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Review

# New Energy Sources: The Enzymatic Biofuel Cell

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A busca contínua por fontes alternativas de energia, imposta por interesses econômicos e ambientais, tem motivado investigações sobre alternativas limpas e eficientes para a produção de energia. As células a combustível são uma estratégia potencialmente eficaz para a conversão de energia. As biocélulas a combustível constituem uma subclasse das células a combustível, que possuem grande potencial para aplicação em dispositivos de baixa potência (geralmente da ordem de micro a mili watts). Ao invés dos tradicionais catalisadores metálicos, as células a combustível biológicas empregam biomoléculas, tais como enzimas, microrganismos, ou organelas para converter energia química em energia eléctrica. As biocélulas a combustível oferecem várias vantagens frente às baterias tradicionais, incluindo o uso de componentes renováveis e não-tóxicos, seletividade de reação, flexibilidade de combustíveis, e a capacidade de operar em temperaturas brandas e pH neutro. De fato, estudos recentes têm demonstrado características promissoras destes dispositivos; no entanto, apesar dos vários avanços obtidos nesta área, alguns desafios ainda precisa ser enfrentados. Este trabalho de revisão tem como objetivo proporcionar aos leitores do *Journal of the Brazilian Chemical Society* uma visão geral das biocélulas a combustível enzimáticas, e o seu desenvolvimento desde a primeira descrição em 1964. Os resultados mais recente da literatura (incluindo a tecnologia implantável), além de uma perspectiva para futuras pesquisas nesta área também são apresentados.

The continuous search for alternative energy sources, imposed by economic and environmental concerns, has motivated investigations into clean and efficient alternatives for energy production. Studies have shown that fuel cells are a potentially efficient strategy for energy conversion. Biofuel cells constitute a subclass of fuel cells with promising application in low-power devices (generally in the order of micro to milli watts). Instead of metallic catalysts, biological power sources employ biological molecules such as enzymes, organelles, or microorganisms to convert chemical energy into electricity. Biofuel cells offer several advantages over traditional batteries, including the use of renewable and non-toxic components, reaction selectivity, fuel flexibility, and ability to operate at lower temperatures and near neutral pH. Indeed, recent papers have demonstrated the promising characteristics of these devices; however, some challenges remains to be faced despite the several advances in this area. This review aims to provide the readers of the Journal of the Brazilian Chemical Society with an overview of enzymatic biofuel cells, their development since its first description in 1964, and the most recent outcomes. The latest papers in this field (including implantable technology) and an outlook for future research in this area are also presented.

**Keywords:** biofuel cell, enzyme immobilization, sustainable energy source, green energy, electrocatalysis

## 1. Introduction

Economic and environmental factors along with the human consumption pattern (which heavily relies on non-renewable fuel sources) have called for "clean" and efficient energy production processes. The increasing energy demand associated with the rapid growth of the world population has engaged authorities, governments, companies, and many research teams worldwide in developing viable processes to obtain efficient and sustainable energy. In this scenario, producing renewable energy may constitute a mean to relieve the worrying issue of global warming and provide new alternatives to the current energy consumption behavior.[1] Currently, alternative fuel sources such as solar

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energy, hydrogen, biomass, biofuels, and fuel cells, are some of the most promising technologies available to generate energy,[2] and many research groups have been devoted to the design of fuel cells over the last decades. These devices consist of a system that can generate electrical energy from electrochemical reactions involving chemical species oxidation and reduction.[3] Characterized as a non-polluting and silent technology, this system converts chemical energy into electricity according to the mechanism illustrated in Figure 1. In general, traditional fuel cells use noble metal catalysts to generate electrons from fuel oxidation (typical fuels are hydrogen or small organic molecules such as methanol, ethanol, and glutaraldehyde, among others).[3] After the oxidation step, an external circuit transfer the electrons to the cathode side where the electrons react with an oxidant molecule (usually oxygen), and generate electrical work as well as water and heat.

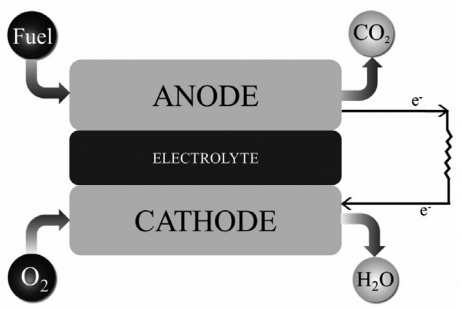


**Figure 1.** Representative scheme of the hydrocarbon fuel cell operation mechanism.

Different types of basic fuel cells exist, depending on the type of electrolyte and operation temperature. This technology offers considerable advantages over other processes, such as high conversion efficiency and generation of substantial power density.[3] Although fuel cells yield good results, some factors limit their large-scale application: high cost and future scarcity of noble metal catalysts (e.g., platinum, employed as base catalyst in many fuel cell devices), issues regarding electrode passivation, and inability to oxidize some byproducts of the employed fuels.[4] Furthermore, hydrogen production, purification, and storage (hydrogen is one of the fuels that is most often employed in traditional fuel cells) also poses major technical challenges.[1]

An alternative to conventional fuel cells based on metal catalysts is to turn to biological fuel cells or biofuel cells. This device employs enzymes (enzymatic biofuel cell) or microorganisms (microbial fuel cell) as catalyst instead of the traditional noble metal catalysts.[5] These devices constitute a system that can directly transform chemical energy into electricity through reactions involving biochemical steps, or even a system in which the activity of the cell (or part of it) stems from the action of biocatalysts.[6] The connection between biology and electricity and the concept of a biofuel cell have been known since 1911,[7] when MC Potter noted that a culture of the bacterium *E. coli* produced electricity in half-cell studies employing platinum electrodes. After a few decades, the interest in this technology increased; in the 1950s and 1960s, the central idea of the United States space program involved the use biofuel cells in two fronts: to treat waste originating from the aircraft and to obtain electricity from the treated waste.[8] Motivated by the possible in vivo application of this device, Yahiro *et al.*[9] were the first to describe a biofuel cell that used isolated enzymes on the surface of an electrode and to show that it was possible to produce electricity using the enzyme glucose oxidase (GOx). Since then, the number of publications in the area has increased.[10] The main advantages of the biological fuel cells are: the use of clean and renewable catalysts (enzymes or microorganisms), the ability to operate at mild temperatures (20-40 °C) and physiological pH conditions, and the possibility to use several fuels because enzymes and microorganisms offer diversity and specificity. Additionally, scaling up the use of biocatalysts tends to reduce production costs, which is not possible for non-renewable metallic catalysts. All these advantages point to an economically viable process, as judged from the growing research in this field all over the world.[8, 11, 12] This review will be only focus on the enzymatic biofuel cell, aiming to provide the readers of the Journal of the Brazilian Chemical Society with an overview of this enzymatic device, their development since its first description in 1964, and the most recent outcomes. The latest papers in this field (including implantable technology) and an outlook for future research in this area will also be presented.

## 2. Enzymatic Biofuel Cells

### 2.1. Application, operation, and key performance parameters

Half a century has elapsed since the first description that enzymes produce electric current.[9] Over the last decade, the development of devices containing enzymes immobilized onto solid surfaces has increased fast,[13-16] and has included large technology companies.[17,18] Besides being potentially applicable as energy source in batteries, biofuel cells can be used in vivo; e.g., pacemakers, neurostimulators, drug carriers, and glucose sensors, among others, which is an attractive feature of this technology.[8]

At this point, the first questions that arise are: (*i*) How can one obtain electrons from an enzyme-catalyzed reaction

in a biofuel cell device? (*ii*) How is the energy produced in this process related to the bioelectrochemical reactions occurring on the surface of an electrode and to the electrons generated in these reactions? Equations 1 to 3 illustrate the electron production mechanism in an enzymatic bioanode that catalyzes ethanol oxidation via $NAD^+$-dependent dehydrogenase:

$$CH_3CH_2OH + NAD^+ \xrightarrow{\text{Alcohol dehydrogenase}} CH_3CH_2O + NADH \quad (1)$$

$$NADH + \text{electrocatalyst}_{(red)} \longrightarrow NAD^+ + \text{electrocatalyst}_{(ox)} \quad (2)$$

$$\text{electrocatalyst}_{(ox)} \longrightarrow \text{electrocatalyst}_{(red)} + e^- \quad (3)$$

The operation of an enzymatic biofuel cell resembles the functioning of conventional fuel cell: first, a fuel undergoes an enzyme-catalyzed oxidation at the anode side. This reaction releases electrons that reach the cathode side through an external circuit. In the cathode, an oxidant (usually $O_2$) is reduced, producing electrical work (Figure 2). In other words, the electric current flows according to a potential difference and, consequently, an enzyme-catalyzed reaction involving a fuel (substrate) generate power.

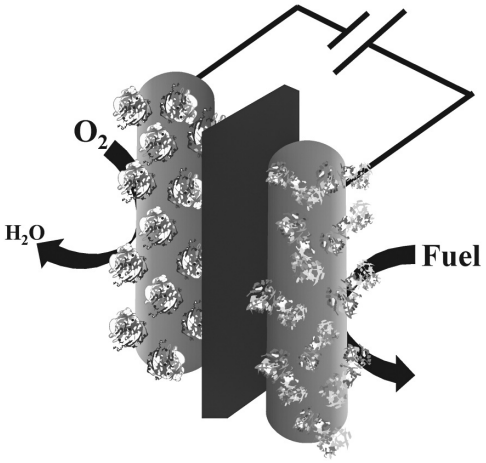


**Figure 2.** Schematic representation of an enzymatic biofuel cell.

An oxidoreductase enzyme can oxidize carbohydrates, alcohols, or even amino acids, and transfer electrons from the fuel to the electrode surface. Considering that enzymatic fuel cells generally employ the aforementioned fuels, it is possible to prepare anode-based electrodes by immobilizing different types of enzymes. For sure, glucose oxidase has been the most often employed enzyme since the first description of a biofuel cell. It's in vivo application is desirable because different human physiological fluids, such as blood, plasma, saliva, and tears, contain sugar.[19, 20] Papers employing enzymes from hydrogen, ethanol, methanol, and Krebs cycle metabolisms also exist in the literature: hydrogenase,[21] alcohol and aldehyde dehydrogenase,[13, 22] cytochrome c,[23] cellobiose dehydrogenase, and D-fructose dehydrogenase,[24] pyruvate dehydrogenase, citrate synthase, aconitase, isocitric dehydrogenase, ketoglutarate dehydrogenase, fumarase, and malate dehydrogenase.[25] Scientists are also testing others enzymes, depending on the target fuel.[26] As for enzyme-based cathodes, laccase or bilirubin oxidase usually perform the oxygen reduction reaction.[16,17,27-43]

The difference between the thermodynamic potential of the cathode and the anode ($\Delta_{Ec\text{-}Ea}$) expresses the cell voltage, but this value can decrease by several orders of magnitude due to overvoltage ($\Delta\eta$). $\Delta\eta$ results from (*i*) slow electron transfer occurring at both electrode sides; (*ii*) ohmic drop ($\Sigma\Omega$), associated with all the resistances in the system (film diffusion, membrane, supporting electrolyte); and (*iii*) electrode wear out ($\Delta$£), a parameter that reflects electrode degradation:

$$E_{cell} = \Delta_{Ec\text{-}Ea} - \Delta\eta - \Sigma\Omega - \Delta£$$

The equation above provides important information about any enzymatic electrode. Maximizing the so-called thermodynamic potential window ($E_c$ - $E_a$) yields better biofuel cell performance. Therefore, enzymatic biofuel cell researchers aims to prepare/achieve bioelectrodes that facilitate the catalyzed reactions, to increase the open cell voltage (OCV). Moreover, these researchers target better cell design and prototypes that can reduce the overall resistances, making electric current flow more easily through the system. To produce commercial devices, it is also necessary to keep $\Delta$£ as low as possible.

