

A glucose/H_2O_2 enzyme fuel cell described by Pizzariello et al. involved composite electrodes constructed by spray painting an inert support with carbon particles modified with ferrocene as mediator and either *A. niger* glucose oxidase or horseradish peroxidase as the biocatalyst.[119] The poor match of the mediator potential for that of the enzymes meant that both electrodes operated at a very high overpotential, and working fuel cell voltages were below 100 mV when the electrodes were operated in compartments (separated by Nafion) containing, respectively, 10 mM glucose or 8 mM peroxide. This report does, however, provide an example of power generation from a crude, bioderived fuel: power generation from hydrolyzed corn syrup was also demonstrated.[119]

A different approach to entrapping *A. niger* glucose oxidase with mediators at an electrode is described by Lim et al.[152] The fuel cell electrodes were assembled by incorporation of glucose oxidase (anode) and bilirubin oxidase (cathode) with multiwall carbon nanotubes into a silica sol−gel matrix. Use of soluble mediators (ferrocene for the anode and ABTS for the cathode) necessitated separation of the electrodes by a membrane (Nafion 117). (We note that the membrane would not be necessary if the mediators were coimmobilized with the enzymes because bilirubin oxidase is unaffected by glucose, and glucose oxidase reacts preferentially with ferrocene rather than O_2.) With the fuel cell operating at room temperature, with 0.1 M glucose at the anode and O_2-saturated electrolyte in the cathode compartment, a power density of 120 μW cm^{-2} (at 240 mV) was recorded, which dropped to 86 μW cm^{-2} in ambient air.[152]

There are several recent reports of sugar/O_2 fuel cells that make use of dehydrogenases with heme electron relay centers and are able to undergo direct electron transfer at carbon electrodes. Sode and co-workers employed a genetic construct involving glucose dehydrogenase from *Acinetobacter calcoaceticus* fused to a cytochrome *c* domain (see section 2.2.1) in a non-mediated glucose/O_2 fuel cell with a Pt cathode. An OCV of 420 mV was recorded.[153] However the current density was very low (<1 μW cm^{-2}), suggesting that few enzyme molecules were engaged in electrocatalysis. Much more impressive currents were achieved in a membraneless fructose/O_2 fuel cell described by Kano and co-workers, Figure 21, in which a heme-containing fructose dehydrogenase (anode) and laccase (cathode) were immobilized on powdered or mesoporous forms of carbon mounted on carbon paper as described in section 2.2.1.[68] The fuel cell was operated at high fuel and oxidant concentrations (0.2 M fructose and saturating O_2, both well above the K_M values for the enzymes), although stirring the

