Enzymatic Biofuel Cells: 30 Years of Critical Advancements

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Abstract

Enzymatic biofuel cells are bioelectronic devices that utilize oxidoreductase enzymes to catalyze the conversion of chemical energy into electrical energy. This review details the advancements in the field of enzymatic biofuel cells over the last 30 years. These advancements include strategies for improving operational stability and electrochemical performance, as well as device fabrication for a variety of applications, including implantable biofuel cells and self-powered sensors. It also discusses the current scientific and engineering challenges in the field that will need to be addressed in the future for commercial viability of the technology.

Keywords: biofuel cells; bioelectrocatalysis; bioanodes; biocathodes; biobattery

Introduction

One common example of a bioelectronic device is an enzymatic biofuel cell, as shown in Figure 1. Enzymatic biofuel cells are a type of fuel cell that utilizes enzymes as the electrocatalysts to catalyze the oxidation of fuel and/or the reduction of oxygen or peroxide for energy conversion to electricity. Most traditional fuel cell electrocatalysts are conductive-metal nanoparticle catalysts that operate at temperatures ranging from 45°C to 150°C. These catalysts have the advantage of high stability and high activity in highly acidic and/or basic environment. However, they are plagued with passivation issues that require simple and high purity fuels (i.e. hydrogen and methanol). On the other hand, living organisms can consume and metabolize complex fuels (i.e. sucrose, fructose, etc.) and fuel mixtures and their catalysts do not have the

same issues. Therefore, the field of enzymatic biofuel cells has expanded over the last 3 decades, and it is now possible to consider applications (i.e. implantable fuel cells, JP-8 fuel, etc.) where traditional metal nanoparticle electrocatalysts have limitations. As shown in Figure 1, enzymatic biofuel cells are more complex than traditional fuel cells, because typically open circuit potentials are significantly lower than theoretical due to cofactor redox potentials, enzyme redox potentials, and mediator redox potentials. This review article will detail the advancements in the field of enzymatic biofuel cells over the last 30 years and discuss the challenges that will face enzymatic biofuel cell development in the next 30 years.

History Before The Debut of Biosensors & Bioelectronics

Although microbial biofuel cells have been studied for over a century, enzymatic biofuel cell history goes back to the early 1960s. In 1964, Yahiro et al. invented the concept of enzymatic biofuel cells with a glucose oxidase bioanode and a Pt cathode (Yahiro et al. 1964). This system had very low open circuit potentials (175-350 mV), but showed the proof of concept that an oxidoreductase enzyme could catalyze a fuel cell half reaction, in this case, the oxidation of glucose to gluconolactone, and transfer the electrons via small molecule redox species to the electrode surface. This pioneering work set the definition of enzymatic biofuel cell as a fuel cell utilizing an enzyme as the electrocatalyst at one of the two electrodes, in this case the anode. Although there are several reports of biofuel cells in the 1960s, the early work all involved the use of a purified oxidoreductase enzyme and a mediator for performing mediated electron transfer (MET) to the electrode surface (Davis and Yarbrough 1967; Hunger and Perry 1966; Yahiro et al. 1964).

Enzymatic biofuel cells rely on enzymatic bioelectrocatalysis, as do amperometric and voltammetric biosensors. Therefore, research in the field has been aided not only by work

specifically on enzymatic biofuel cells, but also by research improving enzymatic bioelectrocatalysis for biosensor applications. In the late 1970s, Berezin et al. made one of the most outstanding contributions to the field in discovering direct electron transfer (DET) (Berezin et al. 1978). Unlike MET systems, direct bioelectrocatalysis does not require the use of an external redox mediator and can transfer electrons directly from the protein to the current collector/electrode. This simplification also results in less potential losses due the potential difference between the enzyme cofactor/active site and the mediator. This work significantly impacted the overall goal of biofuel cells for the future. Although laccase bioelectrodes were invented with this work, it wasn't until 1984 that the concept of utilizing enzymes at the cathode of a biofuel cell was discussed and even then it didn't utilize the enzyme for direct bioelectrocatalysis. Instead, it involved a gold cathode that produced peroxide from oxygen and then the peroxide was consumed by the enzyme chloroperoxidase (Laane et al. 1984). This pioneering work expanded the field of biofuel cells to fuel cells utilizing enzymes at either the anode or the cathode.