Another crucial parameter associated with the performance of any fuel cell is the power density that this system provides. This parameter reflects the electron generation rate in the enzyme-catalyzed reactions. Unlike traditional fuel cells, which afford power densities of the order of milli to kilo watts, enzymatic biofuel cells generate power densities in the order of micro to some milli watts, which is sufficient for applications in some small electronic devices.[17] The representative scheme in Figure 3 shows the power range of some of the alternative methods of energy production.[8]

Despite the various advantages and possible applications of enzymatic biofuel cells, to achieve an efficient practical device, it is necessary to consider some crucial factors when developing this type of system. The first major challenge is the fact that enzymes are proteins; therefore, these biomolecules display a weak three-dimensional structure that must be maintained, to ensure that its catalytic activity is reatained.[5] Although enzymes are highly specific and efficient catalysts,

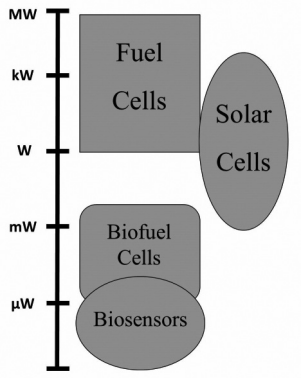


**Figure 3**. Schematic representation of the power range that some of the alternative energy production methods provide (adapted from reference 8)

they have limited lifetime in solution. Hence, their use in biofuel cells requires a critical step: immobilizing the enzyme onto an electrode surface.[44] Achieving electrical contact between the enzyme and the electrode, is also fundamental, because this is one of the most important processes in the field of bioelectrochemistry. Achieving high electron transfer rate from the active site of an immobilized enzyme to the electrode surface is probably the most critical point when constructing an enzymatic biofuel cell.

### 2.2. Enzyme immobilization

Immobilizing biomolecules on solid surfaces is a matter of great scientific interest. Numerous possibilities exist when it comes to using enzymes in different biotechnological areas, particularly in the industrial and analytical fields.[8,26] It is essential to develop and improve immobilization techniques, because this step will greatly influence bioelectrode efficiency and protein lifetime. Removing an enzyme from a three-dimensional environment (where the molecules of the substrate and products molecules easily enter and leave the catalytic site) to a solid surface requires methodologies that ensure retention of protein conformation and catalytic properties. Immobilization methodologies must also provide the enzyme with an adequate microenviroment that enables the protein to resist changes in temperature, pH, and solution composition, which often lead to protein denaturation or inactivation. Furthermore, the immobilization process must furnish a mechanically and chemically stable layer without forming a capacitive region on the electrode surface.[13, 26] The presence of various functional groups in the protein structure allows for one to use different enzyme immobilization strategies. In general, immobilization methodologies include chemical and physical methods, depending on the type of interaction between the enzymes and the anchoring agent.

Among chemical methodologies, which elicit direct binding of the enzymes to a solid support, the covalent linkage and cross-linking procedures are noteworthy.[44,45] In the case of direct covalent bond between the enzymes and the solid substrate, the procedure generally involves modifying or functionalizing electrode surfaces, which can then covalently bind the enzymes. Researchers choose this design when preparing monolayer systems. In most methods, the amino groups of lysine residues constitute the main reactive groups, but this protocol can also employ carboxyl and sulfhydryl groups.[45] Rüdiger *et al.*[21] presented a good example of this strategy: they developed a method to covalently bind Ni-Fe hydrogenase to gold electrodes modified with a self-assembled 4-aminothiophenol monolayer. Klis *et al.*[46] employed a similar protocol to immobilize laccase onto gold electrode by covalent binding the enzyme to self-assembled mercaptoundecanoic or mercaptopropionic acid monolayers. Researchers have also bound an enzyme molecule to an electrode surface via cross-linking agents such as glutaraldehyde. Baravik *et al.*[47] described how they immobilized glucose oxidase in the form of a cross-linked composite prepared by electropolymerizing aniline-functionalized carbon nanotubes and thioaniline-modified glucose oxidase on a gold electrode modified with thioaniline monolayer. Despite being simple, this technique has not received much attention for biofuel cell purposes in recent years because the enzymatic activity usually decreases after immobilization.[48] Sol-gel matrices have been used to manufacture both biosensors and bioelectrodes for enzymatic biofuel cells, where the proteins are encapsulated and directly connected to the electrode surface. The satisfactory results obtained with this methodology stem from the formation of a porous structure that contains several cavities. Lim *et al.*[49] prepared nanostructured electrodes for glucose/$O_2$ biofuel cell based on enzyme encapsulation along with carbon nanotubes into sol-gel silica matrices.

Because physical protocols are generally simpler and more efficient regarding enzyme immobilization, researchers prefer them to procedures that involve the formation of chemical bonds. Accordingly, entrapment in microcapsules and polymer gels as well as adsorption protocols are more commonly used in enzymatic fuel cells.[44,45] Heller's group showed that is possible to immobilize many enzymes into polymeric hydrogels containing an osmium redox center;[16] the employed hydrogels generally consist of cross-linked redox polymers that swell in water and produce known redox hydrogels. Using redox hydrogels has many advantages: they can conduct electrons while retaining the most important features of typical hydrogels, such as conducting ions and allowing diffusion of substrate

species such as glucose.[50-53] Microencapsulation consists in trapping the enzyme molecules in the pores of a membrane. Minteer's research group successfully used this methodology to modify a Nafion® membrane and then immobilize dehydrogenase enzymes onto carbon surfaces.[13,25,54-63] Treatment of the Nafion® membrane with tetrabutylammonium bromide afforded a favorable environment to immobilize biomolecules, maintained the physical properties of the unmodified membrane, reduced its acidity and increased the mass transport through the membrane. Similarly, Klotzbach *et al.*[64,65] described how they immobilized dehydrogenase enzymes in chitosan modified with hydrophobic groups.

One can efficiently immobilize several enzymes and proteins onto solid substrates using multilayer architectures on which biomolecules anchor by either physical (electrostatic) or covalent interactions. The first case uses the self-assembly technique (physical adsorption of biomolecules onto a substrate in a solution with optimized pH and ionic strength) at room temperature to retain their activity for a considerable time.[66] This process involves sequential adsorption of oppositely charged material from a suitable solution that can be repeated according to the desired number of bilayers.[67] Szamocki *et al.*[37] reported the effective sequential immobilization of laccase and an osmium complex onto a mercaptopropane sulfonate-modified gold surface. Rengaraj *et al.*[68] obtained a fully assembled membraneless biofuel cell at graphite electrodes. These authors used the layer-by-layer technique to achieve the anode and the cathode. They employed osmium complexes along with glucose oxidase and laccase to obtain the 3D electrocatalytic structures. Frasconi *et al.*[69] reported on a self-assembled bioelectrode containing genetically engineered glucose oxidase and gold nanoparticles, to obtain multiple enzymatic layers. Aquino Neto *et al.*[22] employed the electrostatic layer-by-layer technique to prepare bioanodes for ethanol/$O_2$ biofuel cells. The authors tested both mono (alcohol dehydrogenase) and bienzymatic (ADH and aldehyde dehydrogenase) systems immobilized onto a carbon paper support along with polyamidoamine (PAMAM) dendrimers.

### 2.3. Electron transfer processes

The redox enzymes employed in biofuel cell studies are generally classified in three main groups, according to their electrical communication.[51] The first group contains the redox center located in a peripheral area of the enzyme, so it can directly transfer electrons to or accept electrons from an electrode surface. This group includes the PQQ-dependent dehydrogenase enzymes. The second group bears a weakly bound cofactor ($NAD^+$ or $NADP^+$) that acts as a mediator at the redox center. This species can diffuse to the electrode surface and carry the electrons from the enzymatic catalysis. The third group involves enzymes with a strongly bound redox center, normally located inside the protein shell. Classification of the electron transfer between enzymes and electrode surfaces depends on the way electrons move from the enzyme catalytic site to the electrode surface.[51] Direct processes are known as direct electron transfer (DET); processes requiring the assistance of a mediator molecule are designated mediated electron transfer (MET) (Figure 4).

