INTRODUCTION AND CHARACTERIZATION OF AN INNOVATIVE BIOFUEL CELL PLATFORM WITH IMPROVED STABILITY THROUGH NOVEL ENZYME IMMOBILIZATION TECHNIQUES

By

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The members of the Committee appointed to examine the thesis of MICHAEL BRYANT FISCHBACK find it satisfactory and recommend that it be accepted.

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Abstract

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Enzyme-based biofuel cells (BFCs) are a promising technology as a small-scale power source, but their practical uses are hampered by their short lifetime and poor power density. In this work, we have developed a miniature BFC consisting of an air-breathing cathode and an enzymatic anode. The miniaturization of BFC was done by adopting the design of stackable proton exchange membrane (PEM) fuel cells, and the smallest dimension of BFC was 12 x 12 x 9 mm. The enzymatic anodes were constituted with stabilized glucose oxidase (GOx) in a form of crosslinked enzyme clusters (CEC) on the surface of carbon nanotubes (CNTs). When these enzyme clusters on CNTs were applied to carbon supports, a high surface area multilayered complex with internal pore structures was formed within the enzymatic anode. We have compared these enzymatic CEC anodes to anode electrodes fabricated by more conventional methods, such as enzyme coating (EC) and covalent attachment (CA), and demonstrated that our novel CEC electrodes far outperform these electrodes both based upon power density output

and stability. We have also demonstrated that the buffer solution plays an important role in determining the performance and stability of BFCs. It was found that when the cell was buffered, the initial performance was very high but its performance quickly dropped due solely to a deactivation of the PEM. On the other hand, when the cell was unbuffered, the initial performance was lower than that of the buffered runs due to the low pH condition but its performance was very stable for a very long operation time. For example, under continuous operation, a potentiostatic measurement of the BFC in an unbuffered solution showed no significant current density drop for more than 16 hours. This unprecedentedly high operational stability of the BFC using the enzyme-CNT hybrid materials opens up a new potential for many BFC applications. Finally, we have explored the effect of glucose and mediator concentrations in the feed solution of the BFC and have shown that maximum power output occurs at concentrations around 100 mM and 10mM, respectively.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Overview

A biofuel cell (BFC) is a device which uses a biological mechanism to catalyze the anode and/or cathode reactions of a fuel cell from which power can be derived [1]. Due to the fact that biological systems are catalyzing the reaction(s), the fuel used in BFCs is most commonly an organic fuel, such as glucose or ethanol. Two major classes of BFCs exist, cells which use whole organisms and cells which use purified enzymes to convert a specific fuel to a given product. For the purposes of this paper, only enzymatic BFCs will be discussed. BFCs can derive their power in either of two ways: 1) As the enzymes catalyze a reaction electrons are captured and transferred to an external circuit, as is most often the case; or 2) BFCs can derive their power by oxidizing the products created through an enzymatic reaction [2]. In the second case, the electrons flowing through the external circuit do not actually come from the enzymatic reaction, but rather from the oxidation of the product created by the enzyme on a separate inorganic (Pd, Pt, Ru, etc.) catalyst.

For those BFCs which derive their power directly through the enzymatic reaction (case #1), there are two possible mechanisms by which the electrons (electricity) can be transferred from the enzymes active site to the electrode surface and thus through external circuitry. These mechanisms are mediated electron transfer (MET) and direct electron transfer (DET). Mediated electron transfer mechanisms involve either diffusive

mediators or stationary conductive polymer matrixes to transfer electrons, while DET is accomplished by placing a conducting material, such as gold nanoparticles or carbon nanotubes (CNTs), within electron tunneling distance from the enzymes' active sites, with a critical distance of around 20 Å of separation maximum [3]. As many enzymes active sites are buried deep within the proteins shell, DET can only be achieved with a handful of enzymes such as cytochrome *c*, peroxidase, ferredoxin, plastocyanin, azurin, and azotoflavin whose active sites are on the enzyme's periphery [4-6].

