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Biofuel Cells: Enhanced Enzymatic Bioelectrocatalysis

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Abstract

Enzymatic biofuel cells represent an emerging technology that can create electrical energy from biologically renewable catalysts and fuels. A wide variety of redox enzymes have been employed to create unique biofuel cells that can be used in applications such as implantable power sources, energy sources for small electronic devices, self-powered sensors, and bioelectrocatalytic logic gates. This review addresses the fundamental concepts necessary to understand the operating principles of biofuel cells, as well as recent advances in mediated electron transfer– and direct electron transfer–based biofuel cells, which have been developed to create bioelectrical devices that can produce significant power and remain stable for long periods.

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1. INTRODUCTION TO ENZYMATIC BIOFUEL CELLS

The production of energy from biologically renewable resources has been a recent focus of many research groups due to the need for cheap, environmentally friendly, renewable fuels and catalysts (1–5). Enzymatic biofuel cells represent one approach for such clean energy production because they involve the use of chemically modified electrode surfaces that can harness the flow of electrons produced and consumed by redox enzymes. These electrons can then be fed into an electric circuit, where they can be used to do work. Microbial and mitochondrial fuel cells are an important part of biofuel cell technology, but they are not addressed in this review. Henceforth, we use the term biofuel cell to refer specifically to an enzymatic biofuel cell. Like a traditional fuel cell, a biofuel cell consists of an anode, where one or more oxidation reactions occur, and a cathode, where a reduction reaction occurs (**Figure 1**). Instead of expensive metal catalysts, biofuel cells use renewable enzymes as the catalysts. These devices also offer a clean energy alternative to fossil fuels in that they can use renewable fuels such as sugar (i.e., glucose, fructose, lactose), ethanol, pyruvate, and lactate to produce electricity.

The parameters by which biofuel cells are evaluated are similar to those used for polymer electrolyte membrane fuel cells. The performance of biofuel cells is evaluated with the open circuit voltage (OCV), polarization curves, and power curves (**Figure 2**). The OCV is the voltage at which no current flows and, as such, is the highest voltage for the electrochemical cell. In principle, this voltage should be equal to the difference in the redox potentials of the fuels at each electrode. In reality, the experimentally measured OCV is affected by characteristics of the biofuel cell, such as the overpotential required to begin electrocatalysis and the use of mediators that shuttle electrons from the enzymes to the electrodes. Most enzymes use cofactors that have lower redox potentials than the reactions they catalyze, so typically, the highest OCV for a biofuel cell is equal to the difference in redox potentials of the two cofactors or the two mediators. As with traditional fuel cells, once the circuit is closed, the voltage drops and current begins to flow. The sources of loss in biofuel cells are similar to those in a typical fuel cell; they include activation losses (due to slow kinetics at the electrode surfaces), ohmic losses (due to resistive losses in the

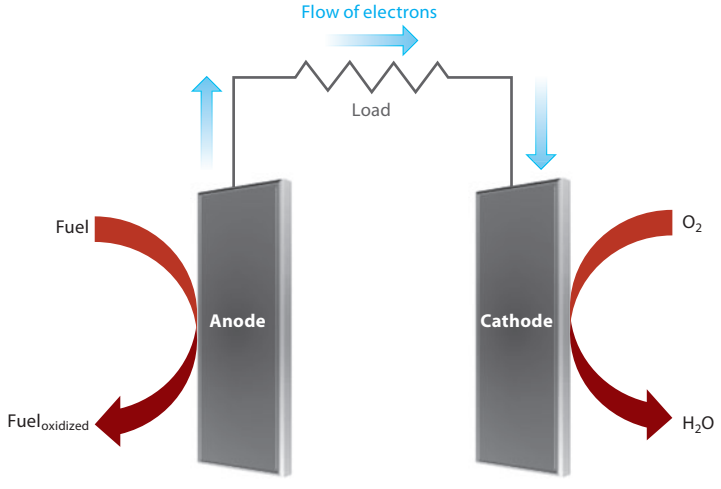

Figure 1

General scheme of a fuel cell, showing fuel oxidation, oxygen reduction, and the flow of electrons across a resistor (the load).

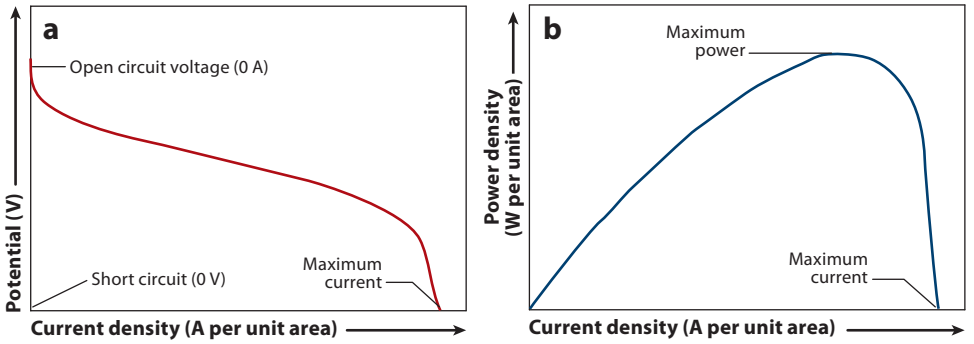

Figure 2

Generic (*a*) polarization and (*b*) power curves obtained from a biofuel cell.

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electrolyte and at the electrode surfaces), and mass-transport losses (due to diffusion limitations at high current densities). The most useful conditions for a biofuel cell are those that give an optimal blend of voltage and current (represented as the highest power output and the peak of the power curve).

Biofuel cells have many advantages that make them attractive as alternatives to batteries and conventional fuel cells. Because they use enzymes as catalysts, biofuel cells can be selective toward the fuel being used and do not typically suffer from fuel crossover and poisoning. These features allow some biofuel cells to be operated in a compartment-less mode, which permits easy fabrication and removes the need for a separator membrane. Biofuel cells operate under mild pH and temperature conditions (pH 5–8, 25–37°C), which is in stark contrast to the highly acidic/basic and high-temperature operating conditions of traditional fuel cells. Also, the fuels mentioned above that are typically used in biofuel cells are renewable and are safer and easier to handle than traditional fuels such as hydrogen gas, methanol, and borohydride.

The applications of biofuel cells are wide ranging. Because of their biocompatible catalysts, biofuel cells could be used as implantable power sources, where the cells are powered by a fuel such as glucose in the bloodstream. Half of this goal has been partially realized with the use of subcutaneous glucose sensors based on wired glucose oxidase (GOx) anodes, but these sensors must be replaced every three to five days (6), which is not an acceptable lifetime for a battery replacement. This application has many exciting possibilities, such as battery-less pacemakers and insulin pumps. However, many hurdles must be overcome to achieve implantable power; such obstacles include long-term enzyme stability and biocompatibility. Although enzymes are biologically "friendly," the body's immune system would respond to an implanted biofuel cell, and such a response must be countered or accounted for in the cell design. Progress is being made in the operation of biofuel cells within living tissue, as evidenced by various reports of biofuel cells operating in plants (7, 8) and animals (9), but a device for long-term human implantation is far from completion.