Although direct electron transfer became a goal for enzymatic biofuel cells, most biofuel cells in the literature continued to utilize mediated electron transfer, because MET systems typically resulted in higher current densities, even though they have higher potential losses and issues with mediator stability. Turner and coworkers were the first to utilize organometallic redox mediators for biofuel cells in the early 1980s (Cass et al. 1984). Some of this work involves redox mediators in solution while some involved immobilization of these ferrocene mediators in conducting polymers (Cass et al. 1984; Dicks et al. 1989). This important discovery led to the incorporation of organometallic redox mediators into redox polymers, which was pioneered by the Heller group and is now used by countless researchers in the field (Barriere et al. 2006;

Barton et al. 2001; Barton et al. 2002; Degani and Heller 1989; Gallaway et al. 2008; Gallaway and Calabrese 2008; Gao et al. 2009; Kavanagh et al. 2009; Mano et al. 2002a; Mano et al. 2002b; Mano et al. 2003; Mano et al. 2005; Mano et al. 2006; Mao et al. 2003; Stoica et al. 2009; Tasca et al. 2009).

Enzymatic biofuel cell research after the first publication of Biosensors & Bioelectronics

Cofactor regeneration

Oxidoreductase enzymes contain or require redox cofactors that change oxidation state during substrate catalysis. There are a variety of natural oxidoreductase organic and inorganic cofactors including: nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate (NADP^+), flavin adenine dinucleotide (FAD), pyrroloquinoline quinone (PQQ), hemes, iron-sulfur clusters, coenzyme Q, coenzyme F420, flavin mononucleotide (FMN) and ascorbic acid. Many of these cofactors are derived from vitamins and some cofactors are bound in the enzyme while others are diffusional. One of most common cofactors for oxidoreductase enzymes is NAD(P), which is a diffusional mediator that becomes NAD(P)H upon oxidation of substrate/fuel and therefore must be regenerated at the electrode. There are thousands of NAD(P)-dependent dehydrogenases in the literature and many have been used for bioanodes of biofuel cells including: glucose dehydrogenase (Persson et al. 1985), alcohol dehydrogenase (Moore et al. 2004b), aldehyde dehydrogenase (Akers et al. 2005), formate dehydrogenase (Palmore et al. 1998), lactate dehydrogenase (Sokic-Lazic et al. 2011), pyruvate dehydrogenase (Sokic-Lazic and Minteer 2009), malate dehydrogenase (Rincon et al. 2010), and many others. However, NAD(P)H has poor electrochemistry on most common electrode surfaces (i.e. carbon, gold, and platinum) with overpotentials of 0.5-1 V and quick passivation of electrode surfaces (Karyakin et al. 1999a). The late 1980s and early 1990s resulted in a wealth of research on

modifying electrodes for improving NADH oxidation, so that NAD-dependent dehydrogenases could be used at the anode of biofuel cells (Gorton 1984; Karyakin et al. 1999a; Karyakin et al. 1999b; Persson et al. 1985). This resulted in a widely expanding choice of fuels from glucose to methanol, ethanol, formate, glycerol, lactate, pyruvate, and malate. One of the most common strategies for decreasing the overpotential of NADH oxidation was the use of electropolymerized azines. This strategy is still used frequently today. However, recent research has shown that electropolymerization is not the only option for immobilization. Specifically, azine electrocatalysts can be immobilized via chemical polymerization of the polymer, adsorption to carbon nanotube structures, and crosslinking within carbon nanotube/polymer composites. (Arechederra et al. 2010; Goran et al. 2014; Meredith et al. 2012). Although NAD-dependent bioanodes are quite complex due to the need for a diffusional cofactor and an electrocatalyst, they are still commonly used in bioanodes today, because they are oxygen independent compared to oxidase-based bioanodes. However, it is important to note that they are not the only type of dehydrogenase that is utilized in biofuel cells today. Specifically, heme-containing dehydrogenases, PQQ-dependent dehydrogenases, and FAD-dependent dehydrogenases are becoming more and more popular in recent years, because they are oxygen independent and do not require diffusional mediators (Coman et al. 2008; Kamitaka et al. 2007a; Milton et al. 2013; Schubart et al. 2012b; Stoica et al. 2006; Tasca et al. 2008; Tkac et al. 2009).