Biofuel cell systems have many advantages over traditional fuel cells. BFCs can be operated at room temperature and at various, including neutral, pHs depending upon the nature of the enzymes used. By comparison, the majority of traditional fuel cells, which rely on precious metal catalysts, need to be operated at elevated temperatures and often at acidic pHs [7]. Biofuel cells also offer a cost advantage over fuel cells which require precious metal catalysts. Furthermore, BFCs are able to oxidize organic fuels which generally have an activation energy barrier too great to be overcome by the use of metal catalysts [8]. However, the largest advantage of BFCs is that the enzymes which are used to catalyze the reaction(s) are extremely substrate specific allowing only the desired fuels to participate in the redox reactions. This also allows for the possibility of eliminating the membrane of the BFC, allowing for very simple BFC designs. This is because the only function of the membrane is to contain the fuel solution so that it does not react at the inappropriate electrode. The expected differences between conventional fuel cells (hydrogen, methanol, etc.) based on noble metal catalysts and BFCs are illustrated in Table 1.1.

Table 1.1. Differences between noble metal PEM-type fuel cells and biofuel cells [9,10].

Fuel Cell	pH	Temp.	Minimum Volume	Maximum Current Density	Lifetime	Cost
Biofuel Cell	Variable	25 – 37 ^{o}C	0.01 mm^3	< 10 mA cm^{-2}	Weeks	Low
General Inorganic PEM Fuel Cell	Acidic	Higher is Better	> 1 cm^3	> 100 mA cm^{-2}	Years	High

Conversely, however, BFCs do poses some intrinsic problems which need to be overcome before their use can become of value. The rate of energy generation within a BFC system is limited by the mass transport rates of fuel and mediators to and from a limited quantity of enzymes. However, whether the bottleneck is poor mass transport or low enzyme densities, rate of energy generation will still be nearly proportional to the number of enzymes participating in redox reactions within the BFC system. The enzyme density within BFC systems is low because researchers are generally only able to coat their enzyme supports with a monolayer of enzyme. This problem combined with thermodynamic potential losses due to the use of mediators results in low power outputs from BFCs, usually less than 10 mW ml^{-1} [9]. Furthermore, BFC systems are renowned for their short working lifetimes due to enzyme denaturation or deactivation [11].

These shortcomings greatly limit the applications for BCFs in the near future. If stability under physiological conditions can be increased to months or years, even with no further improvements made to power output, BFC systems can be used to power devices such as sensors or sensor/transmitter combinations. The powering of sensors is an important potential function of BFCs. For example, it has been purposed that BFC systems could be implanted into humans, being fueled solely off of the body's own glucose, to power glucose sensors to provide diabetics with blood glucose concentration

information [10]. Yet, if engineering problems related to enzyme kinetics and electron transfer can be resolved, the opportunities for BFCs will become seemingly endless. BFCs could then be used to power artificial human hearts, bladder control valves, cardiac pacemakers, or to efficiently retrieve energy from biological wastes.

1.2 Literature Review

The use of purified biological enzymes to produce electrical energy through the catalyzation of a chemical reaction is an idea which has recently received increased attention due to the perceived need for small-scale *in vivo* power generation [2]. However, the broad visions for devices which can carry out this action vary as widely as from powering small diabetic glucose sensors within the human body to powering mobile electronic devices to powering an artificial human heart [9, 10, 12-15]. Due to this vast array of possible, albeit not all probable, applications, devices reported vary greatly in their size, structure, and mechanisms. Yet, the formulation of any BFC system has the same goals: to maximize the power output from the device and to maximize the lifetime and stability of the device. The objective of this review is to examine the strides which have been made in addressing power density, lifetime, and stability issues.