A more realistic application for biofuel cells is the replacement of batteries in small electronic devices. Proper disposal of batteries is expensive, and improper disposal causes toxic waste to leak into the environment, which makes a biobased energy source attractive. The current densities generated by biofuel cells are generally in the microamps to tens of milliamps per square centimeter, which, if generated at a high enough voltage, would be enough power for many portable electronic devices.

Biofuel cells, however, are not without some drawbacks, which must be addressed to make them viable devices for alternative energy production. Electron transfer from enzymes to electrode surfaces is inherently unnatural; therefore, most enzymes require mediators or specialized electrode surfaces to efficiently facilitate the transfer of electrons from enzymes to electrodes. Another drawback to enzymatic biofuel cells is that enzymes are fairly unstable catalysts in the long term, and their progressive degradation under constant operation results in a gradual loss of power. However, significant strides in enzyme stabilization have been made; potential solutions to this problem include micellar enzyme encapsulation (10), genetic modification, and enzyme regeneration in situ (11).

1.1. Electron Transfer Between Enzymes and Electrodes

Oxidoreductase enzymes normally interact with a biological cofactor to transfer electrons between the active site and a substrate. Some of these natural cofactors (FAD, NAD, NADP), although effective in their native biological settings, have poor electrochemical properties at common electrode materials, such as carbon, gold, and platinum, and can be expensive. Therefore, to successfully transport electrons between enzymes and electrode surfaces, natural cofactors are frequently replaced, altered, or removed completely, depending on the enzyme and the type of biofuel cell. Biofuel cells can be categorized by the type of electron transfer used, and two methods have become predominant: mediated electron transfer (MET) and direct electron transfer (DET) (**Figure 3*a*** and ***b***, respectively). In MET, molecules with multiple redox states are used to accept or donate electrons from the enzyme active site and to assist in transporting charge to or from the

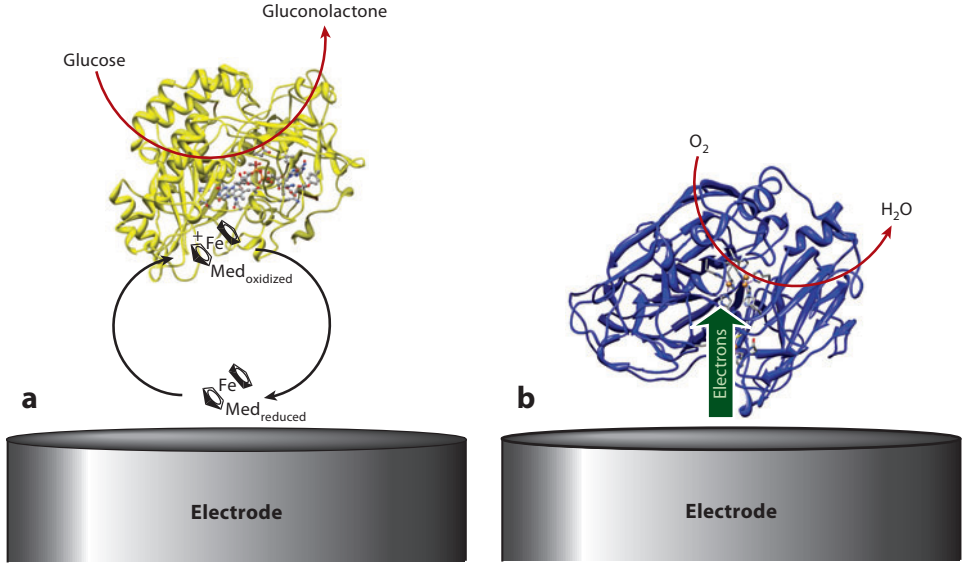

Figure 3

Schematic diagrams showing (*a*) mediated and (*b*) direct electron transfer. (*a*) Ferrocene mediates electron transfer between glucose oxidase and an electrode. (*b*) Laccase accepts electrons directly from an electrode surface and uses them to reduce oxygen to water.

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electrode surface. For example, **Figure 3*a*** shows a ferrocenium moiety accepting an electron from the reduced $FADH_2$ cofactor in GOx, after which the electron can be passed on to the electrode (provided that it is poised at an oxidizing potential relative to ferrocene), thereby regenerating the ferrocenium moiety. In DET, electrons are transferred directly between the active site of the enzyme and the electrode, or to a metallic conductor (nanotube, nanoparticle, etc.) that is connected to the electrode. In the example in **Figure 3*b***, the enzyme laccase accepts electrons directly from an electrode and uses them to reduce oxygen to water. This review investigates recent advances in biofuel cell technology that utilize each of these electron-transfer processes.

1.2. Fundamentals and History of Mediated Electron Transfer

The origins of mediated enzymatic electron transfer lie in early research to find a reliable method for the amperometric detection of blood glucose for diabetic patients. Scientists wanted to utilize the ability of GOx to stoichiometrically oxidize glucose to gluconolactone (Equation 1) but wanted to avoid monitoring the disappearance of O_2 or the production of H_2O_2:

$$\text{Glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2. \quad (1)$$

Nonphysiological molecules with multiple redox states can replace O_2 as the electron receptor in Equation 1, thereby eliminating the need to detect O_2 or H_2O_2 at an electrode surface. Investigators first incorporated these molecules, termed mediators, into glucose biosensors by immobilizing GOx onto an electrode surface while dissolving the mediator in solution, where it could accept electrons from the enzyme, diffuse to the electrode surface, and become oxidized by the electrode to complete enzyme-mediator-electrode electron transfer. The desire for accurate glucose-testing methods has led to the testing of many types of mediators for GOx, including ferrocene derivatives, ferricyanide, quinones, and other organometallic complexes (12–20). A breakthrough in mediated GOx sensors was pioneered by Adam Heller and colleagues (21–24), who effectively coimmobilized mediators on an electrode surface along with GOx. Their mediators (usually osmium bound to various ligands) were covalently attached to polymers [poly(vinylpyridine) or poly(vinylimidazole)] that could be effectively cross-linked in the presence of GOx. Such cross-linked hydrogels swell in aqueous solutions and allow for the facile diffusion of substrates and ions through the films but do not leach mediator molecules into the sample solution. The three-dimensional nature of such redox hydrogels led to a large improvement in the effective number of enzymes that could be electronically linked to the electrode surface (compared with a diffusional mediated electrode). The outstanding success of osmium-based redox polymers as mediators for glucose biosensors led to a wide variety of biosensors based on this technology (25–27), and research into their fundamental properties and the mechanisms by which they transfer electrons from enzyme to electrode has been of great interest. Electron transfer is believed to occur through Marcus-type collisional electron transfer (28, 29), wherein individual redox centers move within finite distances and come close enough to each other to allow for outer-sphere self-exchange reactions to occur. Electrons "diffuse" through the film, and frequently, the rate of electron diffusion is the rate-limiting step in the overall transfer of electrons between enzyme and electrode. In general, redox polymers with the redox center attached to the polymer backbone by long tethers are considered to be superior on the basis of the larger volume element that can be swept out by the redox center (30), but there are exceptions to this phenomenon (31). Many different types of redox polymers based on quinones (17, 32), organometallic compounds (33–35), and conjugated polymers (36–38) have been developed.