First enzyme cascade for deep oxidation

In 1998, Palmore and Whitesides made significant contributions to the field of enzymatic biofuel cells showing that biofuel cell performance was a function of degree of oxidation at the anode with their methanol biofuel cell containing NAD-dependent alcohol dehydrogenase, aldehyde dehydrogenase, and formate dehydrogenase. These enzymes were in solution with diaphorase

and a quinone mediator, but they showed the importance of deep or complete oxidation on current/power density performance (Palmore et al. 1998). This work produced very large current/power densities for the time period without the use of nanomaterials or other high surface area materials.

Membraneless biofuel cells

In 1999, Katz and Willner made another great advancement in the field of biofuel cells by immobilizing a selective enzyme at the anode and a separate selective enzyme at the cathode to form the first membraneless or compartmentless biofuel cell (Katz et al. 1999). As shown in Figure 2, this biofuel cell employs enzyme selectivity to eliminate issues of crossover and allows for the anode and cathode to be as close to each other as possible without physically shorting the two electrodes. Although the performance was low, this rapidly led to a wealth of literature in improving the open circuit potential and current density of membraneless biofuel cells (Deng et al. 2010; Gonzalez-Guerrero et al. 2013; Li et al. 2011; Liu et al. 2010; Mano and Heller 2003; Topcagic and Minteer 2006; Yehezkeli et al. 2011; Zebda et al. 2010). It also led to increased research focused on moving technology toward implantable systems by operating in physiological environments.

Immobilization and stabilization of enzymes at electrode surfaces

Immobilization and stabilization of enzymes at electrodes has been critical for the development of both biosensors and enzymatic biofuel cells. Early enzymatic biofuel cells utilized enzyme in solution, but then the many developments in immobilization and stabilization of enzymes at electrodes for biosensors were utilized by enzymatic biofuel cell researchers to improve the performance and stability of biofuel cells. For instance, most protein film voltammetry and early

studies of direct electron transfer utilized adsorption for immobilization by incubating the electrode in an enzyme solution (Armstrong and Wilson 2000). Although this is very useful for proof of concept of bioelectrocatalysis, it is not a stabilizing technique, because the enzyme can readily leach during use in the enzymatic biofuel cell. Katz and Willner focused on immobilization through covalent binding techniques to gold electrode surfaces (Bardea et al. 1997; Willner et al. 2006), while the Heller group immobilized enzymes by crosslinking redox hydrogels in the presence of enzyme, (Gregg and Heller 1990; Heller 1990) thereby, crosslinking the enzyme into the polymer hydrogel and entrapping it. This technique allowed for high loading of enzyme, facile transport of fuel and product through the membrane, and the entrapment provided some additional stability versus a solution of the enzyme (Barton et al. 2001; Barton et al. 2002; Calabrese-Barton et al. 2001; Calabrese Barton et al. 2001; Chen et al. 2001; Kim et al. 2003; Mano et al. 2002a; Mano et al. 2002b; Mano et al. 2002c; Mano et al. 2002d; Scodeller et al. 2010). However, crosslinking enzyme or covalent binding of enzyme in anyway can decrease the degrees of freedom of the enzyme and therefore decrease the activity, as shown in Figure 3. Figure 3 also shows the many interdependent relationships between composites of redox polymer entrapment-based bioelectrodes that need to be optimized for high performance bioelectrodes. Sandwich and encapsulation techniques have also been designed to immobilize and stabilize an enzyme at an electrode surface either under (sandwich) or within (encapsulation) a polymer layer (Akers et al. 2005; Klotzbach et al. 2008; Klotzbach et al. 2006; Moore et al. 2004a). Over the last decade, these techniques have been combined and utilized for producing stable bioelectrodes. Today, problems with the instability of the mediator or the cofactor is frequently more common than problems with stability of the enzymes (Beilke et al. 2009). A final recent approach to enzyme stabilization is to utilize enzymes from thermophilic microorganisms