The earliest enzymatic BFC was reported by Yahiro et al. in 1964. He and others began to investigate BFCs in an attempt to produce energy at a rate great enough to power a permanently implantable human heart [15-17]. However, it was quickly realized that the power output from these devices was insufficient to power such a large device and that the operational lifetime was inadequate [10]. Thus, very little BFC research appears in the literature until much later. During the 1980's, considerable attention was given to studying enzymes' expressions of bioelectrocatalytic behavior [9]. Research was performed investigating enzyme structures and enzymatic redox mechanisms, culminating in the observation of DET mechanisms and the proposal of electroanalytical applications for bioelectrocatalysts [18-21]. However, BFCs did not receive renewed

interest until the late 1990's. Renewed interest in BFCs arose from new knowledge of enzyme structure and mechanisms and from the advent of enzymatic glucose sensors for diabetics. Enzymatic sensors opened new possibilities for BFCs as they require far less power to operate than other potential implantable devices. For example, a cardiac pacemaker requires only 1 μW to operate compared to power on the order of 10 W for an artificial heart [9, 22].

Since the late 1990's, biofuel cell research has followed closely with biosensor research as many of the technological requirements are related. Henceforth, many papers have been published which have reviewed BFCs and enzyme-based sensors side-by-side [23, 24]. However, one major difference between these two classes of devices exists; BFCs must be capable of generating power while sensors generally consume power. This means that while both systems use enzymes to generate an electrical signal, BFC research seeks to maximize the potential and current output from the device. This means that BFC design aspects which limit power output densities, such as ohmic resistances, slow kinetics, and poor mass transport, must be minimized. On the other hand, sensor research seeks to create devices where the electrical signal is proportional to the concentration of the species of interest, regardless of the current magnitude [9].

Owing to the similarities between enzymatic sensors and BFCs, many BFC designs have been adopted from materials and methods first developed in the sensing field. For example, BFC researchers have tried entrapping enzymes in silica gels, as adopted from the sensing field. The advantage is that the gels keep the enzymes from translocating and the gels have been shown to increase the stability of some enzymes [25, 26]. However, when this entrapment method is applied to BFCs, the transport of

reactants to the enzymes is hampered as well as the enzyme's activities, thus reducing BFCs' performances. This is not, however, a problem in sensing applications because the current generated by the enzymes will generally still vary linearly with the concentration of the reactant, regardless of mass transport limitations or decreased activity.

Still others have tried entrapping enzymes in electron conducting polymers, as also first introduced in the sensing field. However, conductive polymers have the drawback of being susceptible to chemical attack and of poor mechanical stability [9]. Conductive polymers also have the disadvantage of high reactant and product diffusional resistances [27]. The poor lifetime of conductive polymers caused by susceptibility to chemical attack and poor mechanical stability is not a problem for sensors because many sensors are disposable. Furthermore, the poor diffusional resistances within conductive polymers are also not a problem in the design of sensors, so long as the magnitude of the electrical signal is proportional to reactant concentration. However, in the design of BFCs these negative aspects are of significance as they lower the power outputs and working lifetime of the devices.

To illustrate this point, of the BFCs reported which utilize conductive polymers, the best performance reported was a cell which had a daily power decrease of only 5% under physiological conditions, indicating that the conductive polymer support served to stabilize the enzymes. However, when the cell was operated within a grape, the power output fell by 50% over 20 hours due to loss of electron conduction within the redox hydrogel because the polymer had suffered chemical attack. [10]

In addition to silica gels and conductive polymers, other enzyme immobilization methods have been developed. In an attempt to minimize the mass transfer issues seen

when silica gels or conductive polymers are used, researchers have immobilized enzymes on conductive supports, such as gold wires, gold nanoparticles or CNTs. Carbon nanotubes are a commonly employed material due to their high mechanical and chemical stability and their low electronic resistances [28]. Typical attachment methods include covalent attachment, reconstituted enzymes, and tethered cofactors [13, 24, 29-34]. However, these immobilization schemes generally only create a monolayer of enzyme on the surface of the supports. The poor enzyme coating densities then, in turn, result in poor power outputs per volume. Furthermore, the enzymes immobilized by any of these methods are fully exposed to the local environment and have no shield serving to protect them from denaturation, in contrast to enzymes entrapped in silica gels or conductive polymers.