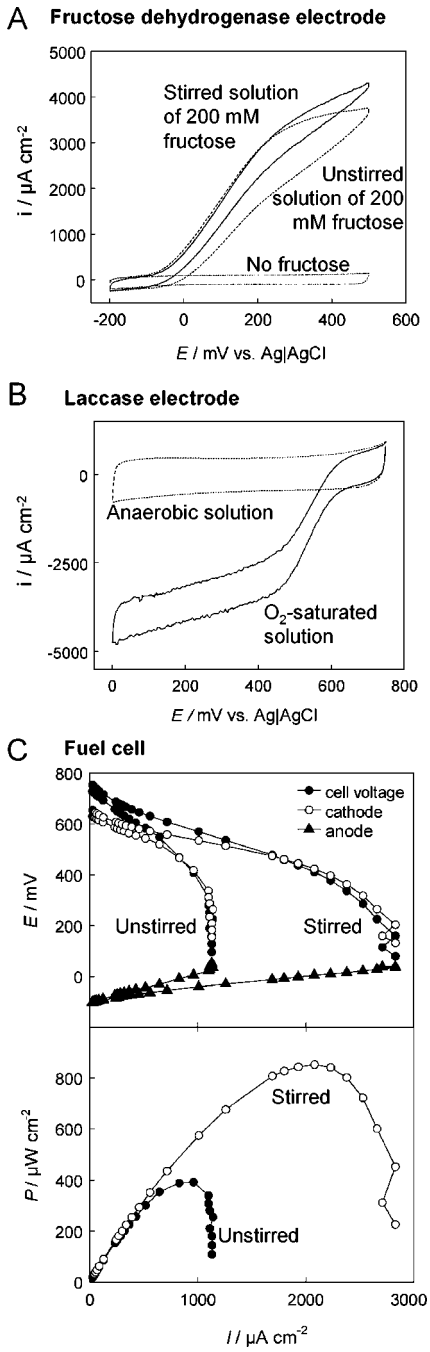


Figure 21. Cyclic voltammetry and fuel cell behavior for electrodes modified with fructose dehydrogenase and laccase. Panel A: Cyclic voltammograms (20 mV s^{-1}) showing direct electrocatalytic oxidation of fructose by fructose dehydrogenase on Ketjen black particles (EC-300J 800 m^2 g^{-1}, average particle size 39.5 nm) on a carbon paper electrode at pH 5. Panel B: Cyclic voltammograms (20 mV s^{-1}) for a carbon paper electrode coated with mesoporous carbon aerogel modified with *Trametes* laccase, showing direct electrocatalytic O_2 reduction at pH 5. Panel C: Behavior of a fuel cell constructed from these electrodes, pH 5, 0.2 M fructose. All experiments were conducted at 25 °C. From ref 68. Reproduced by permission of the PCCP Owner Societies.

solution further increased the current response (Figure 21C), indicating that the cell performance is limited by mass transport. Fuel cells based on direct electron transfer may have advantages for implantable applications because they avoid toxic mediators or byproducts of their breakdown, although they are yet to be demonstrated in this context.

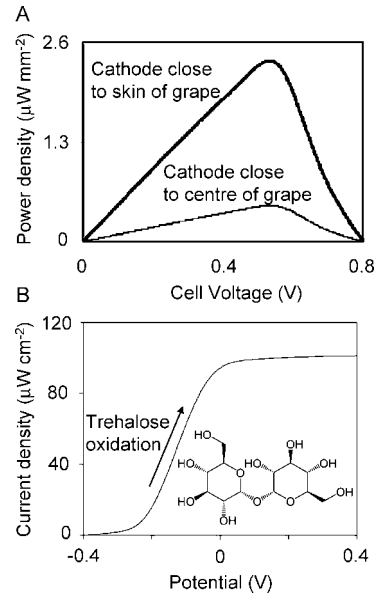


Figure 22. Panel A: Power vs voltage curve for an enzyme-catalyzed glucose/O_2 fuel cell implanted in a grape, showing the dependence upon the positioning of the cathode: either close to the skin of the grape (bold) or near the center of the grape (fine). Panel B: Voltammogram showing the oxidation of trehalose (inset) (32 mM) by glucose-3-dehydrogenase in an Os-containing polymer on a carbon cloth electrode operating at pH 7.4, 37.5 °C. Panel A is reproduced with permission from ref 60 (Copyright 2003 American Chemical Society), and panel B is reproduced from ref 155, Copyright 2006, with permission from Elsevier.

3.1.1. Implantable Fuel Cells

The ability of enzymes to use biologically derived fuels such as glucose and fructose, along with O_2 as an oxidant, directly from their environment under ambient conditions of temperature and pH, suggests niche applications for enzyme fuel cells as implantable power sources in living organisms. (A comparison between potentially implantable enzyme fuel cells and currently available implantable Zn-based batteries was recently presented by Heller.)[7] Possible applications of implantable enzyme fuel cells include self-powered sensors for chemical species in the blood, power sources for an implanted device such as an artificial pacemaker in the heart, and generation of electricity directly from sugars in living plants.