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2. MEDIATED ELECTRON TRANSFER IN BIOFUEL CELLS

In the recent development of biofuel cells, many mediated bioanodes and biocathodes have been created. As explained above, the use of mediators can result in high current production through the linkage of many layers of enzymes to the electrode surface (the highest-performing mediated bioanodes produce maximum current densities of 1 to 25 mA cm^{-2}) (30, 39–44). These currents are more than enough to power a small electronic device (many small batteries have discharge currents of 100 to 200 μA). However, many of today's devices typically require "on-load" voltages of 1.5 to 3.0 V, which presents a significant drawback to many mediated biofuel cell systems because the use of mediators usually lowers the OCV as well as the maximum possible operating potential of the cell. When mediators are immobilized on an electrode surface, the fast kinetics (relative to the kinetics of direct enzyme-to-electrode electron transfer) of electron transfer between the mediator and the electrode surface cause the potential of each electrode to be dominated by the redox potential of the mediator and not by the enzyme or the fuel. This phenomenon results in an operating potential (at maximum power) that roughly equals the difference between the redox potentials of each mediator. This effect limits the types of mediators that can be used to produce practical biofuel cell voltages.

The specific design parameters of a mediated biofuel cell also affect the type of mediators that can be used. If the cell is designed for use as an implantable or single-compartment cell, then a completely immobilized system will probably be necessary to avoid the need for exogenous components in the fuel solution. However, if high current, power, or both are the only requirements, high concentrations of dissolved mediators in separate compartments can be effective. Certain enzymes communicate well with a large number of synthetic mediators [GOx, laccase, bilirubin oxidase (BOD)], whereas other enzymes are extremely selective for their natural mediators or cofactors (dehydrogenase enzymes). These differences can lead to biofuel cells with different requirements, so a large variety of mediated biofuel cells have been developed.

2.1. Mediated Bioanodes Using Glucose as Fuel

The low cost and bioavailability of glucose, as well as the advances in glucose sensors based on mediated GOx electrodes, have translated to a significant focus on GOx-based anodes. Many of the properties of an effective glucose biosensor also translate to an effective biofuel cell anode, in particular the use of mediators that have redox potentials close to that of the FAD cofactor. The Heller group (30, 45, 46) has developed biofuel cell anodes with low-potential osmium mediators [less than 0.0 V versus a saturated calomel electrode (SCE)]. Biofuel cells that use these osmium redox polymers as mediators for GOx have produced up to 400 μW cm^{-2} at voltages as high as 0.88 V (46). In addition, biofuel cells that use osmium redox polymers have been operated in at least two different types of plants: a grape (47) and a cactus (8). Notably, the current/power densities of the biofuel cell implanted in a living cactus responded to dark/light cycles and thereby revealed another possible application of biofuel cells in the monitoring of photosynthetic kinetic processes (**Figure 4**).

Calvo and colleagues (48–50) have used osmium-modified poly(allylamine) (Os-PAA) to create redox polymer films assembled layer by layer. Electrodes coated with positively charged Os-PAA were dipped into solutions of GOx (which is negatively charged) to create an intimate mixture of enzyme and polymer. The thickness, osmium concentration, and enzyme loading of the films can be controlled by the number of adsorption steps (50), which produces reproducible bioelectrocatalytic films that have been used to study the detailed kinetics of mediated enzyme electrodes. In addition, Calvo and colleagues present a molecular theory for describing redox polymer–modified

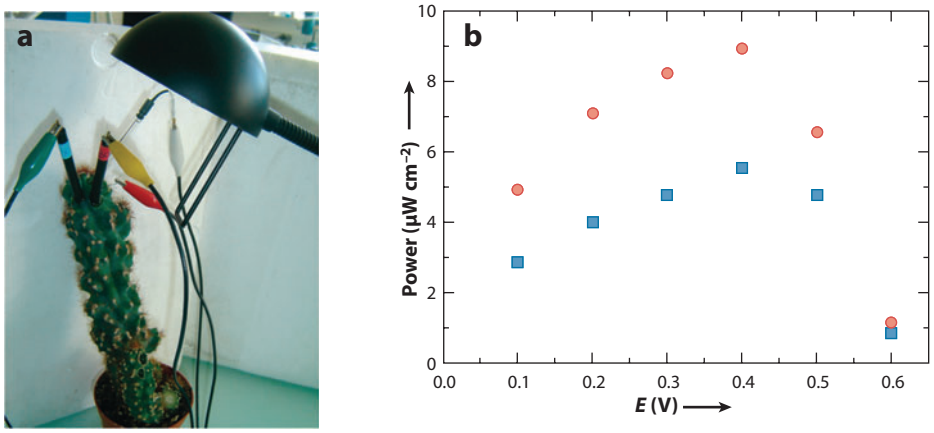

Figure 4

Operation of a biofuel cell inside a living cactus. The biofuel cell employs a bioanode and biocathode that are mediated by osmium redox polymers. (*a*) The optical photograph shows how the electrodes were inserted into the cactus. (*b*) The power curves show the performance of the biofuel cell under light (*red circles*) and dark (*blue squares*) conditions. Reprinted with permission from Reference 8. Copyright 2010, American Chemical Society.

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electrodes that could be useful for the future rational design of redox polymer electrodes with specific desired properties (51).

The versatility of the mediators that can be used with GOx has allowed for the production of many other novel GOx-based anodes, and many such systems continue to appear. Tamaki et al. (32, 44) have used novel hydroquinone-based redox polymers grafted onto carbon black along with GOx to produce bioanodes with high current densities. Ferrocene is generally considered to be an unstable mediator under aqueous, oxidizing conditions, but novel ferrocene-based bioanodes are still being developed due to their low cost and the favorable interaction ferrocenium ions have with the active site of GOx (52). Bunte et al. (40) and Bunte & Ruhe (53) have developed novel redox polymers containing ferrocene, which can be covalently cross-linked with light in the presence of GOx, to yield bioanodes that produce current densities of ~1.2 mA cm^{-2} at 37°C when cross-linked in the presence of GOx and held at 0.45 V versus SCE. Ferrocene's relatively high redox potential is generally regarded as a drawback to its inclusion in biofuel cells due to the low operating potentials that result from its use as an anodic mediator for GOx. To reduce this redox potential and produce a more favorable biofuel cell operating potential, Meredith et al. (39) used a redox polymer based on 1,1′-dimethylferrocene-modified linear poly(ethylenimine) to construct bioanodes that can produce currents of up to 2 mA cm^{-2} when poised at 0.3 V versus SCE. Biofuel cells that use these anodes have produced power densities as high as 146 μW cm^{-2}.