To give an example, the best performing glucose-based BFC which does not incorporate silica gels or conductive polymers produces only 32 $\mu W\ cm^{-2}$ while its power output decreases by 50% over ca. 3 hours [29]. Willner et al. developed this cell by coating a gold wire electrode with a monolayer of reconstituted glucose oxidase (GOx) to form the anode. The poor power output was attributed to the GOx anode and degradation of the biocatalyst was cited as a cause of the poor stability. Thus, future research is needed to improve the power output and stability of BFCs in which enzymes are immobilized on conductive supporting materials, yet which do not require the use of silica or hydro gels. This is the topic of the work presented herein.

However, aside from methods which seek to increase the power output and stability of BFCs by altering the enzyme immobilization methods or materials, an alternative approach is to incorporate genetically altered or thermophilic enzymes. It is

theorized that by genetically altering the characteristics of enzymes it may be possible to decrease their tendency towards deactivation or denaturation, increasing the stability of BFC devices. This would be accomplished by engineering enzymes which are either more thermally stable, that is, capable of operating under a higher temperature range, or by creating enzymes which are more tolerant of a wider range of operating environments, such as high or low pH environments or high salinity environments. Basically, enzymes which are tolerant over a wide range of operating conditions will show an increased activity compared to less tolerant enzymes under optimum conditions. Furthermore, it may also be possible to remove some of the massive protein backbone while still leaving the enzyme's active center in tact, allowing more efficient transport of products and reactants and perhaps allowing conductive materials to reach electron tunneling distance from active centers. However, very little interest has been paid to employing genetically altered or thermophilic enzymes into BFC systems [35].

1.3 Problem Statement

The objective of the work presented herein was to compare the activity and stability of conventional enzyme immobilization techniques with our novel "crosslinked enzyme cluster" immobilization method in an attempt to improve biofuel cell operation. We sought to contribute the BFC community's work by innovating new enzyme immobilization techniques which allow for increased power output density and an increased working lifetime of biofuel cell systems. It was also our mission to study the effects of glucose and mediator concentrations on cell performance as well as to see what effect, if any, the nature of the buffer used and the flow rate of fuel through the cell had on performance. However, in order to reach these goals, we needed to also create and employ a biofuel cell platform which was scaleable, yet well defined and simple, as to allow others to reproduce our system, and to allow for easy manipulation of process variables.

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CHAPTER TWO

MINIATURE BIOFUEL CELLS WITH IMPROVED STABILITY UNDER CONTINUOUS OPERATION

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2.2 Abstract

We have developed miniature biofuel cells (BFCs) with dimensions as small as 12x12x9 mm by adopting the design of stackable proton exchange membrane (PEM) fuel cells. The enzymatic anodes were constructed by using stabilized glucose oxidase (GOx) in the form of crosslinked enzyme clusters (CECs) on the surface of carbon nanotubes (CNTs). The combination of stabilized GOx and unbuffered fuel solution resulted in stabilized performance of miniature BFCs under continuous operation for more than 16 hours. This unprecedentedly high operational stability of miniature BFCs opens up new possibilities for many BFC applications.

2.3 Introduction

Enzyme-based biofuel cells (BFCs) generate electrical energy from biofuels such as glucose and ethanol, which are renewable and sustainable energy sources [1-5]. The advantages of enzymatic BFCs over fuel cells using precious metal catalysts are the substrate specificity and biocompatibility of enzymes, the ability to use a wide range of fuel substances, mild operating conditions, and the cost savings associated with using enzymes compared to expensive metal catalysts such as Pt, Pd or Ru [5]. The advantages of enzymatic BFCs over microorganism-based BFCs are that they do not require nutrients or biomass acclimation, the process can be controlled more easily, they generally produce higher power output densities, and they are easier to engineer for a miniaturized system. Since this paper is related to enzymatic BFCs, we will use the term of BFCs to represent enzyme-based BFCs for further discussion.