An early application of enzymes in a device designed as an implantable power source, described by Ahn et al. in 1976, was the use of hyaluronidase to break down hyaluronic acid from body fluid into *N*-acetylglucosamine and related compounds to enrich the sugar (fuel) content at a Pt-black anode connected to a Ag-black cathode.[154]

A more direct approach used enzymes as electrocatalysts to oxidize naturally occurring sugars. A fuel cell based on carbon fiber electrodes (as described above) with *A. niger* glucose oxidase and *Trachyderma tsunodae* bilirubin oxidase, both wired using (different) Os-containing polymers, as the anode and cathode catalysts respectively, was implanted in a grape in which the glucose concentration exceeded 30 mM.[60] A maximum power density of 0.47 μW mm^{-2} (i.e., 47 μW cm^{-2}) was produced, Figure 22A.

Aiming at a fuel cell suitable for implanting in a living animal, Heller and co-workers reported an electrode based on a flavin-containing glucose-3-dehydrogenase from *Agrobacterium tumefaciens*, an enzyme that catalyzes oxidation of the C-3 hydroxyl functionalities of sugars such as trehalose

(Figure 22B, inset), which is found in insects.[155] Wired to a carbon cloth electrode using an Os-containing polymer, the enzyme oxidized trehalose at a low overpotential (Figure 22B), but has not yet been demonstrated in working fuel cell or a living insect.

3.2. Enzyme Fuel Cells Using Other Alcohols As Fuels

Several early examples of methanol oxidizing enzyme fuel cells were described in section 1.4.[51–53] Palmore et al. observed that although the theoretical maximum cell voltage for a methanol/O_2 fuel cell is close to 1.2 V, the mediators chosen in previous work (such as PES, TMPD) had such high potentials that a maximum cell voltage of only about 0.5 V was possible.[101] More recent developments have focused on improved regeneration systems for NAD^+ for catalysis with NAD^+-dependent dehydrogenases, mediators better matched in potential to increase cell voltage (such as benzyl viologen, BV), and strategies for co-immobilizing mediators and enzymes. Palmore et al. achieved an open circuit voltage of 800 mV and complete oxidation of methanol to CO_2 using a cascade of reactions catalyzed by dehydrogenase and diaphorase enzymes in solution,[101] as shown in Figure 23, but this multicomponent system is incompatible with a membraneless design. Sun and Barton investigated the effect of methanol on the activity of a fungal laccase entrapped in an Os-containing polymer and found that the small effect methanol does have can be attributed mainly to its interaction with the polymer rather than the laccase.[156] A membraneless methanol/O_2 fuel cell should therefore be possible using immobilized enzymes.

Minteer and co-workers have developed a strategy to immobilize enzyme molecules onto carbon electrodes using a Nafion matrix to entrap the protein molecules. The strongly acidic polymer was first neutralized by ion exchange with quaternary ammonium cations.[141] A fuel cell coupling the reduction of O_2 by mediated bilirubin oxidase to ethanol oxidation gave an OCV of 680 mV. The maximum power output was 830 μW cm^{-2} when a membrane was used to separate the anode and cathode; without the membrane, an OCV of 510 mV was recorded, with a maximum power output of 390 μW cm^{-2}. Alcohol dehydrogenase and aldehyde dehydrogenase (catalyzing the NAD^+-mediated oxidation of ethanol to acetaldehyde, and acetaldehyde to acetate, respectively) were co-immobilized at the anode, which was modified with polymethylene green to catalyze NADH/NAD^+ cycling.[135,157]

Electrocatalysts able to achieve full oxidation of ethanol to CO_2 (i.e., C-2 oxidation, as opposed to C-1 oxidation) would be highly desirable because ethanol is an abundant biofuel. In an organism, C-2 oxidation occurs in complex multienzyme pathways such as the tricarboxylic acid cycle.