Another approach for developing efficient bioanodes has been developed by Katz (54) and Willner et al. (55), who created a number of unique electrode architectures based on reconstituted enzymes. In these enzyme electrodes, cofactors are typically tethered to a conductive molecule that is attached to the electrode surface. Apoenzymes (enzymes without cofactors) are then bound to the cofactors to connect them to the electrodes. These novel systems have been used to develop switchable bioelectrodes that can be turned on or off with electric current, magnetic fields, or light (56–58). Another unique redox mediator for GOx, developed by Katz et al. (59), consisted of a reconstituted GOx attached to an electrode by a molecular wire/rotaxane structure, which allowed for the redox active rotaxane to shuttle electrons from the FAD unit to the electrode surface. Katz

BIOFUEL CELLS AS SELF-POWERED SENSORS

Recently, the inhibition and/or activation of the enzymes involved in bioelectrocatalysis has been used to create novel self-powered sensors, which require no external power source. Fundamentally, a biofuel cell is a self-powered sensor for whatever fuel allows it to operate. However, more recent developments have focused on the detection of nonfuel or even non-redox-active substrates. Dong and colleagues (146) used the inhibition of a laccase cathode to create a self-powered cyanide sensor and utilized the Hg^{2+} inhibition of alcohol dehydrogenase and BOD to create a self-powered mercury sensor (147). Although both of these sensors were deactivated in the presence of the target analyte, Meredith & Minteer (148) created a self-powered ethylenediaminetetraacetic acid (EDTA) sensor that was activated in the presence of EDTA. Copper ions were used to deactivate a mediated GOx anode of a biofuel cell and EDTA to reactivate or turn on the sensor. These new developments should lead to a new class of self-powered sensors that can detect various substrates, which then can activate or deactivate enzymatic electrodes.

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and colleagues (60–62) have also developed unique bioanodes that are controlled by enzyme logic systems. The modified electrodes used in these systems undergo physical changes in response to changes in pH (caused by different biochemical signals) that activate or deactivate the electrodes. In one example of a physical change, bioanodes mediated by osmium redox polymers lost all redox activity at pH $\geq$ 7 due to a loss of flexibility in the osmium-modified polymer chains that were covalently linked to the electrode surface (63). At acidic pH, the redox polymers expanded and regained their ability to shuttle electrons between GOx and the electrode. The ability to activate and/or deactivate electrodes by using physical changes in the film or inhibition of an enzyme has also been employed to fabricate a new class of self-powered sensors (see the sidebar).

Until recently, no attention had been paid to the improvement of the GOx enzyme itself due to its low cost, stability, and high efficiency. Courjean & Mano (64) and Mano and colleagues (65–67) have used purified and/or deglycosylated GOx to improve glucose bioanode performance. Mediated biofuel cells using GOx that was purified by hydrophobic interaction, as well as ion-exchange chromatography, produced twice as much power density as did biofuel cells made with commercial GOx due to an increased interaction between the enzyme and redox polymer (66). The Mano group (67) has also reported that deglycosylated GOx can communicate directly with a vitreous carbon electrode, allowing for glucose oxidation with an onset voltage of −490 mV (versus Ag/AgCl). Deglycosylation experiments have also been used to determine that mediated bioanodes using GOx enzymes with varying degrees of negative charge produce higher current densities with more negative charge density on the enzyme surface (65). In another approach, Schwaneberg and colleagues (68–69) have used directed evolution to improve various properties of GOx. One study yielded a GOx mutant with an ~1.5-fold increase in activity toward glucose (68), and another approach using a ferrocenemethanol mediator produced a GOx double mutant with improved thermal resistance and improved basic pH stability (69).

2.2. Other Anodic Fuels

Because of the large amount of early research focused on the glucose/GOx system for the management of diabetes and the versatility of mediators that accept electrons from GOx, the GOx/mediator couple has been the most popular bioanode system. However, the diversity of fuels that can be oxidized by enzymes other than GOx is large and has only begun to be investigated. One of these enzymes, cellobiose dehydrogenase (CDH), is superior to GOx in multiple aspects and is a versatile enzyme that can oxidize various fuels, including glucose, cellobiose, lactose, and

maltose (70). CDH, unlike GOx, can effectively communicate electronically with electrode surfaces through MET or DET (71) due to the presence of both an FAD cofactor and a cytochrome domain. Current densities using MET are higher, and CDH can utilize various dissolved or immobilized mediators, including quinones, azine dyes, ferrocenes, and ruthenium and osmium complexes (70). Also, CDH efficiently catalyzes fuels at pH 4–6 (72), which is advantageous for biofuel cells that utilize laccase as a biocathode enzyme because laccase normally operates optimally at pH 4–5. Mediated CDH bioanodes have been used in biofuel cells that have produced power densities up to 300 $\mu W\ cm^{-2}$ (72).

The oxidation of glucose with GOx removes two electrons from the glucose molecule. From an energy-density perspective, this is an inefficient use of the fuel because a total of 24 electrons are "trapped" in each glucose molecule. A biofuel cell system that completely oxidizes fuels should be able to produce more energy than a single-enzyme system that only partially oxidizes its fuel. Palmore et al. (73) have explored this issue by employing three NAD-dependent dehydrogenase enzymes at one electrode surface for the complete or "deep" oxidation of methanol to CO_2. The Minteer group (41, 74–77) has extensively investigated the deep oxidation of fuels by immobilizing biomimics of multienzymatic pathways (Krebs cycle, glycolysis) onto electrode surfaces to deeply oxidize fuels such as ethanol, methanol, and pyruvate. To perform this deep oxidation, the investigators immobilized as many as six NAD-dependent enzymes in a tetrabutylammonium-modified Nafion® polymer on the same electrode, along with a poly(methylene green) electrocatalyst layer that is used to regenerate NAD. In these biomimics, the addition of each enzyme caused an increase in the power and current of the biofuel cell, but the greatest improvements were observed when a complete cycle was immobilized due to a decrease in product inhibition by the various products of enzymatic catalysis (**Figure 5*a*,*b***). In these bioanodes, NAD and fuel molecules diffuse into the polymer where the enzyme is immobilized, and in the presence of fuel, NADH is generated and is electrocatalytically oxidized by a poly(methylene green) layer on the surface of the electrode. The diffusion of the NAD cofactor into the modified Nafion polymer may be the limiting step in these bioanodes, and efforts to utilize smaller cofactors are under way (76). Recently, an NAD-mediated biofuel cell based on cross-linked mitochondrial enzymes was created; this device demonstrated the first use of a metabolon in a biofuel cell (78). The lysate from the mitochondria, when immobilized onto an electrode, oxidized succinate to CO_2 more efficiently when a cross-linker was added to connect the enzymes together and reduced the average distance that the intermediates in the pathway had to diffuse (**Figure 5*c*,*d***). This methodology holds great promise in the area of multienzyme cascade biofuel cells, as metabolons provide a method for creating enhanced, "intelligent" substrate channeling between immobilized enzymes (79, 80).