(Beneyton et al. 2011; Campbell et al. 2012; Lojou 2011), because enzymes that are stable at high temperatures typically have higher stability at room temperature than the non-thermophilic versions.

Enzyme engineering

Although enzyme engineering has been an emerging subfield of bioprocessing for some time (Kuchner and Arnold 1997), it is only in the last decade that enzyme engineering has been utilized to improve the stability and the performance of enzymatic bioelectrodes for biofuel cells. Early work by Schwaneberg et al. focused on the directed evolution of glucose oxidase for biofuel cell properties (Yu et al. 2011; Zhu et al. 2006; Zhu et al. 2007). This type of induced evolution systems has also been utilized for other enzymes to improve bioelectrocatalytic performance (Gupta et al. 2010; Zulić and Minteer 2011). However, the last 5 years has seen directed evolution move on to site-directed mutagenesis for improved bioelectrocatalytic performance and immobilization on electrode surfaces (Campbell et al. 2012; Durand et al. 2010; Holland et al. 2011; Kamitaka et al. 2006; Wheeldon et al. 2009). These works are providing enzymes that are more stable, more active, and easier to immobilize for bioelectrocatalysis. It is the hope that this technology and other synthetic biology technology will provide new enzymes, new enzyme cascades, enzymes with higher volumetric catalytic activity (i.e. smaller), and enzymes with more efficient direct electron transfer pathways.

Nanostructured bioelectrodes

“Nanomaterials” has become the buzzword in enzymatic bioelectrodes in the last 15 years. Materials ranging from carbon nanotubes to gold nanoparticles have been employed. In the early 2000s, carbon nanotubes and other nanostructured carbon materials were used to increase the

active surface area of enzymatic bioelectrodes, so larger amounts of enzyme could be immobilized (Arechederra and Minteer 2009a; Che et al. 2011; Minteer 2012; Walcarius et al. 2013; Wen et al. 2010; Yan et al. 2006). However, carbon nanotubes and other nanostructured carbon materials have also been used to decrease the overpotential for NADH oxidation. The increased use of nanomaterials has also led to improved direct bioelectrocatalysis of a number of enzymes, including laccase, bilirubin oxidase, heme-containing enzymes (i.e. fructose dehydrogenase), and PQQ-dependent enzymes (Ding et al. 2010; Gupta et al. 2011b; Hussein et al. 2011; Kamitaka et al. 2007b; Lim et al. 2007; Ramasamy et al. 2010; Schubart et al. 2012a; Treu et al. 2009; Wang et al. 2005; Zebda et al. 2011; Zheng et al. 2006). However, it has led to huge controversy in the field, because there have been many false reports of direct electron transfer of the enzyme glucose oxidase (Goran et al. 2013; Wooten et al. 2014). Recent research has utilized nanomaterials as a method for tailoring the biotic-abiotic interface for improved direct electron transfer. There are now many reports of covalent and non-covalent modification of carbon nanomaterials for improved direct electron transfer (Giroud et al. 2015; Giroud and Minteer 2013; Joensson-Niedziolka et al. 2010; Karaśkiewicz et al. 2012; Stolarczyk et al. 2012a). These have been focused toward promoting direct electron transfer for biocathodes, but hopefully these strategies will be expanded to bioanodes in the coming years.