3.3. Enzyme Fuel Cells Using H_2 as a Fuel

The first biologically catalyzed H_2/O_2 fuel cell used whole cells of *D. vulgaris* (Hildenborough) to catalyze methyl viologen-mediated H_2 oxidation, and ABTS-mediated bilirubin oxidase to catalyze O_2 reduction on carbon felt electrodes separated by a membrane and gave an OCV of 1.17 V.[158]

It is only recently that a H_2/O_2 fuel cell based on purified hydrogenase has been described. The O_2 tolerance of the membrane-bound hydrogenase from *R. eutropha* H16 was exploited to produce a membraneless fuel cell in which O_2 reduction was catalyzed by a fungal laccase.[64] Both enzymes were adsorbed at PGE electrodes immersed in buffer, Figure 24A. Streams of H_2 and air were introduced close to the anode and cathode, respectively, and the cell gave an OCV of 950 mV and a power density of approximately 7 μW cm^{-2}, Figure 24B. Introduction of CO had no detectable effect on the power generated. However, the power dropped rapidly when the O_2 stream was brought close to the anode, presumably through O_2 inhibition of the hydrogenase as well as competing O_2 reduction directly at the anode. (Direct O_2 reduction at bare regions of the electrode consumes electrons released from fuel oxidation which diminishes the current flow through the cell; this is a problem in any membraneless fuel cell using O_2 since O_2 is easily reduced at low potential.)

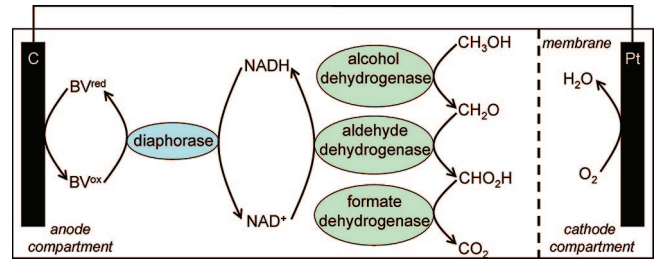


Figure 23. Schematic representation of a fuel cell described by Palmore et al. that couples Pt-catalyzed O_2 reduction to an elaborate sequence of catalytic reactions in which methanol is oxidized to CO_2 by soluble NAD^+-dependent dehydrogenases.[101]

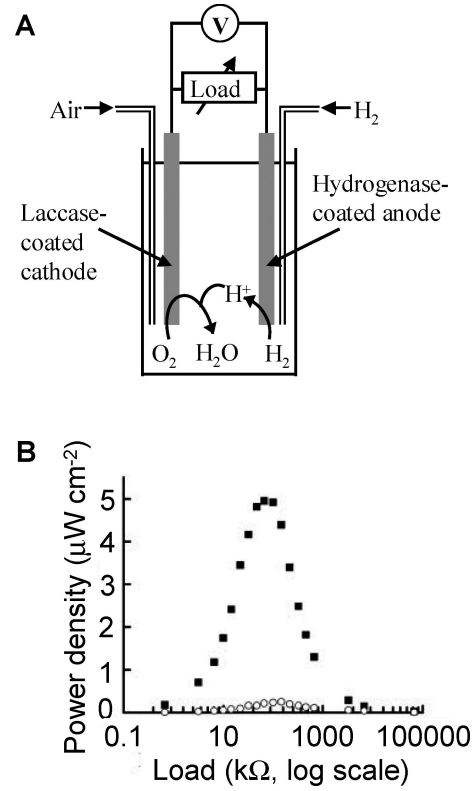


Figure 24. Panel A: Schematic representation of a membraneless fuel cell in which H_2 oxidation is catalyzed by the membrane-bound hydrogenase from *R. eutropha* H16 and O_2 reduction is catalyzed by laccase from *T. versicolor*. Panel B: Power vs applied load curve for the cell shown in Panel A (■) and for a control experiment (○) in which the anode is modified with the O_2-sensitive *A. vinosum* [NiFe]-hydrogenase. Reproduced with permission from ref 64. Copyright 2005 National Academy of Sciences, U.S.A.