Many potential applications of BFCs have been reported or proposed for implantable devices, remote sensing and communication devices as a sustainable and renewable power source [2, 3, 5-7]. The amount of attention given to BFCs is growing very rapidly due to the recent advances, which make the practical applications of BFCs ever more promising. Especially, a lot of effort has been dedicated to the development of membrane-less and microchip-based BFCs in order to miniaturize their total size by orders of magnitude [7, 8]. However, there are no BFC design formats or templates that allow for the production of a working device with a size on the order of 1 cm^3, which are needed for several real applications. Herein, we report the development of a miniature

BFC with a footprint of 1.4 cm^2, by adopting the design of stackable proton exchange membrane (PEM) fuel cells.

For the practical application of miniature BFCs, two key issues need to be addressed: (1) How the power densities can be maximized and what the important parameters affecting the BFC performance are, and (2) How enzymes can be stabilized so that high cell performance can be maintained for a reasonably long operating time. To address these two key issues, various enzyme immobilization methods have been attempted for constructing BFCs, such as adsorption, entrapment, and covalent attachment. Recent advances in bionanotechnology are promising to improve the performance and stability of immobilized enzymes beyond the scope of these traditional approaches [4]. The large surface area provided by nanomaterials for the attachment of enzymes will increase the enzyme loading and possibly improve the power density of BFCs. Additionally, various nanostructured materials have shown great potential for stabilizing enzyme activity, which can be further employed in improving the lifetime of BFCs [4]. For example, the synergetic ship-in-a-bottle approach of crosslinked enzyme aggregates in mesoporous media with bottle-neck structures has been proven to be effective in achieving both intrinsic and operational enzyme stabilities [9-11].

Recently, we have demonstrated unprecedented stability of glucose oxidase's (GOx's) activity by attaching crosslinked enzyme clusters (CECs) onto the surface of carbon nanotubes (CNTs). The enzyme stabilization by CEC approach was so effective that CEC-GOx showed no activity decrease for 250 days. In addition, the activity of CEC-GOx was 51 times higher than that of covalently-attached GOx due to the improved GOx loading in the form of CECs [12]. In this paper, we report the use of CEC-GOx in a

miniature BFC device under continuous operating conditions. We have demonstrated that the stability of miniature BFCs can be greatly improved if an unbuffered solution is used and that the poor selection of buffer salts can have devastating ramifications. This allows a vantage point from which cell behavior in real applications can be further understood, as there is no guarantee to the availability of buffer in real applications.

2.4 Experimental

Reagents and Materials

Glucose oxidase (GOx) from *Aspergillus niger* (E.C. 1.1.3.4), D-(+)-Glucose, glutaraldehyde (GA), benzoquinone (BQ), ammonium sulfate, *o*-dianisidine and horseradish peroxidase (HRP) were purchased from Sigma (St Louis, MO, USA). Carbon nanotubes (CNTs, multi-walled, 30 ± 15 nm in outer diameter and 1~5 μm in length, purity > 95%) were supplied from Nanolab Inc. (Newton, MA, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC) was purchased from Pierce (Rockford, Il, USA) while N-Hydroxysuccinimide (NHS), platinum black and gold mesh were bought from Alfa Aesar (Ward Hill, MA, USA). Nafion® 115 membrane and 5% Nafion® solution (1100 EW) were obtained from Ion Power, Inc. (Castle, DE, USA) and Solution Technology Inc. (Mendenhall, PA, USA), respectively. Carbon felt from Alfa Aesar (Ward Hill, MA, USA) was used for the electrode supporting material. All other reagents were purchased from Aldrich Chemical (Milwaukee, WI) and were of the highest grade commercially available.