Enzyme cascades and metabolons

Although Palmore and Whitesides first introduced the importance of deep oxidation in improving the current/power density of biofuel cells, the last decade has seen an increase in the use of natural cellular pathways and minimal enzyme cascades for deep oxidation. This has included incorporating natural metabolic pathways (i.e. the Krebs's cycle) into enzymatic biofuel cells (Sokic-Lazic et al. 2011; Sokic-Lazic and Minteer 2008; Sokic-Lazic and Minteer 2009) as

shown in Figure 4, as well synthetic metabolic pathways (Tasca et al. 2010; Zhu et al. 2014; Zhu et al. 2011). These synthetic metabolic pathways have led to the evaluation of promiscuous enzymes for multi-step oxidation of biofuels in biofuel cells for enhanced efficiency and energy density (Shao et al. 2013; Xu and Minteer 2012; Xu and Minteer 2014), as shown with the promiscuous enzymes PQQ-dependent alcohol dehydrogenase, PQQ-dependent aldehyde dehydrogenase, and oxalate oxidase in the deep oxidation of glycerol in Figure 5. Many of these metabolic enzymes in living cells form supercomplexes of sequential enzymes in the cascade, called a metabolon. Figure 6 shows a natural metabolon formed in the metabolic pathway of the Krebs's cycle and the substrate channel in the metabolon that channels substrate over a short distance between one enzyme and the next (Wu and Minteer 2015). Recently, researchers have shown that high electrochemical performance requires not only enzyme cascades, but proximal organization of sequential enzymes (Moehlenbrock et al. ; Moehlenbrock et al. 2010; Van Nguyen et al. 2014). This can be done utilizing either chemical strategies (i.e. crosslinking or tethering) or biological strategies (i.e. DNA or protein scaffolding).

Hydrogen fueled enzymatic fuel cells

Although many biofuels (i.e. glucose, sucrose, ethanol, etc.) have been studied as fuels for enzymatic biofuel cells, hydrogen is another possible fuel. Hydrogenases are capable of oxidizing hydrogen, but many are oxygen sensitive. Recently, there has been a great deal of interest in hydrogenase bioelectrochemistry for enzymatic biofuel cell applications (Leger et al. 2002; Ruediger et al. 2010; Vincent et al. 2007). Part of this research has focused on oxygen tolerant hydrogenases (Ciaccafava et al. 2012) or enzyme engineering oxygen tolerant hydrogenases (Dementin et al. 2009), and other strategies have focused on engineering materials to protect hydrogenases from de-activation (Ciaccafava et al. 2010; Lojou 2011). Probably the

most impressive of these materials strategy has been the redox hydrogel designed by the Schumann group to protect the hydrogenase from oxygen (Plumeré et al. 2014), as shown in Figure 7. Overall, oxygen tolerance has resulted in few hydrogen/oxygen biofuel cells in the literature, but these recent advances have made it possible for an explosion of new biofuel cells in this area.

Air-breathing bioelectrodes

As enzymatic biofuel cells have evolved, there has been concern about the low concentration of oxygen in most aqueous solutions. Therefore, there has been a need for major advances in transitioning from a flooded biocathode (biocathode submerged in solution) to an air-breathing biocathode, as shown in Figure 8. Although there are few examples of gas diffusion biocathodes (Ciniciato et al. 2012; Gellett et al. 2010b; Gupta et al. 2011a; Gupta et al. 2011b; Rincon et al. 2011; Zloczewska and Jönsson-Niedziolka 2013), they are emerging as an important technological advancement for the field of enzymatic biofuel cells. The early work focused on laccase in non-physiologically relevant pHs, while the more recent work by the Atanassov group utilizes bilirubin oxidase (Gupta et al. 2011b). Biocathodes are not the only gas diffusion bioelectrode that needs to be developed. Gas diffusion electrodes for hydrogenase bioanodes are also needed as hydrogenase bioanodes are further developed.