2.3. Enzymes for Biocathodes

To date, most biocathodes utilize laccase or BOD to reduce oxygen at the cathode surface. These two enzymes fall into a broader category of enzymes known as multicopper oxidases (MCOs) because they use coordinated copper centers to catalyze the reduction of oxygen to water. The three different types of copper in MCOs are classified as T1, T2, and T3. In many laccases, the T1 copper is close to the surface of the enzyme and is coordinated to two histidine residues and the sulfur from a cysteine residue (81, 82). T1 copper is also known as blue copper because it has an absorption band at approximately 600 nm that arises from a charge transfer between the cysteine sulfur atom and the copper atom. T2 copper is known as normal copper and is typically coordinated by histidine residues. T3 copper is a binuclear complex with a hydroxide bridging ligand between the two copper atoms. The T2 and T3 copper sites form a trinuclear cluster in which oxygen is reduced to water. The mechanism by which these enzymes reduce oxygen is conserved throughout

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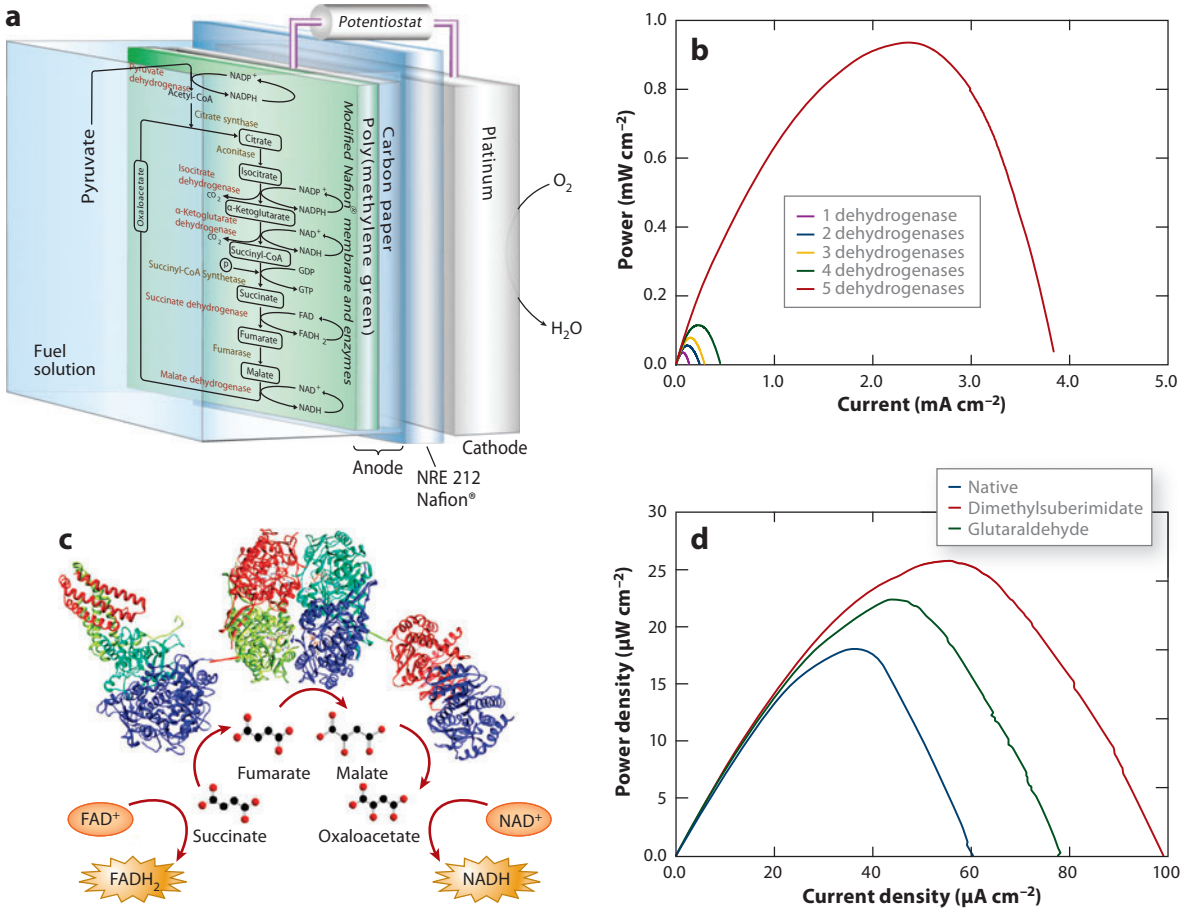

Figure 5

Krebs cycle enzymes are immobilized on an electrode surface to achieve the complete oxidation of pyruvate, and a metabolon is used to enhance substrate channeling in multienzyme cascades. (*a*) All the enzymes utilized in the study; the dehydrogenases are highlighted in red. (*b*) The effect of each additional enzyme on the biofuel cell performance. (*c*) A hypothetical representation of a metabolon formed by cross-linking a portion of the Krebs cycle enzymes. (*d*) Representative power curves obtained from native and cross-linked mitochondrial electrodes. Panels *a* and *b* reprinted with permission from Reference 74. Copyright 2009, the Electrochemical Society. Panels *c* and *d* reprinted with permission from Reference 78. Copyright 2010, American Chemical Society.

all MCOs and is quite complex, so we do not describe it in full here (82–84). A general mechanism involves the T1 copper site accepting electrons from substrates that act as electron donors, then passing those electrons to the T2/T3 tricopper cluster, in which O_2 is coordinated and reduced to water (**Figure 6** shows laccase accepting electrons from an osmium mediator and reducing oxygen to water). Because the T1 copper site in the enzyme is responsible for electron transfer between substrate and enzyme, its redox potential determines the maximum potential of the enzymatic cathode, and this redox potential can change depending on the source or type of the enzyme (85, 86). Interestingly, the electron tunneling distance from the T1 copper site to the T2/T3 tricopper cluster is ~13 Å, which is the same as the distance between the FAD cofactor in GOx and the surface of the enzyme. In GOx, the 13-Å distance makes DET extremely difficult, but MCOs can easily transfer electrons across this distance due to a large electron coupling matrix element (83).

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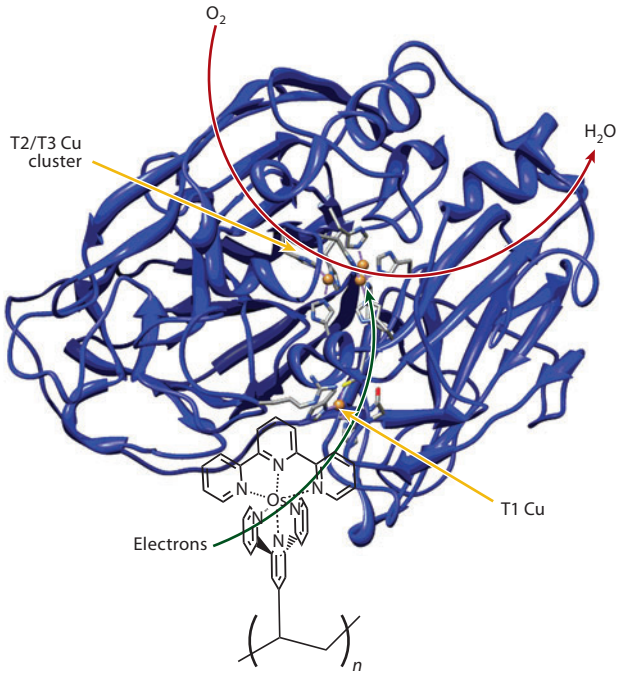

Figure 6

Mediated electron transfer from an osmium complex to a laccase enzyme; the important catalytic copper complexes in the laccase are highlighted.