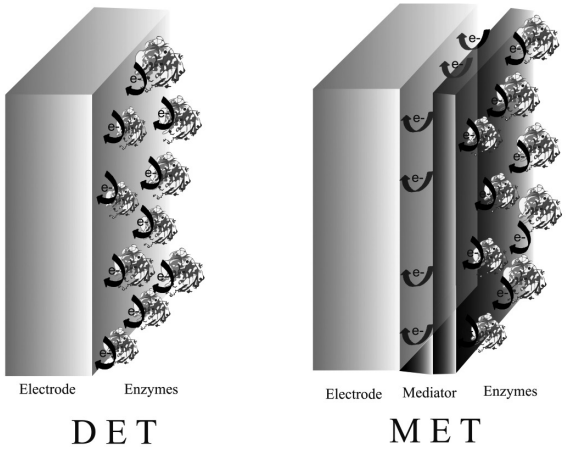


**Figure 4.** Types of electron transfer processes between enzymes and electrode surfaces in enzymatic biofuel cells.

#### 2.3.1. Mediated electron transfer

##### Bioanodes performing MET

Considering that the majority of immobilized proteins cannot electrically communicate with the electrode surface by the DET mechanism,[70] it is essential to use mediator molecules in many bioelectronic devices. The electrical communication between the enzyme redox center at the electrode surface in an enzyme-based biofuel cell regulates bioelectrocatalysis efficiency. However, the redox center of most oxidoreductases enzymes is generally buried inside the protein matrix, so the electrical communication with the electrode surface is hindered.[71, 72] In an enzymatic biofuel cell, a mediator corresponds to a reversible redox species that facilitates electron transfer between the coenzyme and the electrode surface.[5] Although MET-based bioelectrodes require additional species during biofuel cell preparation, MET is generally preferred over DET, because it can generate higher output power with often large orders of magnitude than the direct mechanism. The possibility of using commercially available enzymes, such as many $NAD^+$-dependent alcohol dehydrogenases and $FAD^+$-dependent

glucose oxidase and dehydrogenases, is another advantage of this methodology. The mediator molecules can be either anchored onto the electrode surface (e.g., in the form of a polymeric film), free in solution, or even linked to the structure of the enzyme; it withdraws the electrons generated during the enzymatic catalysis and transports them to the electrode surface.[8,72,73] These mediator species must be able to efficiently perform the enzyme/electrode connection, to rapidly react with the reduced form of the enzyme, and be soluble in both its reduced and oxidized forms, so that it can diffuse to the electrode/enzyme fast. Moreover, they should be non-toxic, stable, and biocompatible.

The efficiency of a mediator depends on its redox potential range, which should be as close to the redox pair of the enzymes as possible.[19] When the electrode potential is higher than the redox potential of the mediator species, mediator oxidation occurs at the electrode surface; if the opposite situation is true, mediator reduction takes place. In these circumstances, the electrode provides a continuous electron flow for both the oxidized and reduced mediator species. In a practical MET mechanism, the potential of the mediator couple controls the system; consequently, the potential window of both mediators determines the OCV of a fully mediated enzymatic device. Considering this premise, the thermodynamic driving force of an MET biofuel cell lies between the redox potentials of the mediator species and the enzyme redox center; therefore, it must be different from zero.[19] In other words, the redox potential of a mediator limits its application in enzymatic bioelectronics. The redox potential of this species must be as close as possible to the redox potential of the employed enzyme. Literature suggests that an enzymatic device would have optimal performance if the potential difference between the mediator and enzyme redox centers lay around 50 mV.[16] Figure 5 illustrates the redox potential range of some of the most often employed metal-based mediators and of the oxidoreductase enzymes glucose oxidase, and laccase. These potentials may vary according to the enzyme structure, the organism from which is extracted, and the pH, among other factors.

The redox potential of some of the enzymes that are generally used in biofuel cells lies around –0.35 V *vs* Ag/AgCl for glucose oxidase from *Aspergillus Niger*,[74] 0.58 V *vs* Ag/AgCl for laccase from *Trametes Versicollor,*[75] and 0.67 V *vs* Ag/AgCl for bilirubin oxidase from *Myrothecium Verrucaria*.[76] Literature shows that ferrocene (redox potential of 0.2 V *vs* Ag/AgCl)[77] and quinone derivatives possess the desired characteristics to function as good enzymatic mediators.[73] Osmium and ruthenium complexes, polypyrrole, phthalocyanine, organic dyes, and other molecules can also serve as mediators in enzymatic devices.[78] Indeed, a variety of osmium and ruthenium complexes find application in enzymatic biofuel cells. By changing the substituents on the ligands of the complex, it is possible to modify their redox potential, which normally ranges from –0.17 to 0.79 *vs* Ag/AgCl.[19,51,79-82]

Ohara *et al.*[83] were one of the first to describe the use of an osmium-based redox mediator in enzymatic biofuel cells. Besides displaying unique electron diffusion coefficients, these redox hydrogel films were also permeable to water-soluble species, such as substrates and products of enzymatic reactions.[51] These mediators provided efficient electrical connection between glucose oxidase and the electrode surface.[83] Dónal Leech's research group extensively reported the use of osmium-based complexes.[84-89] Zafar *et al.*[87] described a wide-range mediator containing five different osmium-based redox polymers that efficiently connected the oxidoreductase pyranose dehydrogenase with graphite electrodes. The prepared polymers covered a potential range from –0.270 to +0.160 mV *vs* Ag/AgCl.

Rengaraj *et al.*[84] prepared a complete enzymatic biofuel cell using glassy carbon and graphite electrodes modified with osmium redox polymers, crosslinked with poly (ethylene glycol) diglycidyl ether. By changing only the donor/receptor behavior of the substituting groups, the

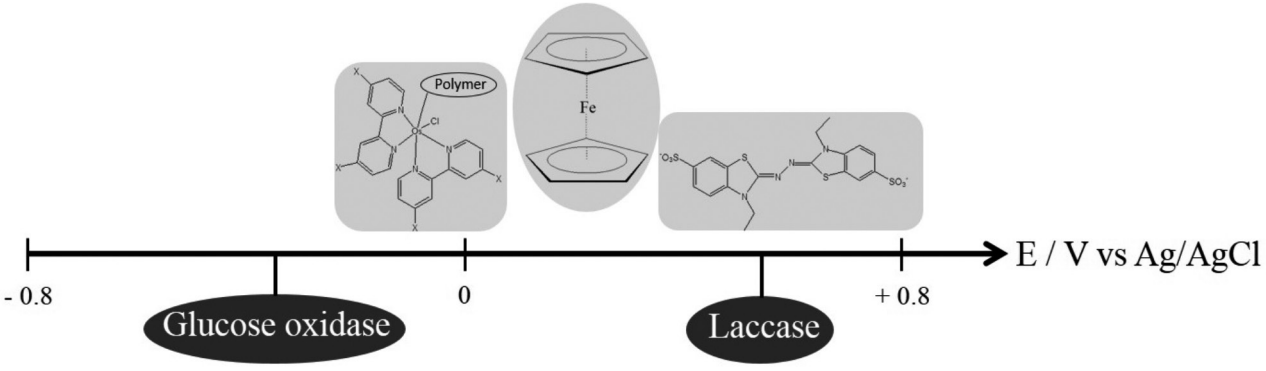


**Figure 5.** Redox potential range of some of the most often employed metal-based mediators and of the oxidoreductase enzymes glucose oxidase and laccase.

authors achieved an enzymatic device with large potential window. Recently, Ó Conghaile *et al.*[85] reported a versatile approach to prepare bioanodes for mediated glucose/$O_2$ biofuel cell. They immobilized glucose oxidase crosslinked in biofilms onto graphite electrodes containing different functionalized osmium complexes. In 5 mmol $L^{-1}$ glucose, the bioelectrodes prepared with dimethoxy or dimethyl-substituted bipyridines provided glucose oxidation currents around 30 and 70 μA $cm^{-2}$ at 0.2 and 0.35 V under pseudo physiological conditions, respectively. Nevertheless, the stability signals proved that the electrodes were inadequate for long-term operation.[85]

Ferrocene has also been effectively employed as a mediator in enzymatic fuel cells. For example, bioelectrodes in the form of cylindrical pellets, prepared by mechanical compression of a mixture of graphite, glucose oxidase, and ferrocene, effectively wired the enzymes onto the electrode surface.[90] The resulting membraneless glucose/$O_2$ biofuel cell generated an OCV of 0.45 and a power density of 80 μW $cm^{-2}$. Kim *et al.*[91] prepared a glucose/$O_2$ biofuel cell based on polypyrrole nanowires along with glucose oxidase and 8-hydroxyquinoline-5-sulfonic acid hydrate. This nanowire-type enzymatic biofuel cell exhibited higher power density compared with film-type biofuel cells.

The NAD-dependent dehydrogenases are one of the most employed enzymes in MET biofuel cell studies. These enzymes transfer electrons and protons to the oxidized form of the cofactor, to produce its reduced form, NADH.[5] A key point to build an efficient mediated system employing NAD-dependent dehydrogenases is to regenerate the oxidized species ($NAD^+$). Despite having a formal redox potential of −0.52 V *vs* Ag/AgCl, NADH oxidation overvoltage is around 1 V at glassy carbon electrodes and even higher at platinum surfaces.[92-94] This energy barrier hinders the application of NAD-dependent dehydrogenases when a electrocatalyst system is not available. The literature contains many examples of compounds that can lower the overpotential of the NADH oxidation reaction, to make the reaction energetically favorable. [95-97] Indeed, electrocatalysts based on organic compounds such as quinones and phenazines reduce the NADH oxidation overpotential, enhancing the $E_{cell}$ output.[13,95] Among various phenothiazine derivatives, organic compounds such as methylene green, methylene blue, and neutral red efficiently oxidize NADH at lower potentials. They can also form stable films, such as poly-methylene green, on the electrode surface upon electropolymerization, facilitating electrode fabrication and affording long-term stability during biofuel cell studies.[62,93]

We must emphasize that, in this type of bioelectrodes, the pair $NAD^+$/NADH acts as the diffusional mediator system and the azines function as the electrocatalysts, to lower the NADH oxidation overpotential. On carbon paste electrodes, for example, methylene green (MG) has a formal redox potential toward NADH oxidation of −0.122 V *vs* Ag/AgCl, pH 7.[98] This data represents a reduction in the overpotential of around 0.4 V, which enables their use in enzymatic fuel cells employing NAD-dependent enzymes. The Michaelis-Menten kinetics can describe the principle of the azine electrocatalytic activity toward NADH;[5,99,100] i.e., the reaction mechanism indicates that NADH and methylene green form an intermediate compound followed by proton abstraction and subsequent $NAD^+$ formation. The use of MG as a freely diffusing electrocatalyst for NADH catalysis imposes restriction to the fabrication of a stable bioelectrode;[101] therefore, researchers have seeked immobilization techniques that provide stable layers of NADH catalyst. In the past decade, Minteer's group successfully employed MG as an electrocatalyst to regenerate NADH in a wide range of dehydrogenase-based biofuel cells.[13,25, 54,58,59,62,102] Using the NADH electrocatalyst in the form of an electropolymerized film, Akers *et al.*[13] obtained a high-current-density bioanode containing poly(methylene green) coated with a layer of tetrabutylammonium bromide salt-treated Nafion® and dehydrogenase enzymes. Klotzbach *et al.*[65] employed the hydrophobically modified chitosan and Nafion® membranes to obtain enzyme modified electrodes for biofuel cell applications. Sokic-Lazic and Minteer also described the use of poly(methylene green) to prepare bioelectrodes on carbon platforms that mimic one of the main metabolic pathways in living cells, the citric acid cycle.[54] More recently, Meredith *et al.*[62] presented a methodology to co-immobilize dehydrogenase enzymes, different NADH electrocatalysts including MG, carbon nanotubes, and polymer hydrogels. The authors claimed that the so-called "one-pot" mixing and casting procedure effectively produced bioelectrodes that promote NADH oxidation at low overpotentials. Recently, our research group obtained good results employing MG as electrocatalyst for NADH oxidation in the form of a stable thin film layer in alcoholic biofuel cell using commercially available enzymes, NAD-dependent alcohol dehydrogenase, and aldehyde dehydrogenase.[22,103] Forti *et al.*[103] introduced the use of PAMAM dendrimers in an ethanol/$O_2$ biofuel cell, by immobilizing alcohol dehydrogenase and PAMAM dendrimers onto a carbon cloth platform. Similarly, Aquino Neto *et al.*[22] used a poly(methylene green) film to prepare bioelectrodes for ethanol/$O_2$ biofuel cell the layer-by-layer assembly.