Enzyme Anode Preparation

GOx was immobilized on CNTs in the form of crosslinked enzyme clusters (CECs) (Figure 2.1A) [12]. The synthesis procedure is as follows. CNTs were treated with acids to functionalize their surface with carboxyl groups, and then further treated with N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for the functionalization of amine-reactive esters on CNT surface. Covalently-attached GOx

(CA-GOx) was prepared by adding GOx to the functionalized CNT suspension and incubating the mixture in a refrigerator overnight. To prepare CEC-GOx samples, ammonium sulfate was added to the mixture of CA-GOx samples and excess GOx, which precipitates GOx clusters in the vicinity of the CNTs. GA was then added to crosslink the enzyme cluster precipitates, which were entangled around CNTs leading to the formation of CEC-GOx on the surface of CNTs.

The enzyme anodes were prepared by casting CEC-GOx onto disk-shaped carbon felt electrodes, which had an area of 0.332 cm^2 (Figures 2.1). First, the CEC-GOx on CNTs was dispersed in a buffered Nafion® solution. Then, the carbon felt electrode was submerged in the solution for 10 minutes and the CNT-coated supports were dried under ambient conditions overnight. All enzyme anodes were stored in an aqueous buffer solution (100 mM sodium phosphate, pH 7.0) at 4 ^oC. SEM images of enzyme anodes were taken using a Zeiss Supra 35 VP FESEM.

Membrane/Cathode Electrode Preparation

All membrane cathode electrode assemblies (MCEA) were prepared in house by painting a Nafion® / Pt slurry directly onto commercially-available Nafion® membranes. A mixture of 5% Nafion® solution, Nanopure water and Pt black was quickly (< 20 sec) ultrasonicated in order to make a good suspension of Pt. The Pt suspension was then painted onto the Nafion® 115 membrane; the Pt loading was estimated to be 7-8 mg cm^{-2}. More detailed procedures for the MCEA preparation have been reported previously [13].