Although laccase and BOD operate with similar catalytic mechanisms, their properties differ. Laccase (usually from *Trametes versicolor*) has been more thoroughly studied, and its T1 copper site has a higher redox potential than that of BOD. However, laccase has an optimal pH of ~4.5 and can be inhibited by halide ions, especially F^- and Cl^-. Conversely, BOD operates optimally at pH 7 and is not inhibited by halide ions, but its activity from most commercial sources is lower than that of commercially available laccase.

2.4. Mediated Biocathodes

Both BOD and laccase accept electrons from numerous different mediators. Osmium-based redox polymers similar to the ones originally developed by the Heller group (87, 88) for use in GOx bioanodes are very effective mediators for laccase and BOD. According to one study, osmium-mediated laccase cathodes reduce O_2 to water much more efficiently (at a lower overpotential) than does platinum metal, which is used in conventional fuel cells as the cathode material (46). Also, the use of some osmium redox polymers in laccase biocathodes results in reduced inhibition by chloride ions due to a proposed tight binding of the osmium complexes to the hydrophobic T1 copper active site (89). A recent study by Gallaway & Calabrese-Barton (90) utilized osmium redox polymers with various redox potentials to show that the optimal potential for a mediator in a laccase biocathode is 0.66 V [versus a standard hydrogen electrode (SHE)]. Redox polymers with potentials higher than 0.66 V had slow electron-transfer kinetics between enzyme and mediator due to the lack of a thermodynamic driving force for electron transfer, and polymers with redox

potentials lower than 0.66 V produced high currents, but at lower power densities due to an increase in the difference between enzyme and mediator redox potential.

One problem that commonly arises with the use of metallic platinum as a cathode material in direct methanol fuel cells (DMFCs) is that the platinum can become poisoned by methanol, which can cross over the polymer electrolyte membrane and reach the surface of the cathode. Laccase can tolerate fairly high concentrations of methanol, which has been exploited in the exploration of biocathodes as replacements for platinum cathodes in DMFCs (91, 92). Laccase cathodes can operate efficiently with methanol feed concentrations up to 10 M.

Mediated cathodes have also been used to investigate the fundamental elements of enzymatic oxygen reduction. A recent study by Calvo and colleagues (93) used scanning electrochemical microscopy to determine that mediated laccase cathodes actually produce small amounts of H_2O_2 when operated, which can inhibit the biocatalytic O_2 reduction current if the electrodes are not operated in a hydrodynamic mode. Although H_2O_2 had been proposed as an intermediate in the catalytic reduction of O_2 by laccases, it had not been detected in mediated cathodes, so this finding was surprising. This observation is relevant to the rational design of enzymatic biocathodes because H_2O_2 inhibits the very laccase enzymes that produce it (93).

An alternate mediator to the osmium polymers is 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), a redox active organic molecule with reversible electrochemistry which has a high activity with laccase and BOD. ABTS has a favorable redox potential for use in biocathodes (0.62 V versus an SHE) and has been used as a diffusional and immobilized mediator (94–96). In a study by Fei et al. (97), pyrrole was polymerized in the presence of laccase and ABTS to form a conductive polymer biocathode. When this cathode was rotated at 500 rpm, current densities of 3 mA cm^{-2} were produced at pH 4.

2.5. Nanomaterials to Improve Mediated Electron Transfer

The availability of many different types of nanostructures has recently increased. It is now fairly inexpensive to purchase or synthesize many different types of nanotubes, nanoparticles, and nanofibers. Due to their large surface areas, high aspect ratios, and high conductivities, these materials have found applications in many areas of chemistry, including biofuel cells. The most common use of nanostructures in mediated biofuel cells is to enhance the rate of electron transfer between an enzyme and an electrode by incorporating highly conductive nanotubes or nanoparticles into a matrix, in which the enzyme and mediators are immobilized.

Some of the most common nanomaterials used in biofuel cells are carbon nanotubes (CNTs), which can be either single walled (SWCNTs) or multiwalled (MWCNTs). In many cases, one incorporates nanotubes into mediated bioelectrodes by mixing them with an enzyme/polymer/mediator mixture, then casting them onto an electrode surface, which yields an enhanced bioelectrocatalytic performance compared with similar electrodes without nanotubes (98–102). CNTs have also been used to immobilize NAD through a noncovalent π-π stacking interaction with the adenine group (103). Such NAD-modified CNTs have been coupled with glucose dehydrogenase to create glucose bioanodes that do not require dissolved NAD in the fuel solution. Baura et al. (104) immobilized hydrogenase enzymes and methyl viologen onto CNTs by using a novel polypyrrole entrapment method to create mediated bioanodes for the oxidation of H_2. Mano and colleagues (105) recently developed a "nanoweb" of entangled CNTs as a scaffold for immobilizing BOD or GOx, along with various osmium-based redox polymers, to enhance the performance of redox polymer–mediated electrodes.

Incorporation of nanotubes into redox polymer–mediated bioelectrode surfaces causes an increase in the electrode surface area, which could be solely responsible for the increases observed

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in catalytic current densities (in most studies, current density is measured with the geometric area of the electrode, and additional surface area from the use of nanomaterials is not typically considered). However, the results of a study involving GOx bioanodes mediated by a ferrocene redox polymer indicate that when CNTs are well dispersed in a redox polymer/enzyme matrix, they can decrease the number of electron-transfer steps between the enzyme active site and the electrode surface by providing alternative, faster routes for electron transfer from enzymes or mediators that are far away from the electrode surface in the immobilization matrix (106).

3. DIRECT ELECTRON TRANSFER BIOFUEL CELLS

The fabrication of high-power, high-stability biofuel cells that undergo efficient DET with electrode surfaces is desirable because of the drawbacks (instability, voltage loss, possible toxicity, and leaching) of using mediators to electronically communicate with enzymes. However, achieving respectable electrocatalytic currents by use of DET is not as simple as coating an electrode with a layer of enzyme. Two primary hurdles must be overcome to create an effective DET biofuel cell: active-site orientation and sufficient loading without overinsulating. Even when the active site of an enzyme is near enough to its outer surface to rapidly transfer electrons with a metallic substrate, simply coating onto an electrode orients the active sites in random directions, thereby eliminating most of the enzymes from the possibility of DET. In addition, this method coats only one monolayer of enzymes onto the electrode, which is not enough to generate sufficient current even if every active site is available. Therefore, to create an efficient DET electrode, the architecture of a modified electrode should be engineered to orient many of the active sites within DET distance of the electrode surface, and the electrodes should provide electronic access to more than one monolayer of enzyme. Two approaches for enzyme orientation have been explored: (*a*) preferential docking of the enzyme active site in close proximity to a high–surface area electrode and (*b*) immobilizing the enzyme inside a highly conductive matrix, in which DET can occur in any direction.