### Biocathodes performing MET

Concerning MET bioelectrode systems for the cathode side, once again osmium-based compounds are the most

often-employed redox species to electrically wire the enzymes (generally laccase and bilirubin oxidase) with the electrode surface. Barrière *et al.*[86] thermodynamically evaluated osmium and ruthenium-based mediators for laccase-catalyzed oxygen reduction. The targeted redox polymers displayed redox potentials around 0.40 and 0.63 V *vs* Ag/AgCl for the osmium and ruthenium-based polymer, respectively. Using poly(vinylimidazole) as the stabilizing agent for the redox species, the authors prepared the biocathodes on a glassy carbon surface along with laccase and the cross-linking agent polyoxyethylene bis(glycidyl ether).[86] Gallaway *et al.*[104] obtained biocathodes containing a series of osmium-based redox polymer mediators. To determine the optimum redox potential toward laccase from *Trametes versicolor* and maximize the power output of a hypothetical biofuel cell, these authors synthetized mediators covering a range of redox potentials from 0.11 to 0.85 V.[104] Szamocki *et al.*[37] presented another strategy to use osmium-based mediators: they immobilized the complexes at the bioelectrode surface along with the laccase enzyme from *Trametes trogii* using the layer-by-layer self-assembled technique. The catalytic oxygen reduction current increased linearly with the number of bilayers in the self-assembled bioelectrode, reaching a maximum catalytic current of 150 μA $cm^{-2}$.[37] More recently, Shen *et al.*[105] fabricated biocathodes based on the laccase and electrodeposited thin films containing Os(4,4'-dicarboxylic acid-2,2'-bipyridine)$_2$ on carbon electrodes. The authors claimed that laccase was readily incorporated in the electrodeposited redox polymer through coordination between the enzyme amine and histidine groups. This system efficiently catalyzed the four-electron oxygen reduction to water at 0.58 V *vs* Ag/AgCl.

One of the most often employed mediators in biocathode studies based on laccase immobilization is 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS). Several literature reports have pointed out that this compound efficiently mediates oxygen reduction to water. Palmore *et al.*[34] presented one of the first reports on the use of a biocatalyst for a $H_2/O_2$-based biofuel using a fungal laccase and ABTS as redox mediator, in the cathode side, to effectively reduce dioxygen to water. Tsujimura *et al.*[106] also described the electrochemical reduction of oxygen to water using ABTS and bilirubin oxidase as the biocatalyst at the cathode side; the authors achieved the targeted reaction at 0.4 V *vs* Ag/AgCl in pH 7.0 and ambient temperature. Smolander *et al.*[107] published an interesting paper focusing on the dioxygen four-electron reduction through concomitant oxidation of the phenolic aromatic compound, to prepare printable laccase-based biocathodes. The half-enzymatic fuel cell tests, along with a Zn anode, furnished an OCV between 1.4 and 1.5 for the fresh samples and maintained an OCV in the range of 0.6 to 0.8 for three days. [107] More recently, Cardoso *et al.*[108] described how a mediated electron transfer biocathode performed in a methanol/$O_2$ biofuel cell. They employed PAMAM dendrimers to immobilize laccase along with ABTS entrapped into polypyrrole films. The electrochemical characterization tests confirmed that the electropolymerized polypyrrole film entrapped the ABTS molecules. Additionally, laccase-mediator system enhanced catalytic oxidation current as compared with the control sample containing lacase-PAMAM dendrimer only. The use of ABTS as mediator entrapped into the biocathode layers generated a power density around 25 μW $cm^{-2}$.[108]

Besides adding functional groups at conducting surfaces, covalent linkage leads to MET between the enzyme and the electrode surfaces. Covalently linked glucose oxidase and bilirubin oxidase as the anodic and cathodic catalyst, respectively, produced a biofuel cell with maximum power density of 13 μW $cm^{-2}$ at 0.25 V.[109] Table 1 summarizes some MET investigations on enzymatic biofuel cells.

### 2.3.2. Direct electron transfer

#### Bioanodes performing DET

Over 1300 oxidoreductases enzymes are known to date,[111] and these enzymes are potentially applicable as biocatalysts in enzymatic biofuel cells. However, less than 100 of these enzymes can directly connect with solid substrates and transfer electrons from the enzyme active site to the electrode surface.[70] Indeed, DET only occurs in enzymes that act as a molecular transducer; i.e., enzymes that can convert a chemical signal into an electric signal via charge transfer, in the presence of a stable redox species.[112] In other words, in the DET mechanism, the enzymatic catalysis and the electrochemical reaction are not separate reactions, but a unique process where the electron functions as a second substrate.[5] The tunneling mechanism of the electrons in a DET enzymatic system will depend on the enzyme structure, the redox center location, the enzyme orientation on the electrode surface, and the distance of the electron transfer.[113] Hence, a good electron transfer rate between enzymes and electrodes will only be possible if all these conditions are met.[114] One great advantage of this type of electron transfer process is that it eliminates the issues related to mediator species, avoiding performance losses that may arise from the potential difference between the enzymes and the mediator species. Moreover, DET enhances selectivity and mass transport rate at the electrode surface,[20] facilitates bioelectrodes construction, and favors

**Table 1.** Summary of MET studies on enzymatic biofuel cells

| Mediated System | Enzyme | Reference |
|---|---|---|
| Redox hydrogel - poly(1-vinylimidazole) / $Os(bpy)_2Cl^+$ cross-linked with poly(ethylene glycol) diglycidyl ether. | Glucose oxidase | Ohara *et al.*[83], 1993 |
| ABTS | Laccase | Palmore *et al.*[110], 1999 |
| ABTS | Bilirubin oxidase | Tsujimura *et al.*[106], 2001 |
| $[Os(bipyridine)_2(poly\{N\text{-}vinylimidazole\})_2]Cl_2$, and $[Ru(bipyridine)_2(poly\{N\text{-}vinylimidazole\})_{10}Cl]Cl$ | Laccase | Barrière *et al.*[86], 2004 |
| Electropolymerized MG / $NAD^+$ | Alcohol Dehydrogenase | Akers *et al.*[13], 2005 |
| Bis(4,4'-diamino-2,2'-bipyridine)Cl / poly(vinylimidazole) or polyacrylamide copolymer | Glucose oxidase | Barrière *et al.*[88], 2006 |
| ABTS | Laccase | Smolander *et al.*[107], 2008 |
| Electropolymerized MG / $NAD^+$ | Dehydrogenase enzymes in cascade | Sokic-Lazic and Minteer,[54] 2008 |
| Serie of osmium-based redox polymer mediators | Laccase | Gallaway *et al.*[104], 2008 |
| Polypyrrole nanowires along with 8-hydroxyquinoline-5-sulfonic acid | Glucose oxidase | Kim *et al.*[91], 2009 |
| Osmium-based mediators self-assembled with poly(allylamine) | Laccase | Szamocki *et al.*[37], 2009 |
| Five different flexible osmium-based redox polymers | Pyranose dehydrogenase | Zafar *et al.*[87], 2010 |
| Osmium redox polymers, cross-linked with poly (ethylene glycol) diglycidyl ether | Glucose oxidase | Rengaraj *et al.*[84], 2011 |
| Six different enzyme/tetrabutylammonium bromide modified Nafion® | Lactate dehydrogenase | Sokic-Lazic *et al.*[25], 2011 |
| Mix of graphite particles and ferrocene | Glucose oxidase | Zebda *et al.*[90], 2012 |
| MG, MWCNTs, and polymer hydrogels | Glucose dehydrogenase | Meredith *et al.*[62], 2012 |
| Os-polymer wired on graphite electrodes | Glucose dehydrogenase | Zafar *et al.*[72], 2012 |
| Dimethoxy or dimethyl-substituted bipyridines osmium complexes | Glucose oxidase | Ó Conghaile *et al.*[85], 2013 |
| $Os(4,4'\text{-dicarboxylic acid-}2,2'\text{-bipyridine})_2$ | Laccase | Shen *et al.*[105], 2013 |
| ABTS / polypyrrole | Laccase | Cardoso *et al.*[108], 2013 |

miniaturization of the enzymatic devices.[112] Although researchers have increasingly targeted DET, the later process often leads to lower power values as compared with mediated systems. This happens because it is difficult to electrically connect a large amount of enzyme to obtain satisfactory power density values. Moreover, most of the enzymes that can accomplish this type of electron transfer (such as PQQ (pyrroloquinoline quinone)-dependent enzymes) are not commercially available, so laboratory extraction and purification steps are necessary to obtain these proteins.[60]

DET between the enzymes and different electrode materials, such as carbon and gold, can occur by using different immobilization techniques and nanostructured materials that appropriately orient the anchored enzymes. In an attempt to enhance DET, Yan *et al.*[115] described a glucose/$O_2$ biofuel cell in which they designed the bioelectrodes architecture along with single-walled carbon nanotubes (SWCNTs), polyethylene blue, and glutaraldehyde as the cross-linking agent. The authors obtained high OCV (800 mV), catalytic reduction of $O_2$ at 0.60 V, and oxidation of glucose at –0.15 V *vs* Ag/AgCl. Coman *et al.*[116] fabricated and characterized glucose/oxygen DET bioelectrodes to operate in neutral buffer and human serum. The authors prepared the electrodes using *Corynascus thermophilus* cellobiose dehydrogenase and *Myrothecium verrucaria* bilirubin oxidase as anodic and cathodic biocatalysts, respectively. Polydopamine can adsorb to a wide variety of surfaces and serve as an adhesion layer to immobilize biological molecules. Wang *et al.*[117] prepared multifunctional carbon nanotubes composites via dopamine oxidation at room temperature; their results evidenced high sensitivity for glucose oxidation. Strategies to build bioelectrocatalytic interfaces by electrochemically functionalizing the MWCNTs surface exist, and this creates a conductive matrix to immobilize the enzyme.[118] Such approach provides an interface where glucose oxidase can perform direct electron transfer, affording a net current peak potential for the immobilized enzyme that lies close to the potential of the FAD/$FADH_2$ pair (0.4 V *vs* Ag/AgCl).