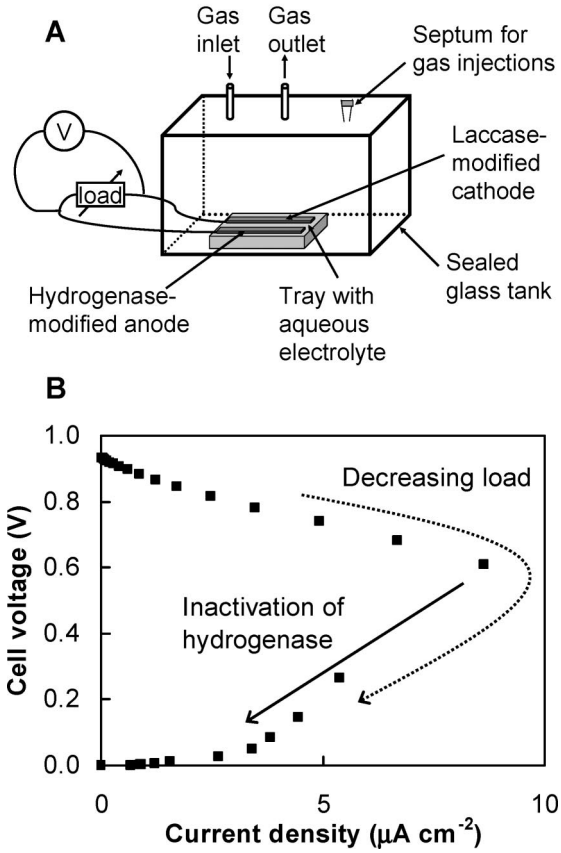


Figure 25. Panel A: Schematic representation of an enzyme fuel cell open to a gas atmosphere of 3% H_2 in air contained within a glass tank.[65] The anode is modified with the *R. metallidurans* [NiFe]-membrane-bound hydrogenase and the cathode with *T. versicolor* laccase. Panel B: The cell voltage vs current plot for this cell was obtained by progressively stepping the resistance to lower values.

In a later experiment, the membrane-bound hydrogenase from *Ralstonia metallidurans* CH34 was used as the anode catalyst in a fuel cell producing electricity from levels of H_2 in air that are too dilute to burn (<4%), Figure 25A.[65] Running on a mixture of just 3% H_2 in air, an OCV of 0.88 V and a power output of about 5 μW cm^{-2} were measured. The power drops sharply at cell voltages below about 500 mV; this is consistent with the inactivation of [NiFe]-hydrogenases observed in electrochemical experiments at high electrode potential (section 2.2.2). This leads to the unusual relationship between cell voltage and current shown in Figure 25B (cf., Figure 1). Again, CO had no detectable effect on the performance of the fuel cell.

Although the power outputs of these hydrogenase-catalyzed fuel cells are tiny, improvements should be possible using strategies such as increasing the enzyme loading on the electrode (see section 2.2.2). Even at their present state, however, they demonstrate the feasibility of generating power from highly contaminated fuel/oxidant mixtures in a manner that is simply impossible using conventional Pt-based catalysts.

4. Photoelectrochemical Cells for H_2 Production

Finally, we mention examples that exploit enzymes as electrocatalysts in cells that use the proton as an oxidant rather than H_2 as a fuel according to the generalized scheme shown in Figure 26. This concept is relevant for identifying possible new routes to *renewable* H_2 production. There is a considerable amount of early literature on H_2 production using chemically mediated electron transfer between enzymes in solution. One report by Woodward et al. described H_2 production from glucose using an NAD^+-dependent glucose dehydrogenase coupled to NADH-dependent H^+ reduction by a hydrogenase.[159] A recent example, described by Amao et al., uses NADH, again generated by glucose dehydrogenase, to release electrons to Mg-chlorophyll-*a* from the blue-green alga *Spirulina*. Under visible illumination, electrons are passed via the mediator methyl viologen to colloidal Pt, which is the H_2 production catalyst.[160]

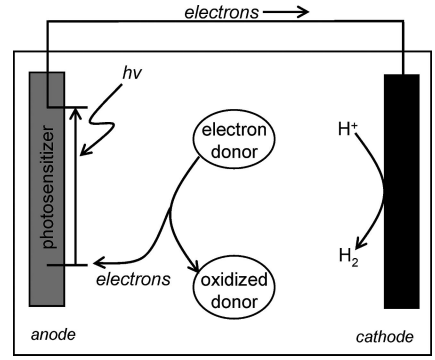


Figure 26. General scheme showing light-induced electrocatalytic H_2 production.