3.1. Direct Electron Transfer Bioanodes

Bioanodes that undergo DET with an electrode surface have been slow to appear due to the ease of creating mediated GOx electrodes and the advanced electrode modifications required to connect enzymes directly to an electrode. The FAD cofactor in the GOx active site has been shown to be buried deep within the enzyme, approximately 13 Å from the surface of the enzyme (107), allowing for small-molecule mediators to easily approach the active site and undergo electron transfer. Until recently, it was assumed that GOx could not undergo DET with metallic electrodes. However, a few reports have recently claimed that GOx can undergo DET with the use of CNTs or metallic nanoparticles, which directly wire the flavonoid active site to an electrode surface (108–113). The most notable of these reports shows that current densities of up to 8 mA cm^{-2} can be reached when GOx is mixed with MWCNTs and pressed into a pellet using a pressure of 1×10^4 N (114).

Most DET bioanodes are based on other enzymes, such as the family of PQQ-dependent dehydrogenases, which can oxidize simple alcohols, aldehydes, fructose, and glucose. These enzymes have a PQQ group that oxidizes the substrate and, depending on the enzyme, can then pass the electrons on directly to an electrode or to heme *c* groups near the surface of the enzyme where they can undergo DET with an electrode surface (115). PQQ-dependent glucose dehydrogenase (GDH) has been utilized in commercial glucose biosensors due to its oxygen independence (6) and has been suggested as a viable replacement for GOx in biofuel cells (116). A recent study by

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Flexer et al. (117) utilized carbon cryogel electrodes to immobilize PQQ-dependent GDH and produce catalytic current densities of almost 1 mA cm^{-2}. Furthermore, they showed that a mutant GDH with only one different amino acid (relative to the native GDH) was able to produce almost twice as much current under similar conditions. The Minteer group (118, 119) has developed enzyme cascade DET bioanodes based on PQQ-dependent alcohol and aldehyde dehydrogenases and oxalate oxidase for the complete oxidation of glycerol to CO_2. This group is also developing enzyme cascades based on other PQQ-dependent enzymes that will be able to completely oxidize glucose to CO_2, which would enable biofuel cells to utilize all of the energy bound up in glucose (120).

Fructose dehydrogenase (FDH) is a favorable enzyme for DET bioanodes as it has a pH optimum of 4 to 5, which is similar to that of laccase, the most common cathodic enzyme. FDH has been incorporated with nanomaterials to facilitate DET (121–123), and a recent study by Miyake et al. (124) showed that incorporation of FDH into a CNT forest yielded bioanodes with limiting currents up to 16 mA cm^{-2}. When utilized in a DET biofuel cell with laccase that was similarly incorporated into a CNT forest, an exceptional maximum power density of 1.8 mW cm^{-2} was obtained.

Cellobiose dehydrogenase, which has both a FAD cofactor and a heme domain, has been utilized by Gorton et al. as an anodic DET enzyme in bioanodes (70, 72, 125). When no mediators are present in solution, the FAD cofactor can undergo interdomain electron transfer with the heme domain, which in turn can undergo DET with an electrode surface. Recently, a bioanode using a CDH enzyme from *Phanerochaete sordida* produced current densities of up to 500 μA cm^{-2} when wired to a glassy carbon (GC) electrode surface with *p*-aminophenyl-modified SWCNTs (125).

3.2. Direct Electron Transfer Biocathodes

As discussed above, the T1 copper site of an MCO oxidizes an electron donor to acquire the electrons required for the reduction of oxygen. Because this T1 copper site is close to the surface of the enzymes, it can undergo DET with metallic electrode surfaces. Some simple examples of DET bioelectrodes involve the adsorption of laccase and/or BOD onto the surface of carbon electrodes (85, 126–128). These studies have proven useful in the characterization of the fundamental electron-transfer processes between MCOs and electrodes and in the investigation of the redox potentials of the different copper sites in the enzymes; however, for the most part these studies have not proven useful for producing significant amounts of bioelectrocatalytic current. To do so, one of the aforementioned modified electrode fabrication processes must be used.

In the area of preferential enzyme docking or binding to an electrode surface, the work of Armstrong and colleagues (129) is notable; these authors have modified electrodes with various aromatic groups to influence the orientation of MCOs and to produce enhanced oxygen reduction catalysis. In one study, anthracene groups that were covalently attached to a graphite electrode surface enhanced the catalytic activity of the laccase enzyme relative to an unmodified electrode (129). The enhanced bioelectrocatalysis was thought to arise from a hydrophobic binding pocket surrounding the T1 copper active site that oriented itself near the aromatically modified electrode surface. The aromatic groups were hypothesized to act as mimics for the enzyme's natural phenolic lignin substrates. Additional studies involving laccase revealed that carbon cloth modified with anthracene or chrysene groups also enhanced bioelectrocatalysis (130). A later study by Armstrong's group (131) revealed that bioelectrocatalytic reduction of oxygen by BOD can also be enhanced by aromatic modification of electrodes but that the aromatic groups needed to have carboxylate groups attached. Further investigation into aromatic modification of electrodes by Thorum et al. (132) revealed that anthracene-modified gold electrodes showed a similar increase

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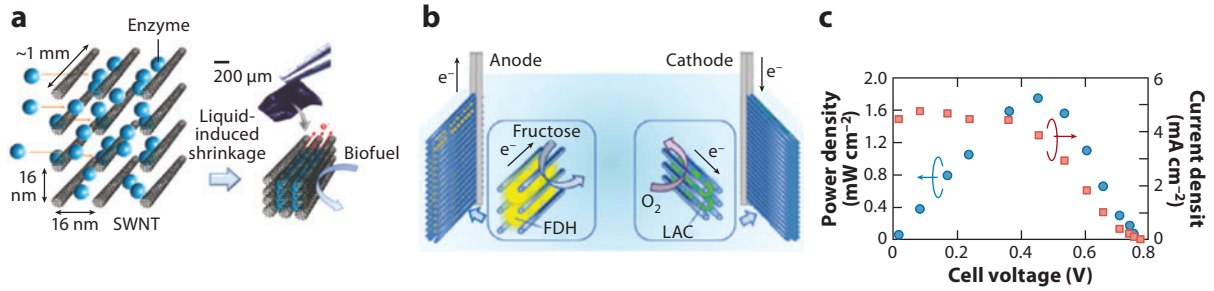

Figure 7

Direct electron transfer (DET) bioelectrocatalysis through the entrapment of enzymes in carbon nanotube forests. (*a*) The entrapment process. (*b*) The biofuel cell using fructose dehydrogenase (FDH) and laccase (LAC) enzymes trapped inside carbon nanotube forests. (*c*) The resulting polarization and power curves from the DET biofuel cell. Reprinted with permission from Reference 124. Copyright 2010, American Chemical Society. Abbreviation: SWNT, single-walled carbon nonotube.