Apart from using CNTs, introducing metallic nanoparticles is also a good strategy to enhance electronic conduction through the electrode surface. Holland *et al.*[119] applied site-specific gold nanoparticle conjugation in a glucose/$O_2$ biofuel cell. They attached a genetically modified glucose oxidase enzyme containing a free thiol group near its active site to a maleimide-modified gold nanoparticle, to obtain direct electrical communication

between the conjugated enzyme and an electrode surface. Wang *et al.*[40] inserted metallic gold nanoparticles in the bioelectrode structure, to obtain a mediatorless sugar/oxygen biofuel cell operating in human physiological fluids, such as blood and plasma. The authors registered a maximum current density of 40 μA $cm^{-2}$ and an OCV of 680 mV in a sugar-containing neutral buffer. When the authors continuously operated the biofuel cell in physiological buffer for 12 h, they observed only a 20% drop in power density. The authors emphasized that the operational stability in the buffer was as good as the storage stability of the biocathode and the bioanode.[40] Despite the large number of papers claiming DET between glucose oxidase and different functionalized electrode materiais (some of which are mentioned above), this situation is controversial and is still a subject of debate in the literature. Such discussion stems from the fact that DET due to bioelectrocatalytic glucose oxidation can be mistaken for other processes that might suggest the same behavior. Naturally MET due to FAD species that are not bonded to the enzyme active site, hydrogen peroxide mediation, or even FAD species released from denatured enzymes are some of the situations that may take place at the electrode surface and lead to erroneous interpretation of DET.[20]

PQQ-dependent enzymes also provides direct electrical connection between proteins and solid surfaces. The cofactor of these enzymes consists of PQQ covalently linked to the protein structure, along with multiple heme-C complexes. The suggested action mechanism of these proteins indicates that substrate catalysis occurs at the PQQ active site; then, the electrons move on to the heme redox sites and finally to the electrode surface (Figure 6).[120]

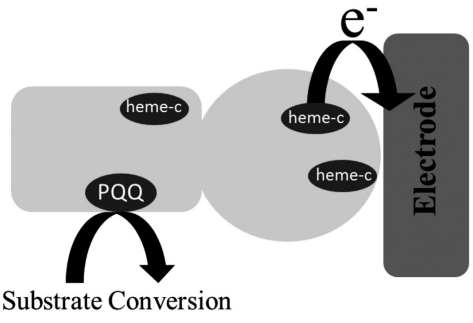


**Figure 6.** Illustration of the electron transfer mechanism in a PQQ-dependent enzyme.[120]

This kind of electron transfer is feasible on different electrode surfaces employing PQQ-containing enzymes such as alcohol, lactate, and D-fructose dehydrogenase.[121,122] The main sources of these enzymes are the bacteria *Acetobacter*, *Gluconobacter*, *Pseudomonas*, and *Commamonas*. It is generally preferable to extract and purify the enzyme from *Gluconobacter*, because these bacteria thrive in easily controlled conditions and in the presence of concentrated sugar solutions and low pH. These enzymes are not commercially available, so most papers describe their isolation from *Gluconobacter sp. 33* and their further purification in the laboratory.[60,70,123,124] Flexer *et al.*[125] showed that PQQ-dependent glucose dehydrogenase from *Acinetobacter calcoaceticus* underwent DET at carbon cryogel electrodes. Treu *et al.*[56] developed a method to first isolate and purify PQQ-dependent enzyme pyruvate dehydrogenase from *Gluconobacter*. They found that the purified PQQ-dependent enzyme underwent DET at carbon electrode surfaces, thus being applicable in a pyruvate biofuel cell.[56] Aquino Neto *et al.*[124] compared the performance of a DET bioanode containing both PQQ-dependent alcohol dehydrogenase and PQQ-dependent aldehyde dehydrogenase immobilized onto different modified electrode surfaces; these authors employed either a tetrabutylammonium-modified Nafion® membrane polymer or PAMAM dendrimers to immobilize the enzyme. Electrochemical characterization showed that the prepared bioelectrodes underwent DET on glassy carbon surface in both the presence and absence of MWCNTs. A self-assembled bioelectrode prepared on gold surfaces modified with dendrimers afforded similar redox potential, indicating that both methodologies provided an environment that favored DET in the PQQ-dependent enzymes. The biofuel cell tests confirmed that the DET process was easy, and that the performance enhanced in the presence of carbon nanotubes. These electrode modifications represent effective methods to immobilize quinohemoproteins on electrode surfaces and obtain direct electrical connection. Literature papers also exist on glucose/$O_2$ biofuel cell using PQQ-dependent enzymes, where the PQQ-dependent glucose dehydrogenase covalently binds to SWCNTs.[126] Cyclic voltammetry of the enzymes immobilized onto the modified carbon electrode evidenced two redox peaks related to the PQQ cofactor of the enzyme. The authors demonstrated not only direct electron transfer but also biocatalytic activity toward glucose. In other words, they obtained a molecular-size electronic nanowire between the active site of the enzyme and the carbon microelectrode surface.[126]

### Biocathodes performing DET

Despite the difficulty in achieving electric connection between the active sites of the enzymes and electrode surfaces, many cathode-based materials are found on literature, in which the immobilized oxidoreductase enzymes are able to undergo DET. Gupta *et al.*[42] presented a gas-diffusion electrode based on bilirubin oxidase and hydrophobized carbon black. The authors claimed an oxygen

reduction reaction onset potential around 0.55 V *vs* Ag/AgCl. Besides, the prepared device maintained an OCV around 0.65 V, which was close to the redox potential of the enzyme. Vaz-Dominguez *et al.*[127] covalently bound laccase from *Trametes hirsuta basidiomycete* to graphite electrodes previously modified with aminophenyl derivatives. In the absence of redox mediators, the authors achieved catalytic current toward oxygen reduction around 500 μA $cm^{-2}$, which suggested that the T1 Cu catalytic center of the enzyme was appropriately oriented. Furthermore, the prepared biomaterial displayed high operational stability and resisted inhibition by chloride.

Using carbon nanotubes to achieve better enzyme attachment/orientation and consequently higher DET efficiency is probably the most often employed strategy to prepare cathode materials. Many literature papers have dealt with this nanomaterial to study biofuel cells. The goal of functionalizing the carbon nanotubes surface with different active groups is to induce interactions with the catalytic site of the target enzymes via covalent bonds or hydrophobic interactions. Lau *et al.*[128] prepared Teflon®/MWCNTs composites modified with tethered crosslinkers, which can covalently bind to the targeted biocatalysts, to give a gas-diffusion device. Nazaruk *et al.*[129] applied a hybrid biofuel cell consisting of a Zn anode and laccase/SWCNTs to catalytically reduce oxygen. The highly efficient anode material furnished a biofuel cell with a power density of almost 1 mW $cm^{-2}$; the hybrid system gave an OCV of 1.5 V. Martinez-Ortiz *et al.*[130] described 4-(2-aminoethyl) benzoic acid-functionalized graphite electrodes in which the benzoic acid moiety interacted with the laccase T1 site and induced a DET environment between the T1 site and the graphite electrode surface. The authors also prepared a semi-enzymatic fuel cell using a zinc anode and the functionalized graphite electrodes with a substrate-like molecule, to obtain high electron transfer rate and a power density of 1.1 mW $cm^{-2}$ at 0.41 V.[130] Anthracene-modified CNTs also constitute scaffolds for DET in enzymatic oxygen reduction. Stolarczyk *et al.*[131] compared the efficiency of SWCNT cathodes modified with phenyl, naphthyl, and terphenyl moieties. This set of electrodes reached the onset of oxygen reduction at ca. 0.60 V *vs* Ag/AgCl and generated a current density of 200 μA $cm^{-2}$ at 0.2 V. Meredith *et al.*[61] prepared a biocathode by mixing anthracene-modified nanotubes along with laccase immobilized with a modified-Nafion® membrane. In the tested conditions, a compartmentless enzymatic device using a DET fructose dehydrogenase anode produced an OCV of 707 mV, a maximum power density of 34.4 μW $cm^{-2}$, and a maximum current density of 201.7 μA $cm^{-2}$.[61] Table 2 summarizes some DET studies on enzymatic biofuel cells.