Computational modeling

Finally, as researchers have started designing biofuel cell prototypes, there has been a renewed interest in computational modeling and simulation of enzymatic biofuel cells. This started with early work that evaluated single rotating disk bioelectrodes (Bartlett and Pratt 1995) and has moved on to simulations of biofuel cells and biofuel cell electrodes (Calabrese-Barton 2005; Kar

et al. 2011; Osman et al. 2013; Wang et al. 2011). These simulations and modeling are critical to the future of the field as we move beyond fundamental science into applications and prototypes for applications.

Applications of Enzymatic Biofuel Cells

There are a variety of applications for enzymatic biofuel cells. When researchers consider the low current/power density of enzymatic biofuel cells, the first application that comes to mind is power sensors. In 2001, Katz and Willner invented the concept of self-powered biosensors that utilized a biofuel cell as a biosensor for the fuel (Katz et al. 2001). This was later translated to include inhibition and activation based self-powered biosensors (Meredith and Minteer 2011; Wen et al. 2011). Katz has further focused this area in recent years to utilize biological logic gates for self-powered biosensing (Amir et al. 2009; Katz 2010; Katz and Pita 2009; Tam et al. 2009; Zhou et al. 2009; Zhou et al. 2012), with an almost limitless opportunity for applications. The last 5 years have brought about a great deal of phenomenal research at translating enzymatic biofuel cells from the lab bench to implantable systems. The first biofuel cell operating in a living organisms was the work of Mano and Heller implanting their biofuel cell in a grape in 2003 (Mano et al. 2003). In 2010, Cosnier et al. implanted the first glucose biofuel cell in a rat (Cinquin et al. 2010). In 2012, Rasmussen et al. implanted a trehalose biofuel cell in a cockroach (Rasmussen et al. 2012) and Katz et al. implanted a glucose biofuel cell in a snail (Halámková et al. 2012) and a clam (Szczupak et al. 2012). In 2013, Katz et al. scaled up to implanting their glucose biofuel cell in a lobster and Cosnier's group made further developments in rat implantation (Cosnier et al. 2014), as shown in Figure 9. Although not all researchers are considering invasive implantation, some researchers have studied the ability to fabricate an

enzymatic biofuel cell on a contact lens (Falk et al. 2012; Falk et al. 2013; Reid et al. 2015) or patches (Miyake et al. 2011; Ogawa et al. 2015).

Other researchers have been more focused on this use of enzymatic biofuel cells for powering portable devices (Gellett et al. 2010a). This research has led to a variety of different prototype designs, including biobatteries (Gomez et al. 2010; Stolarczyk et al. 2014; Stolarczyk et al. 2012b), microfluidic prototypes (Bedekar et al. 2008; Gonzalez-Guerrero et al. 2013; Lee and Kjeang 2010; Lim and Palmore 2007; Reid et al. 2013; Togo et al. 2007; Togo et al. 2008; Zebda et al. 2010; Zebda et al. 2009a; Zebda et al. 2009b), and paper-based biofuel cells (Narváez Villarrubia et al. 2014; Shitanda et al. 2013; Zhang et al. 2012). Most of these designs were focused on minimizing the internal resistance of the fuel cell compared to the traditional H-cell systems of early years. Decreasing the internal resistance allows for higher current density and power density. However, probably one of the most exciting advances in prototyping has been recent work by the Cosnier group on hybrid supercapacitor/biofuel cell technology for future energy storage and conversion (Agnes et al. 2014).