It is also possible to combine power generation with H_2 production in a fuel cell, the ultimate goal being to achieve photoelectrocatalytic H_2 production from water. In a test of a dye-sensitized TiO_2 anode, Hambourger et al. coupled ethanol oxidation by an NAD^+-dependent alcohol dehydrogenase (from the yeast *S. cerevisiae*) with electrocatalytic proton reduction by a Pt electrode.[161] This work has been extended to encompass electrocatalytic enzymatic H_2 production. The [FeFe]-hydrogenase from *Clostridium acetobutylicum* attached to a carbon electrode was recently shown to be a very good catalyst for H^+ reduction, Figure 27A, and this property was further demonstrated in a device in which light-dependent H_2 production was observed when this hydrogenase electrode was coupled to a TiO_2 photoanode with a sacrificial electron donor, Figure 27B.[11]

A related nonelectrochemical experiment was carried out by Okura, Friedrich, and co-workers. The membrane-bound hydrogenase from *Ralstonia eutropha* was fused to photosystem I (PSI) from *Thermosynechococcus elongates* via coupling of the PSI subunit PsaE (Figure 28A). Upon illumination, electrons produced by PSI are transferred to the hydrogenase where they are used to reduce H^+ to H_2 (Figure 28B).[162]

5. Inspiration from and Outlook for Biological Fuel Cell Catalysts

Many enzymes are now proven to be extremely good electrocatalysts. Along with very high turnover rates driven by minimal overpotential, their specificity renders them suited for miniature, membraneless fuel cells that can produce electrical power from a fuel/oxidant mixture. The drawbacks for enzymes are that they are large molecules that cannot make the best use of space on an electrode surface, they are not robust, and their operating conditions are limited in terms of temperature and pH. Their applications are therefore limited to niche situations where they enjoy a particular

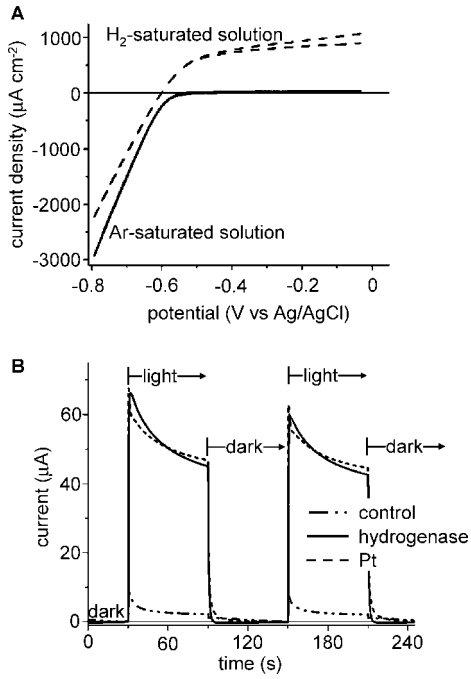


Figure 27. Panel A: Electrocatalytic H^+ reduction and H_2 oxidation by the [FeFe]-hydrogenase from *Clostridium acetobutylicum*, pH 7.0, 30 °C, 50 mV s^{-1}. Panel B: Demonstration of photo-H_2 production using a photosensitized TiO_2 anode with NADH as electron donor and either hydrogenase or Pt as the cathode catalyst. Reproduced with permission from ref 11. Copyright 2008 American Chemical Society.

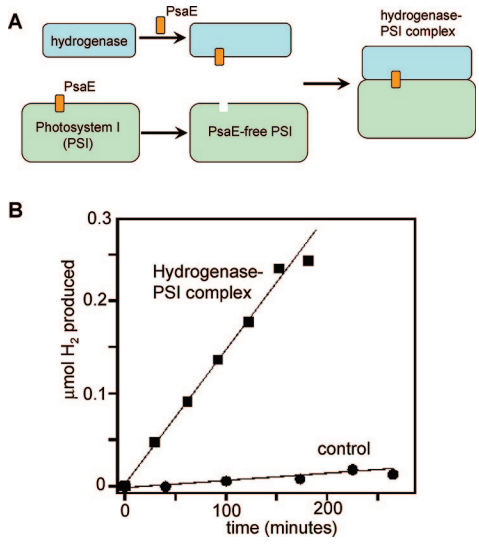


Figure 28. Panel A: Generation of a complex between *R. eutropha* membrane-bound hydrogenase and PSI from *T. elongates*. Panel B: This complex exhibits light-dependent H_2 production with ascorbate as electron donor. Panel B is adapted with permission from the authors of ref 162. Copyright 2006 Wiley-Blackwell Publishing Ltd.