**Table 2.** Summary of DET studies on enzymatic biofuel cells

| Bioelectrode Architecture | Enzyme | Year |
|---|---|---|
| SWNTs, polyethylene blue, and glutaraldehyde | Glucose oxidase | Yan *et al.*[115], 2006 |
| Covalent attachment with SWNTs | PQQ-dependent glucose dehydrogenase | Ivnitski *et al.*[126], 2007 |
| Covalent binding to aminophenyl derivatives-modified graphite electrodes | Laccase | Vaz-Dominguez *et al.*[127], 2008 |
| Spectrographic graphite electrodes | Cellobiose dehydrogenase | Coman *et al.*[116], 2010 |
| Carbon paper and modified-Nafion® | PQQ-dependent pyruvate dehydrogenase | Treu *et al.*[56], 2010 |
| Multifunctional CNTs composites / dopamine | Glucose oxidase | Wang *et al.*[117], 2011 |
| 4-(2-aminoethyl) benzoic acid functionalized graphite electrodes | Laccase | Martinez-Ortiz *et al.*[130], 2011 |
| Site-specific gold nanoparticle conjugation | Glucose oxidase | Holland *et al.*[119], 2011 |
| Anthracene-modified MWCNTs | Laccase | Meredith *et al.*[61], 2011 |
| Carbon cryogel electrodes | PQQ-dependent glucose dehydrogenase | Flexer *et al.*[125], 2011 |
| Gas-diffusion electrode based on hydrophobized carbon black composite | Bilirubin Oxidase | Gupta *et al.*[42], 2011 |
| Arylated-Modified CNTs | Laccase | Stolarczyk *et al.*[132], 2012 |
| Aminoethyl residues-functionalized SWCNTs | Laccase | Nazaruk *et al.*[129], 2012 |
| Three-dimensional gold nanoparticle-modified electrodes | Glucose oxidase | Wang *et al.*[40], 2012 |
| Tethered crosslinkers-functionalized MWCNTs | Laccase | Lau *et al.*[128], 2012 |
| Ferrocene-modified MWCNT | Glucose oxidase and catalase | Stolarczyk *et al.*[131], 2012 |
| Electrochemically functionalized MWCNTs | Glucose oxidase | Moumene *et al.*[118], 2013 |
| MWCNTs-modified carbon paper | PQQ-dependent alcohol dehydrogenase | Aquino Neto *et al.*[124], 2013 |

### 2.4. Bioelectrode characterization

#### Electrochemical techniques

Electrochemistry focuses on the relationship between the chemical and electrical effects of a target process.[133] Among the various available electrochemical techniques, enzymatic biofuel cell studies generally use cyclic voltammetry, polarization curves, and impedance measurements to gain better insight into the enzymatic system. Researchers employ these techniques to evaluate both semi-cell and complete biofuel cell and to asses mediator species by voltammetry and amperometry. Cyclic voltammetry is a simple and fast technique to initially characterize the reversibility of redox processes occurring on an enzymatic-based device. Normally, analysis of the voltammetric profile of a given system furnishes information about anodic and cathodic processes and gives the amount of electrons transferred in each case.[133] Electrochemical characterization reveals how mediator species behave in enzymatic biofuel cells performing MET; (i.e., in cells where the enzymes do not establish direct contact with the electrode surface). As discussed earlier, the thermodynamic driving force of an MET biofuel cell lies on the difference between the redox potentials of the mediator species and the enzyme redox center. Hence, electrochemical characterization, which generally relies on the cyclic voltammograms of the targeted species, helps one to obtain better electronic shuttle between the enzymes and the electrode surface. Many literature papers on enzymatic biofuel cells employ this electrochemical tool to characterize different mediator species such as osmium and ruthenium-based complexes, ferrocene, and organic dyes with distinct voltammetric behavior.[5, 16, 86] When the enzymes are in direct electric contact with the electrode surface, the electrochemical profile refers to the catalytic redox process of a particular biomolecule. Several literature examples have used cyclic voltammetry to confirm the direct electrochemistry of redox proteins and different electrode materials, such as carbon and gold.[124, 134-136] At this point, it is worth highlighting the need to improve the electrochemical characterization of the active site of the enzyme. Indeed, this can promote better catalytic activity and electron transfer rate within the biodevice. Such feat constitutes a future goal in protein engineering, and shall help mimic active sites instead of regular biocatalysts on the electrode surface.[137]

The most often employed electrochemical tool in enzymatic biofuel cells is the power curve. Such curve displays the potential or the current profile as a function of the power density of a given system; it simplifies the most significant result expected from a fuel cell; i.e., the generation of electrons. Osman *et al.*[10] elegantly described the whole process occurring in a power curve, from the OCV to zero. In general, this curve includes three steps: the first potential decrease corresponds to activation overpotential; the second decline refers to the ohmic drop; and the third process results from mass transport effects.[10] Recently, electrochemical impedance spectroscopy has also received considerable attention in the field of bioelectrodes characterization, mainly because it is sensitive and non-destructive.[138-140] This technique clarifies many of the electrical properties of a biological device and the resistances involved in a biofuel cell, specifically in the case of the electron-transfer processes occurring on both the anode and the cathode sides. Such situations are critical during the fabrication of prototypes, because all the internal resistances of the operating device will directly affect the system power.[141]

#### Spectrophotometry, fluorescence and electronic microscopy

The Michaellis-Menten model provides one of most relevant qualitative descriptions of the relationship between enzymes and substrates. This model is widely used in enzymatic kinetic characterization studies, and the function extracted from the curve of reaction rate *vs* substrate amount is a rectangular hyperbola.[142] Not all the enzymes fit the Michaellis-Menten model, even though cannot be considered a standard mechanism for all enzymatic reactions, it is useful in most cases. Using statistical calculations, the Michaellis-Menten equation furnishes both the maximum susbtrate conversion rate and the well-known Michaellis-Menten constant, $K_m$, which indicates the enzyme specificity toward a given substrate. Another parameter of great importance in enzymatic kinetics is the turnover number ($K_{cat}$), which provides the maximum number of substrate molecules that is converted to product per active site of enzyme per unit of time. Considering that all these kinetic data are normally determined for the enzyme in solution, an intersting way to obtain more clues about how the immobilization procedure affects the enzyme is to determine the kinetic parameters using the immobilized enzyme itself. This kind of assay can be very helpful to compare the effectiveness of the different enzyme methodologies and to calculate the amount of enzyme that remains active after application of different protocols. Considering the immobilization effect, literature data have suggested that the kinetic behavior of an immobilized enzyme follows a particular pathway or retains the same substrate affinity after the anchoring procedure.[143, 144] This assay simply involves immersing the substrates containing the immobilized enzymes inside a cuvette and then follow the absorbance changes due to conversion of the substrate

or oxidation or reduction of the cofators (such as the very often employed $NAD^+$ and ABTS).[143-145]

In studies involving enzymatic biofuel cells, fluorescence measurements help assess how the enzyme and the support interact. These measurements also provide information about enzyme occupation, distribution, and stability.[5] The interaction between dehydrogenase enzymes and hydrophobically modified chitosan is a good example of how the fluorescence technique aids bioelectrode characterization. Lau *et al.*[146] investigated the microenvironment of the immobilized enzyme malate dehydrogenase. These authors bound the fluorophore *acrylodan* to the enzyme structure and measured the fluorescence emission in the immobilized state. The fluorescent emission of the probe shifted towards the red as a result of increased dipole moment, indicating that the local environment became more hydrophilic. The authors claimed that appropriate modification of the enzyme microenvironment could enhance both the enzymatic activity and power density.[146] Konash *et al.*[147] studied how different fluorophore species interact with the enzyme alcohol dehydrogenase and immobilizing agents. These authors demonstrated that this fluorescence technique coupled with polarization and confocal microscopy was potentially applicable to visualize the polymer structure as well as the distribution of the incorporated species. Therefore, it is a very useful characterization tool to investigate how enzymes interact with polymeric matrixes.

The surface morphology of bioelectrodes has also been the target of much research. For this purpose, the main techniques employed in enzymatic biofuel cells are the physicochemical methodologies known as BET and Langmuir isotherms. X-ray diffraction analysis and scanning electron microscopy images (usually performed to evaluate film formation and thickness) are also described elsewhere. Isotherms normally help investigate bioelectrodes based on porous structures; they also provide the coverage profile.[148] Reports on the composition and morphology of bioelectrodes based on hydrogenase and functionalized MWCNTs,[149] as well as papers on the microstructure of the polypyrrole film containing entrapped ABTS molecules exist in the literature, too.[108]

### 2.5. Nanomaterials applied in bioanode structure and cell design

Advances in nanoscience and nanotechnology have enabled scientists to develop novel micro and nano electrodic materials. Moreover, immobilization methodologies can help improve both the biosensors and biofuel cells.[150] To solve one of the main limitations of enzymatic biofuel cells; i.e., the low efficiency of the electron transfer between the active site of the enzyme and the electrode surface, new trends in biofuel cell catalysts design include incorporating materials at the nanoscale, such as carbon nanotubes, nanofibers, nanocomposites, as well as metallic nanoparticles, in the bioelectrode structure. The use of nanotechnology to develop bioelectrodes for enzymatic biofuel cells has recently received much attention.[10] Nanostructured materials are an optimal environment to immobilize macromolecules: they provide larger surface area for enzyme immobilization, besides enhancing the efficiency of kinetic processes and allowing incorporation of higher enzyme load. Furthermore, the presence of these nanomaterials in the bioelectrode structure significantly enhances the bioelectrode electroactivity and provides efficient electrical contact between the enzyme active site and the electrode surface.[10] Nanomaterials also improve diffusional processes and reduce the redox potential of many mediator species used in MET enzymatic devices.[137] The sum of these contributions tends to significantly raise the output power density values furnished by a biofuel cell, besides increasing the lifetime of the immobilized enzyme.[20]

Carbon nanotubes (CNT) have attracted considerable attention from the scientific community since their discovery in 1991. Their dimensions and extraordinary electronic, thermal, and mechanical properties have encouraged several studies, which showed that the presence of CNT significantly improves material properties.[117, 151] The high CNT electrical conductivity and its biocompatibility allows for their use as electrode constituent, so they are a promising material for enzymatic biofuel cells. CNT can favor both DET (by decreasing the electron transfer distance) and MET (by introducing redox molecules that assist the electron transfer process in CNT via functionalization or electropolymerization).[20,117,150,151] To preserve the conformational structure of immobilized enzymes, anchoring protocols employing CNT generally involve non-covalent interactions instead of chemical procedures. Hence, adsorption methods based on the hydrophobic interactions between the enzymes and CNT are usually employed.[151] Furthermore, chemical modification of CNT with carboxylic acid groups, to obtain better dispersion, increases their biocompatibility through coupling with the amine groups of the enzyme.[152, 153] Enzymatic biofuel cells containing CNT have primary been developed on two fronts – associated with metal nanoparticles or polymers. A synergistic effect arises from the combination of CNT and polymers, culminating in increased mechanical stability, enhanced electrical conductivity, and three-dimensional structure with high electroactive area. Both compounds

have the ability to strongly interact with biomolecules, furnishing a highly conductive porous matrix. This feature favors diffusional processes and prevents enzyme loss, thus affording multifunctional materials.[136, 154-157]