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in laccase-catalyzed O_2 reduction. Another method to preferentially orient a MCO enzyme near the electrode surface is to use a covalent linker to attach the enzyme directly to an electrode surface (133–137). In theory, this method could allow for a more stable attachment of the enzymes to the electrode surfaces than a noncovalent method could, but the attachment in these cases could be random (with respect to the active site), given that reactive linkers such as carbodiimides, aldehydes, and succinimidyl esters are typically used for the covalent linkages.

Instead of fabricating modified electrodes that preferentially orient the active sites of enzymes near the electrode surface, investigators have also mixed or encapsulated MCOs into highly conductive matrices based on metallic nanomaterials. This methodology produces composite electrode materials that contain randomly oriented enzymes, but if the encapsulation of the enzymes is effective, the orientation should be irrelevant because the active sites of the enzymes are near to the conductive matrix of interest. A few biocathodes fabricated with this method have yielded current densities well above 1 mA cm^{-2}. Murata et al. (121) used three-dimensional gold nanoparticle–modified electrodes to immobilize BOD and produce catalytic current densities up to 5.2 mA cm^{-2}. These biocathodes were remarkably stable and retained 90% of their initial activity after 48 h of continuous operation. Miyake et al. (124) developed another novel approach for creation of high-performance biocathodes: They utilized the liquid-induced shrinkage of CNT forests to entrap and immobilize laccase (**Figure 7*a***). The shrinkage of the nanotubes around the enzyme was hypothesized to optimize the spacing between enzymes and nanotubes, thereby enabling the connection of many enzymes to the conductive nanotube forest. Coupled with an FDH anode constructed in the same manner to form a complete DET biofuel cell, this approach yielded a maximum power density of 1.8 mW cm^{-2}, which was enough to power an LED device (**Figure 7*b*,*c***). Zebda et al. (114) recently described another development in the area of high-performance biocathodes, wherein the authors fabricated biocathodes by simply pressing laccase/MWCNT composite mixtures into solid pellets. These pellet electrodes were coupled with DET GOx bioanodes fabricated in the same manner to create a compartment-less biofuel cell that produced 1.3 mW cm^{-2} of power at 0.6 V. This result is quite remarkable when considering the operating pH of 7, which is far from the optimal laccase pH of 4 to 5. Typically, compartment-less biofuel cells that use laccase and GOx are operated at pH values of ~5 due to the almost complete loss of laccase activity and the retention of a considerable amount of GOx activity at pH 7 (39, 46, 124, 138).

Flexer et al. (139a) fabricated another novel biocathode material by adsorbing BOD inside highly porous, three-dimensional carbonaceous foam electrodes. These electrodes produced up to 2.1 mA cm^{-2} of current density at 1,000 rpm and retained almost 100% of their activity after 24 h of operation. This study makes an interesting point about the calculation of current densities for high–surface area electrodes. The authors reported that the true current densities produced by their electrodes was only 1.58 μA cm^{-2}, which they calculated by dividing the current by the true surface area as determined by mercury intrusion porosimetry. This current density was similar to that (2 μA cm^{-2}) obtained for a flat GC electrode with adsorbed BOD in the same study, which suggests that the high–surface area carbonaceous electrodes do not actually improve the efficiency of DET between enzyme and electrode. Our opinion is that most studies involving high–surface area electrodes that report current densities based on geometric surface area alone would probably find similar results if a detailed surface area analysis were carried out as in the case of Flexer et al.; this conclusion highlights the need for standardized electrode geometries in the field of bioelectrocatalysis (140).

3.3. Air-Breathing Biocathodes

A new development in the area of DET biocathodes is the fabrication of enzymatic biocathodes that can operate using gas-phase O_2. Because the concentration of O_2 in air is much higher than in oxygen-saturated water, air-breathing enzymatic biocathodes should be able to produce much more current than biocathodes that utilize dissolved oxygen. One of the first examples of air-breathing cathodes used osmium-based redox polymers to mediate electron transfer between laccase and the electrode, thereby producing current densities up to 1 mA cm^{-2} (141). The biocathodes in this study were used in H_2 fuel cells and were fairly unstable due to water loss from the cathode.

The next generation of air-breathing biocathodes utilize DET for enzyme/electrode communication. Gellett et al. (142) immobilized laccase in carbon black with the aid of tetrabutylammonium bromide–modified Nafion to create an ink that could be painted onto electrodes and utilized in H_2 or DMFCs. The laccase/H_2 fuel cells produced current densities of up to ~38 mA cm^{-2}, and the laccase/methanol fuel cells performed even better, producing current densities above 50 mA cm^{-2} and power densities of ~10 mW cm^{-2} while maintaining stability for more than 350 h. Atanassov and colleagues (143, 144) have also developed air-breathing enzymatic biocathodes based on laccase and BOD by simply drop-casting the enzymes onto the high–surface area carbon black pellet electrodes. These DET air-breathing cathodes produced current densities up to 1 mA cm^{-2} and were stable for up to 30 days.

4. CONCLUSIONS AND OUTLOOK

Since biofuel cell research entered the mainstream in the late 1990s, biofuel cell technology has significantly advanced in the areas of electrode engineering, performance, and stability. Once topping out in the tens of microwatts per square centimeter, the power densities of biofuel cells now reach into the milliwatts per square centimeter. New advances in nanomaterials and the use of novel redox polymers have been used to wire many different enzymes to electrode surfaces to create biofuel cells that operate at high potentials, which makes the use of these devices in real-world applications more of a possibility. Novel immobilization polymers have increased the lifetime of enzymes many times over by creating favorable microenvironments for enzymes, while allowing for the facile diffusion of water, substrates, and ions to the modified electrode surfaces.

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Although these advancements are significant, major improvements in power density and fuel cell stability (both storage and operational) must be achieved before enzymatic biofuel cells can be used as implantable power devices or as replacements for batteries in small electronic equipment. One way that each of these issues can be addressed is the rational genetic modification of enzymes to create biocatalysts that are extremely stable, have more favorable redox potentials, and/or take up less space on electrode surfaces (to fit more catalyst per unit area). In fact, research is already under way to improve the redox potential of laccase by altering the amino acids that surround the T1 copper active site (145), and site-directed mutagenesis has been used to develop an alcohol dehydrogenase that utilizes an NAD biomimic (nicotinamide mononucleotide) as a cofactor (76). Further research into these areas should yield more efficient, stable enzymes that will greatly improve the performance of the biofuel cells in which they are used.

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DISCLOSURE STATEMENT

S.D.M. is a scientific cofounder of Akermin, Inc. She has filed a number of patents related to biofuel cells. The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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