Challenges

Significant progress has been made in the field of biofuel cells in the last 30 years, but several issues still need to be addressed for the commercial viability of enzymatic biofuel cells for the applications discussed above. Currently, enzymatic biofuel cells are still plagued with lower than desirable stability and electrochemical performance. Performance is a vague term that can mean lower current density, power density, volumetric catalytic activity, efficiency, energy density, or power density. The next decade will see considerable effort in improving stability through the combination of biotechnology strategies (extremophilic enzymes and enzyme engineering) and materials engineering strategies to improve the chemical microenvironment for enzymes at

electrode surfaces. However, the challenges for performance improvements will be interrelated, because frequently materials strategies for enzyme stabilization result in lower catalytic activity and therefore lower electrochemical performance.

Improving electrochemical performance will occur in a variety of research areas. Open circuit potentials will be improved by utilizing more direct electron transfer systems, as shown in the entirely DET-based fructose/oxygen biofuel cell in Figure 10, and designing new mediator-based systems with potentials closer to the enzyme/cofactor redox potentials. Current density and power density will be improved by incorporating strategies for faster electron transfer between enzymes and electrodes, as well as improving the catalytic activity of the enzymes and the ability to load high quantities of enzymes on electrode surfaces. High loading can occur via the use of high surface area electrode materials, but can also occur through enzyme engineering of smaller protein structures, so the volumetric catalytic activity of the enzyme is higher. Finally, efficiency and energy density can be improved through a variety of strategies. Synthetic biology has taught us that minimal enzyme cascades can be designed for reaction pathways, but these minimal enzyme cascades will need scaffolds for improving the proximity between sequential enzymes and ensuring substrate channeling between catalysts. These scaffolds will need to address electron transfer pathways to the electrode surface as well.

One of the major hurdles of enzymatic biofuel cells are the oxygen cathodes. The concentration of oxygen is low in buffer or biological fluids. This is further plagued by the fact that oxidase enzymes at the anode produce peroxide that can inhibit oxygen cathodes (Milton et al. 2013, 2014) and therefore decrease performance even more. There have been successful strategies for increasing the oxygen concentration in aqueous environments (Karaskiewicz et al. 2013), but air-breathing biocathodes would resolve these issues. Air-breathing cathodes are used in traditional

fuel cells and there has been a wealth of literature on designing and improving the three-phase boundary for metal nanoparticle-catalyzed oxygen reduction reaction (ORR) in traditional fuel cells, but little is known about how to design three-phase boundaries for laccase or bilirubin oxidase-based cathodes. This will need to be studied in the coming decade in order to realize the commercial applications of implantable biofuel cells and portable power biobatteries and biofuel cells.

Conclusions

Although enzymatic fuel cells predate the first issue of Biosensors & Bioelectronics, the field was very young and focused toward only mediated electron transfer. The last three decades have seen major advancements in the field to address low open circuit potentials, low current and power densities, low fuel efficiency, and low stability. These advances have resulted in open circuit potentials increasing from 175 mV to almost 1 V and current densities increasing from nA/cm² to mA/cm², as shown in Table 1. These increases come from minimizing mediator and overpotential losses for mediated electron transfer or moving to direct electron transfer for enzymes capable of direct electron transfer. It has also resulted from increasing electrochemical assessable surface areas with nanomaterials, increasing degree of oxidation of anodes with enzyme cascades and metabolons, and better cell design to minimize internal resistance. The advances have been dramatic in the last decade and have led the way for implantable applications and bio-batteries with performances that can compete with traditional batteries in the portable power realm. However, further research into improving stability and performance are still needed to improve open circuit potentials, operational lifetimes, and the ability to operate in air-breathing mode.

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345 Table 1: Comparison of Enzymatic Biofuel Cells with High Performance