Likewise, metallic nanoparticles have also drawn the attention of researchers in various fields, including bioelectrochemistry. The most important characteristics of this material include increased surface area per volume, excellent electron transport, and high catalytic power. In the case of enzymatic biofuel cells, the incorporated metallic nanoparticles act as electron "carriers" between the enzyme and the solid substrate, improving the bioelectrocatalytic process. This undoubtedly constitutes the major advantage of incorporating metallic nanoparticles along with biomolecules. Furthermore, enzymes and nanoparticles have similar sizes, so it is possible to reduce the electron transfer distance without affecting the enzyme activity.[158] Therefore, combining nanoparticles that exhibit unique electronic and catalytic properties with biomaterials that display incomparable catalytic and specificity behavior can furnish high-performance hybrid nanobiomaterials.[26] The literature brings different possibilities to incorporate metal nanoparticles in systems containing biomolecules. Most papers have focused on the preparation of hybrid bioelectrodes containing gold and platinum nanoparticles.[26] Gold nanoparticles present high biocompatibility and excellent conductivity; metallic platinum has similar properties, as well as excellent electrocatalytic power.[159] Amperometric glucose sensors and sugar/$O_2$ enzymatic biofuel cells are good examples of incorporation of platinum nanoparticles along with biomolecules.[137,160] The literature brings good examples on the preparation of biosensors and mediatorless enzymatic biofuel cells based on glucose and fructose oxidation, along with gold nanoparticles.[40,137,161,162]

### 2.6. Implantable enzymatic biofuel cells

The possible applications of enzymatic biofuel cells cover low-power portable devices; their use as battery for implantable devices is the ultimate aim. Enzymes normally operate in physiological conditions, catalyzing complex reactions in many organisms, so applying them in implantable devices makes much sense.[19] Since the first description of electricity generation as the sole result of glucose oxidase catalysis,[9] scientists have seeked application of biofuel cells in cardiac pacemakers (used to regulate the cardiac rhythm by applying small electrical charges to the myocardial tissue) that employ glucose as fuel. Currently pacemakers operate with a lithium-iodine battery at a power output around 10 μW, which represents an energy density of 1 W h $mL^{-1}$. The lifetime of these pacemakers is higher than 10 years.[163-165] The advantage of an implantable enzymatic biofuel cell is that it would continuously furnish electricity, because it could be possible to continuously replace the substrate.[19] Despite the lower power density generated by implantable biofuel cells, which normally lies in the range of some microwatts, these cells can satisfactorily provide the required energy inside many living organisms. At this point, the biological stability of the employed enzyme is the major drawback and is obviously the parameter that requires the most attention for practical purposes. Mass transfer issues also matter in an implantable enzymatic device. The output power depends on the amount of fuel, because substrate concentration in fluids is normally very low. The electrical connection and the surgical procedures involved in battery implantation also demand attention. Finally, the toxicity of some compounds employed in an enzymatic fuel cell, such as osmium-based mediators, must be overcome, so that viable implantable devices can be achieved.[19] An excellent review by Barton *et al.*[19] has covered good examples of implantable enzymatic devices, such as the preparation and characterization of a DET glucose/$O_2$ biofuel cell operating in human serum presented by Coman *et al.*[116] The complete biofuel cell supported on rods of spectrographic graphite electrodes reached a power density of 4 μW $cm^{-2}$ and an OCV of 0.58 V. Implantable technology is challenging, and obtaining electrical power from a small living organism is even more difficult. Cinquin *et al.*[166] fabricated a functional implantable glucose/$O_2$ biofuel cell working in the retroperitoneal space of freely moving rats. The innovation of this work lay on the simple mechanical confinement of various enzymes and redox mediators. Sales *et al.*[167] also prepared an implantable enzymatic fuel cell in a living rat. The authors conducted assays under physiological conditions using glucose from the rat blood as the anodic fuel and dissolved oxygen as the oxidizing agent in the cathode side.[167] Rasmussen *et al.*[168] implanted an enzymatic biofuel cell based on trehalose oxidation and oxygen reduction in a living insect. The authors designed the bioelectrodes on the basis of a bienzymatic trehalase/glucose oxidase in the anode side; the cathode material consisted of bilirubin oxidase grafted along with osmium complexes on thin carbon fibers. Halamkova *et al.*[169] employed PQQ-dependent glucose dehydrogenase and laccase as biocatalysts immobilized on a buckypaper (nanostructured composition consisting of densely packed CNTs). The cross-linking agent (1-pyrenebutanoic acid succinimidyl ester) connected the enzymes to the CNT via covalent binding with amine groups of lysine residues. The implanted biofuel cell gave an OCV of 530 mV and

maximum power density around 30 μW $cm^{-2}$, which was comparable to values obtained for other systems implanted in rats and rabbits. MacVittie *et al.*[170] implanted biocatalytic electrodes into the hemolymph between the exoskeleton and the stomach of an American lobster; employing immobilized PQQ-dependent glucose dehydrogenase in the anode compartment and a laccase-based cathode in a buckypaper conductive support, the authors produced the desired energy. Southcott *et al.*[171] demonstrated an implantable biofuel cell operating under conditions that mimicked the human physiology for a continuously operating pacemaker. A buckypaper support was employed as electrode material to immobilize PQQ-dependent glucose dehydrogenase and laccase on the anode and cathode respectively, along with 1-pyrenebutanoic acid succinimidyl ester. The prepared pacemaker produced a profile of electrical pulses similar to those usually registered with a commercial device operating on the basis of standard lithium-based battery.[171] Implantable enzymatic biofuel cells can also function as power supply for electronic contact lenses. Falk *et al.*[172] reported a DET-based biofuel cell that generated significant electrical energy in the human lachrymal liquid. Cellobiose dehydrogenase and bilirubin oxidase worked as the anodic and cathodic bioelements, respectively. The authors claimed an OCV of 0.57 V, a power density of about 1 μW $cm^{-2}$, and an operational half-life of over 20 h.

### 2.7. Enzymatic biofuel cell development and recent outcomes

Fuel cells employing enzymes as the main catalysts have emerged from the desire to obtain well defined and specific reactions on the surface of an electrode.[8] Although the first description to obtain electricity from a biological species dates from 1911,[7] only in 1964 did Yahiro *et al.*[9] demonstrate the first example of an enzymatic device. Figure 7 illustrates how enzymatic biofuel cells evolved since the first descriptions.

For a long time, glucose remained as the standard fuel in enzymatic biofuel cell studies, motivated mainly by the possibility of applying it as pacemaker battery in vivo. In the first work on glucose, the authors demonstrated that two flavoenzymes, glucose oxidase and d-amino acid oxidase, produced electricity. Although this system gave low current density, the glucose/$O_2$ assay using platinum as cathode in contact with air provided OCV in the range of 300-350 mV.[9] In the 1970s, just a few reports dealt with biofuel, most descriptions involved mainly metallic electrodes. For example, the work of Rao *et al.*[173] aimed to achieve glucose oxidation for possible application in implantable

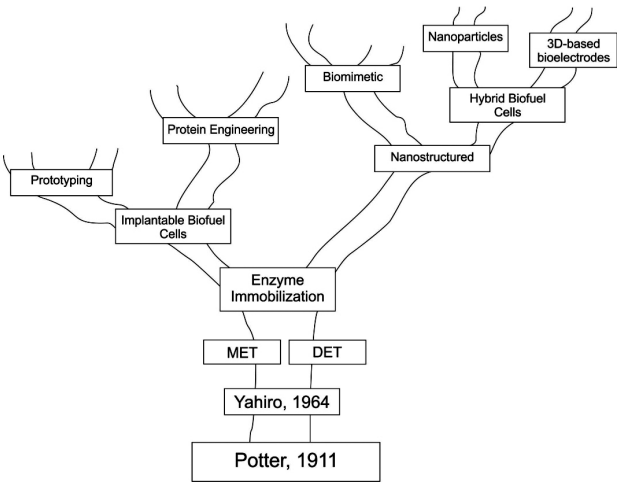


**Figure 7.** Development of Enzymatic biofuel cells profile.

electronic devices. Yeh *et al.*[23] presented an example of enzymatic electrochemistry when they described the reversible electron transfer characteristics of cytochrome c9 by electrically contacting it with indium oxide electrodes. Plotkin *et al.*[174] reported on a methanol-based biofuel cell with an OCV of 0.3 V when they employed bacterial methanol dehydrogenase along with ethosulfate phenazine as the redox mediator. In the 1980s, Aston and Turner[175] published a review paper concerning both biofuel cells and biosensor devices, thus reviving the interest in connecting biological systems with electrochemical devices. Many elegant papers were published in the 1990s, among which the report of Palmore *et al.*[176] stands. These authors used three dehydrogenase enzymes in cascade, to completely oxidize methanol. Willner and Katz[177] described a membraneless biofuel cell employing PQQ and microperoxidase.

In the beginning of the 21st century, biofuel cell researchers began to attempt to obtain microdevices for implantable technology. Katz and Willner[178] prepared an electroswitchable enzymatic biofuel cell based on glucose and cytochrome oxidase in a $Cu^{+2}/Cu^{0}$-polyacrylic acid hybrid matrix. Professor Adam Heller, a great enthusiast for the bioelectrochemistry field, greatly contributed to knowledge about enzymatic biofuel cells. This review will highlight some of these contributions hereafter. Professor Heller designed a tiny enzyme-based biofuel cell for micro-sized implantable medical devices. The bioelectrodes consisted of two electrocatalysts with 7-μm diameter deposited on carbon fiber electrodes in a polycarbonate support.[15] The anode side comprised glucose oxidase covalently bound to a reducing-potential copolymer based on osmium complexes along with a laccase-based cathode. Such bioelectronic device furnished 600 nW at 37 °C, which was enough to power small

silicon-based microelectronics. Mano *et al.*[179] prepared a miniature biofuel cell to operate in a physiological buffer. These authors obtained the bioelectrodes by immobilizing the redox enzymes with the respective redox hydrogel mediators on a carbon fiber support. For a similar bioelectrode configuration, the same authors described an implantable miniature biofuel cell operating in grape, which produced 2.4 μW at 0.52 V.[32] In another paper, these authors reported another miniature biofuel cell employing osmium-based mediators; they achieved a power density of 2.68 mW $mm^{-2}$ at 0.78 V. [180] Employing a highly efficient cathode prepared with laccase and an osmium-based redox mediator, Soukharev *et al.*[181] obtained a miniature membraneless glucose/$O_2$ biofuel cell that operated at high voltage, 0.88 V, and produced 350 μW $cm^{-2}$.