advantage over Pt-based catalysts—we are unlikely to see enzyme fuel cells powering buses! These niches are interesting and important; they include short-term implantable devices in medical applications (such as wound-healing), using glucose and O_2, self-powered sensors, and miniature fuel cells for electronic devices (ideally using methanol or H_2) where the electrodes could even be disposable.

Another way to view the role of enzymes in fuel cell development is that they are benchmarks for performance. Although they lack stability and place a large footprint on an electrode surface, were we able to "strip" enzymes down to a volume little more than the active site, it is almost certain that they would be better electrocatalysts than all chemical counterparts, with the possible exception of Pt. There are continual efforts to improve the performance and selectivity of electrocatalysts and reduce the need for precious metals. Most of these catalysts, such as bimetallic coatings (Pt/Ru, Pt/Bi etc) and various molecular systems (Fe, Ni, Co) for H_2 production and oxidation, and Ru bipyridyl complexes for O_2 reduction, bear little resemblance to the active sites of enzymes. But research on other catalysts, such as Fe and Co porphyrins for O_2 reduction, are following nature's line. The reason for relatively low catalytic activity in these systems may be that synthetic strategies tend to ignore the supramolecular aspect of the enzyme's active site. Not only is the metal coordination sphere important but also the surrounding environment that provides acid−base groups, directional electrostatics, spatial confinement, and protection against water and inhibitors (including O_2 in the case of hydrogenases).

Certain obstacles must be overcome to further develop enzyme fuel cells beyond the proof of concept work that has been done so far. Some of these challenges are common to all enzymes. Stability is one such challenge, both inherent stability (protein conformation) and the ability to stay attached to the electrode. So far these aspects are not good enough for implantable devices. Another problem is the large footprint of enzyme molecules and the need to render electrodes 3D, that is, to have numerous electronically coupled enzyme layers extending out from the electrode surface while providing full access for substrates.

Further issues arise that are specific to individual enzymes. For example, although hydrogenases are extremely efficient catalysts (high activity, low overpotential) for both H_2 oxidation and H_2 production, most of the enzymes so far characterized are inactivated or damaged by traces of O_2, and this hinders their application. There is no example so far of an enzyme showing high activity for H_2 oxidation (or particularly) H_2 production and tolerance to O_2. There is also much interest in electrocatalysts for oxidizing primary alcohols, particularly in fuel cells for small electronics (alcohols are very convenient liquid fuels) and new enzymes might be identified or engineered to perform this function. However, so far, no suitable enzyme has been identified that combines a neutral or acidic pH optimum with facile cofactor regeneration.

In addition to applications, the study of enzymes in a fuel cell environment provides a good deal of fresh insight about how enzymes operate as part of biological power production. We therefore reiterate our earlier idea that a *pair* of enzymes is characterized not only by by their individual properties, but also by an additional parameter, *power*.

6. Abbreviations

ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)
AglAgCl	standard silver/silver chloride electrode ($E_{AglAgCl} = E_{SHE} - 0.197$ V at 25 °C)
FAD	flavin adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
PEM	proton exchange (or polymer electrolyte) membrane
PGE	pyrolytic graphite edge
PQQ	pyrroloquinoline quinine

PSI	photosystem I
RDE	rotating disk electrode
RHE	reversible hydrogen electrode
SCE	standard calomel electrode ($E_{SCE} = E_{SHE} - 0.241$ V at 25 °C)
SHE	standard hydrogen electrode

7. Acknowledgments

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