Anode	Cathode	OCV (V)	Current density ($\mu\text{A}/\text{cm}^2$)	Power density ($\mu\text{W}/\text{cm}^2$)	Reference
Methanol MET	Pt cathode	0.8	2600	680	(Palmore et al. 1998)
Methanol MET	Pt cathode	0.65	26400	-	(Kim et al. 2013)
Ethanol MET	Pt cathode	0.62-0.82	3-5.4	1160-2040	(Akers et al. 2005)
Glucose MET	Laccase MET	1.0	-	350	(Soukharev et al. 2004)
Fructose DET	Laccase DET	0.79	2800	850	(Kamitaka et al. 2007b)
Glucose MET	Laccase MET	0.8	11000	1450	(Sakai et al. 2009)
Glycerol DET	Pt cathode	0.73	4200	1210	(Arechederra et al. 2007)
Maltodextrin MET	Pt cathode	0.6	6000	800	(Zhu et al. 2014)
Glucose DET	Laccase DET	0.95	-	1300	(Zebda et al. 2011)
Glucose MET	Laccase DET	0.76	4470	1540	(Reuillard et al. 2013)
Fructose DET	Bilirubin oxidase DET	0.7	4900	870	(Murata et al. 2009)

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Figure 1: Schematic of an enzymatic biofuel cell utilizing a mediated bioanode and a direct electron transfer-based biocathode.

Figure 2: Schematic of the first membraneless and compartmentless enzymatic biofuel cell.

Reproduced with permission from Elsevier (Katz et al. 1999).

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359 Figure 3: Schematic of a redox polymer-based bioelectrode. Blue text are properties that affect
360 potential, red text are properties that affect current density, and purple text are properties that
361 affect both.

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Figure 5: Schematic of the artificial metabolic pathway for oxidation of glycerol in a biofuel cell.
 Reproduced with permission from Wiley(Arechederra and Minteer 2009b).

Figure 6: Substrate channeling in an in-vivo Krebs cycle metabolon where mMDH is malate dehydrogenase, CS is citrate synthase, and ACON is aconitase (Wu and Minter 2015). Reproduced with permission from Wiley.

Figure 7: **a**, Catalytic cycle of hydrogenase and deactivation pathways via O_2 inhibition and high potential (dotted lines). Ni-C, Ni-SI_a and Ni-R (in blue) are the reduced catalytically active states, and Ni-A (in red) and Ni-B (in green) are the oxidized inactive states. In the absence of O_2 , high-potential inactivation proceeds mostly to the Ni-B (ready) state, from which reactivation is fast once the potential is lowered. If the enzyme is exposed to O_2 at high potentials, the Ni-A (unready) state is formed. Reactivation of Ni-A is a very slow process. Exposure to O_2 at more reducing conditions will lead mainly to the Ni-B state. At lower potentials, an O_2 -sensitive hydrogenase can sustain H_2 production in the presence of small amounts of O_2 . **b**, Proposed scheme for double protection of hydrogenases by the redox hydrogel. Open circles represent active hydrogenase and filled circles represent inactive hydrogenase. Assumed steady-state concentration profiles of reduced viologen (blue), H_2 (green) and O_2 (red) are shown. **c**, Chemical structure of the viologen-modified polymer. Reproduced from Reference (Plumeré et al. 2014) with permission from Nature Chemistry.

Figure 8: Schematic of an air-breathing cathode. Reproduced with permission from Elsevier (Zloczewska and Jönsson-Niedziolka 2013).

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407 Figure 9: Most advanced implanted biofuel cell in a rat. (Left insert) Micro-connectors are
408 inserted in the rat skull (right insert) the sealed biofuel cell after autopsy of the rat(Cosnier et al.
409 2014). Reproduced with permission from Elsevier.

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Figure 10: (A) Polarization curves of the fructose/oxygen biofuel cell utilizing DET of fructose dehydrogenase at the anode and DET of bilirubin oxidase at the cathode. The cell voltage ($\diamond$), the cathode potential ($\blacksquare$) and the anode potential ($\blacktriangle$) are plotted as functions of the current density (j). The measurements were performed in 1 M citrate buffer (pH 5.0) containing 500 mM D-fructose under quiescent and air atmospheric conditions (in a passive mode) at room temperature (23 ± 2 °C). (B) The power density (P) as a function of the current density (j) of the biofuel cell. Reproduced from Ref.(So et al. 2014) with permission from the PCCP Owner Societies.

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