Despite the many reports dealing with glucose/$O_2$ biofuel cells, research teams have also targeted alcoholic-based biofuel cells. Topcagic and Minteer[59] described a biofuel cell based on alcohol dehydrogenase and bilirubin oxidase for ethanol oxidation at the anode and oxygen reduction at the cathode, respectively. Results showed a 30-day lifetime, power density of 0.46 mW $cm^{-2}$, and OCV in the range of 0.68-0.83 V. Our research team has also prepared bioanodes for ethanol/$O_2$ biofuel cell by immobilizing alcohol dehydrogenase and PAMAM dendrimers onto a carbon cloth platform via passive adsorption or the layer-by-layer technique.[22, 103]

Most studies in the biofuel cell literature employ only a single enzymatic system, with a fuel oxidation step involving one or two electrons. However, researchers have also focused on enzymatic biofuel cells dealing with complete substrate oxidation, to take full advantage of the energy density of the substrate. Palmore *et al.*[176] designed the first multi-enzymatic biofuel cell system to oxidize methanol; they immobilized the enzymes alcohol, aldehyde, and formate dehydrogenases onto graphite electrode as the anode and used it along with a platinum cathode. Sokic-Lazic and Minteer[54] reported on an enzymatic biofuel cell mimicking the citric acid cycle, which is the main metabolic pathway that living cells employ to convert carbon fuels. The authors immobilized dehydrogenase enzymes on carbon electrode in cascade and verify increased current density according to the number of enzymes. This same group also mimicked the Krebs's cycle: they prepared bioanodes with dehydrogenases in cascade to completely oxidize pyruvate.[55] Immobilization of sequential dehydrogenase enzymes in cascade to complete oxidize lactate in a lactate/air biofuel cell has also been described.[25]

Another interest in the development of enzymatic biofuel cells is protein engineering, which affords biocatalysts with a specific design or desired characteristic. To this end, scientists have employed several strategies to rationally design redox enzymes.[182, 183] Protein engineering can provide changes in the structural framework improving substrate access to the active site via proper orientation of the enzyme at the electrode surface,[184] direct introduction of some redox mediators into the enzyme structure,[185] and enhancement of enzyme stability,[186] among other strategies. Campbell *et al.*[187] coassembled bifunctional protein building blocks, to create multifunctional biomaterials. The authors prepared multifunctional hydrogels that efficiently catalyzed dioxygen reduction to water at neutral pH using both a metallopolypeptide (which displays cross-linking functionality) and a modified polyphenol oxidase (a small laccase genetically engineered to exhibit cross-linking functionality). The prepared material also allowed the bioelectrocatalytic system - enzyme and redox mediator - to act as the physical structure of the hydrogel, making it applicable in a wide range of systems.[187] Despite the high applicability of PQQ-dependent glucose dehydrogenase in enzymatic biofuel cells, mainly due to its oxygen tolerance and high catalytic efficiency, this enzyme is not stable in the long term. Yuhashi *et al.*[186] reported engineering of this enzyme in a glucose/$O_2$ cell, and the results pointed to enhanced stability of the bioelectrodes prepared with the mutant enzyme. Another paper dealing with a hybrid bioelectrode described a site-specific attachment of a genetically modified glucose oxidase. It was possible to obtain a free thiol group near this active site, with gold nanoparticles performing DET with the electrode surface.[119]

Confirming the growing interest in enzymatic biofuel cells over the past decades, several important review papers exist in the literature, some of which we highlight here. In 2004, Barton *et al.*[19] presented a complete overview of immobilization techniques, applications, peculiarities, and differences as compared with biosensors with emphasis on MET processes. In 2006, Bullen *et al.*[8] reviewed works published between 1994 and 2006, focusing mainly on performance parameters such as power density, OCV, and development to that date. In 2007, Minteer *et al.*[12] highlighted the practical application parameters of enzymatic biofuel cells, such as stability and lifetime, besides future trends in the development of this device. In 2008, Cooney and Minteer[5] reviewed the electron transfer processes, the aspects related to bioelectrodes configuration, and characterization techniques. In 2010, Ivanov *et al.*[188] discussed the bioelectrode preparation methodologies, and modeling as well as aspects that still limit the commercial application of this bioelectronics device. In 2011, Osman *et al.*[10] presented the recent progress in the development of enzymatic biofuel cells, as well as advances

in new electrodic materials, immobilization methods, nanostructuring, and aspects related to the construction of these devices from an engineering viewpoint. Still in 2011, Opallo and Bilewicz[189] presented the latest aspects regarding the development of nanostructured bioelectrodes, also focusing on catalysis of the oxygen reduction reaction. In early 2012, Yang *et al.*[26] reviewed aspects associated with the immobilization of enzymes onto electrode surfaces, whereas Falk *et al.*[20] presented a mini review that discussed DET processes in enzymatic devices.

Enzymatic biofuel cells provide a means to obtain clean and renewable energy, so they have gained growing scientific and technological importance in recent years. Indeed, many studies have attested to the promising features of this device. For sure, many have been the achievements in the field of implantable technologies, as stressed in the previous topic; at this point, some recent outcomes deserve to be highlighted. In 2009, Sony® Electronics Corporation introduced the first prototype of a biobattery based on a glucose/$O_2$ biofuel cell, to provide energy for low-power portable devices.[17, 18] The developed passive-type biofuel cell displayed a multi-stacked structure and was able to generate 100 mW with a total bioelectrode area of 80 $cm^3$. The maximum power density was 1.45 mW $cm^{-2}$ at 0.3 V, the OCV was 0.8 V, and the short-circuit current density was 11 mA $cm^{-2}$. The multi-stacked structure provided sufficient energy to continuously operate a Walkman and a radio-controlled car for 2 h.[17] Trying to improve two of the most rate-limiting steps in enzymatic bioelectronics (especially in miniaturized devices); i.e., electron transfer rate and mass transport of substrates, Gao *et al.*[190] prepared engineered porous microwires as electrode support for enzymatic biofuel cells. They obtained this support by a coagulation spinning process. The CNTs furnished a highly porous structure to immobilize the enzyme and a much higher ratio of available redox polymer centers. The miniature membraneless glucose/oxygen MET biofuel cell was able to generate 740 μW $cm^{-2}$ at 0.57 V. Zebda et al[136] published another remarkable paper on high-power glucose-based biofuel cell, in which they employed compressed carbon nanotube electrodes to efficiently wire the enzymes. Both the bioanode and biocathode were based on three-dimensional CNTs mechanically compressed with the glucose oxidase and laccase, respectively. The authors also added catalase to the mixture, to diminish peroxide contamination. When associated, the bioelectrodes delivered a high power density of up to 1.3 mW $cm^{-2}$ and an open circuit voltage of 0.95 V in the mediatorless biofuel cell test. This same group claimed an operational power density of about 1 mW $cm^{-2}$ in the case that the cell operated for one month under physiological conditions.[136]

Hybrid biofuel cells employing zinc as the anodic material are also a possibility to obtain high power density devices.[129] This biofuel cell is generally constructed by using a zinc bar or wire as electron source along with a biocathode based on laccase or bilirubin oxidase to catalytically reduce oxygen. This type of biofuel cell dismisses the need for compartmentalization and membrane separation; also, it can work in direct contact with the environment. Jensen *et al.*[191] described a type of zinc biobattery that can power a 1.5 V household device for 38 days. The Zn/$O_2$ biofuel cell device, prepared in the absence of any mediators, yielded 0.44 mW $cm^{-2}$ at 0.5 V. Stolarczyk *et al.*[132] reported other impressive power density and OCV values employing a DET cathode using SWCNT modified with anthracene and anthraquinone species: 1.5 V and 2 mW $cm^{-2}$, respectively. Using a hybrid biobattery, Zloczewska *et al.*[192] achieved an OCV of 1.75 V and generated a maximum power density of 5.25 mW $cm^{-2}$ at 0.4 V.

## 3. Conclusion and Future Outlook

A few years ago, one could say that the applicability of the enzymatic biofuel cell as an alternative energy source was questionable or a dream that would be difficult to come true. Nowadays, although metallic-based fuel cells still stand out in terms of research and power output, the use of enzymes to efficiently convert energy from chemical substrates to electricity has increased. These systems still need to meet the requirements of practical commercial application, though. Significant improvements in terms of enzyme immobilization, power density, stability, cost of the employed materials, and issues related to the electron transfer between enzymes and electrode surfaces still need to be achieved. Over the last years, there has been many outcomes both in terms of MET and direct electronic connection between enzymes and electrode surfaces. These efforts have increased the number of papers describing enhanced electron shuttle through different electrode surfaces. Moreover, elegantly designed bioelectrodes have also been reported to enable direct electrical connection between several enzymes and solid supports.

To achieve higher power density output, the use of hybrid zinc-based biobattery has emerged as an interesting strategy to obtain high power devices. Indeed, this possibility has been a goal in the recent specialized literature. In terms of protein engineering, despite the many advances seen in the past years and even though some good results in enzymatic biofuel cells using mutant enzymes have been achieved, challenges still exist in this field. Fundamental studies on protein structure-function relationships are

still necessary to obtain better electron transfer rate and substrate conversion at electrode surfaces. Considering implantable technology, this area has witnessed many advances, including the promising results in terms of the generated current density. However, the in vivo use of enzymatic biofuel cells still requires further investigation, especially with regard to operational stability tests, to attend to the desirable durability. Developing hybrid nanocatalysts containing enzymes, carbon nanotubes, and metallic nanoparticles is another strategy employed to prepare high-performance enzymatic biofuel cells. In this kind of bioelectrode architecture, it is always important to consider the maintenance of the characteristics that make biofuel cells devices environmentally and economically attractive, i.e., the costs of the prepared device and the amount of non-renewable compound must be borne in mind. Whereas, researchers focused on understanding the chemistry of enzymes on electrode surfaces, current efforts are more directed towards the development of methodologies and materials integrated with the biocatalysts. This trend aims to maximize enzyme distribution, abandoning a classic two-dimensional condition to obtain a three-dimensional structure with highly ordered biocatalysts in a high-performance biomaterial. Finally, standardizing stability and operation tests is crucial to obtaining consistent data on enzymatic activity retention over long periods. Hence, in the near future, besides investigating performance parameters researchers of enzymatic biofuel cells must also consider interface engineering; i.e., they must evaluate the prepared biomaterials in prototype devices, to better visualize them under operational conditions.

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