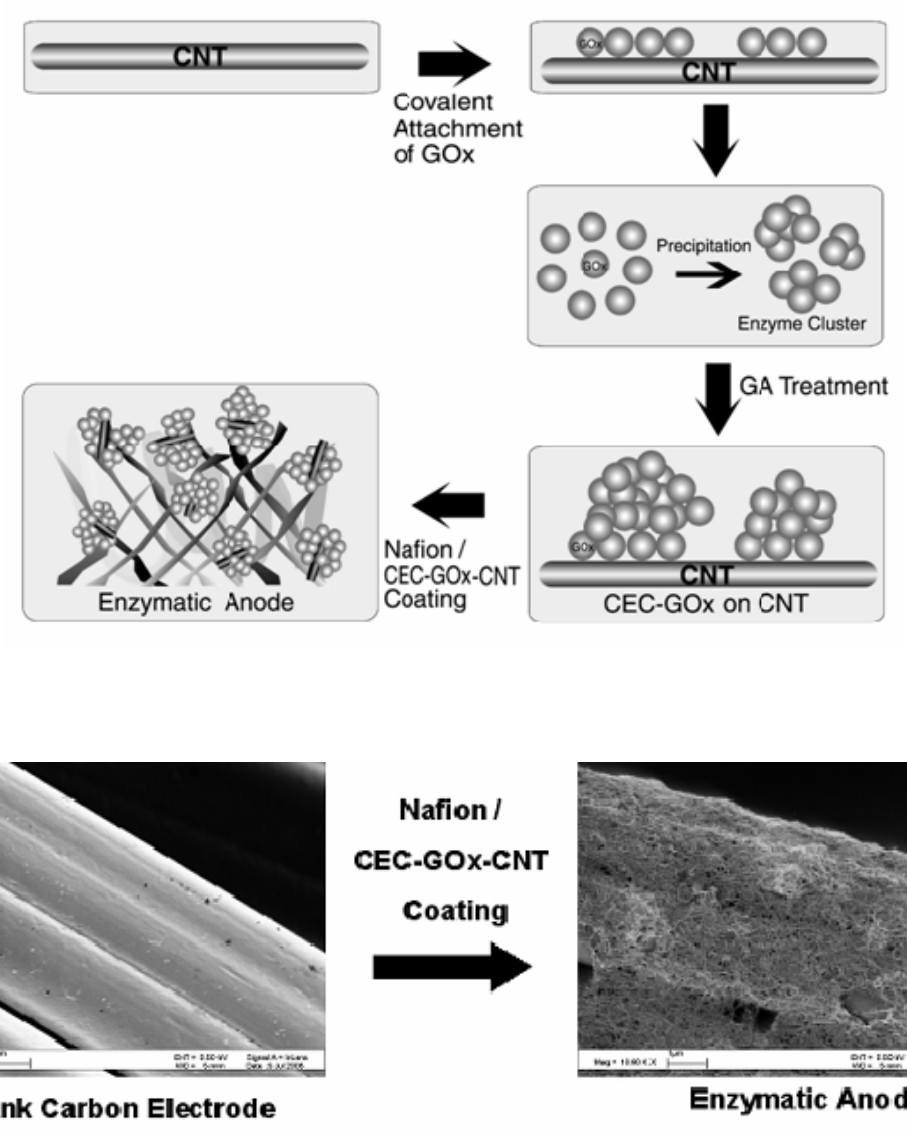

Figure 2.1. A) Schematic of the preparation procedure for CEC-GOx and enzymatic anode. B) SEM images of blank carbon electrode (left) and enzymatic anode (right).

Biofuel Cell Assembly and Operation

A PEM-type cell design was employed in this research, which consists of an air-breathing Pt cathode and a carbon supported GOx anode. A schematic diagram of the miniature BFC is shown in Figure 2.2A. The cell's body, i.e., anode fuel reservoir and cathode clamp, was constructed with Teflon and the anode and cathode current collectors

were made of titanium. A piece of 1 cm^2 gold mesh was placed between the anode electrode and the anode current collector to ensure firm contact and improve electron transfer. All junctions were sealed with silicone gaskets, and the cell was held together with four nylon screws to prevent short-circuiting.

The BFC was operated at room temperature and was fed with a fuel solution consisting of 200 mM glucose, 10 mM BQ and sometimes buffer salts, at a flow rate of 68 µl min^{-1}. A 200 ml volume of this fuel solution was recycled through the cell during operation. All solutions were made with Nanopure water (> 18 MΩ-cm), and glucose stock solutions were prepared at least one day in advance to allow for equilibration of anomers.

Electrochemical and Activity Measurements

All electrochemical measurements were performed using a Solartron® 1287a potentiostat (Farnborough, Hampshire, UK). Potentiostatic analysis was performed by measuring the current response as a function of time at fixed BFC potential. Activity measurements were performed using a UV-Vis spectrophotometer (Shimadzu, Japan), with a protocol as follow [14]. One milliliter working reagent containing 0.17 mM o-dianisidine and 1.72 wt% D-(+)-glucose was added in a disposable cuvette. Ten microliters of 0.2 mg ml^{-1} HRP and 50 µl of GOx were mixed with the working solution in the cuvette right before measurement was initiated. The increase in absorbance at 500 nm was monitored and the initial GOx activity was calculated from the absorbance increase per unit time.

2.5 Results and Discussion

CEC-GOx was prepared by first precipitating GOx molecules to form enzyme clusters in the vicinity of CA-GOx, followed by GA treatment to crosslink these clusters and attach the crosslinked enzyme clusters to the CA-GOx on the CNT surface (Figure 2.1A). To fabricate the enzymatic anodes, these CEC-GOx complexes with CNTs were coated onto a carbon fiber matrix (blank carbon electrode) using Nafion® binder. The scanning electron microscopy (SEM) image of the blank carbon electrode shows the individual carbon fibers with no fibrous enzyme-CNT complexes. However, the SEM image of the enzymatic anode reveals a thick multilayer of the Nafion®/CEC-GOx-CNT matrix on the carbon fiber surface (Figure 2.1B), which creates complex internal pore structures adding a large amount of additional surface area. We anticipate that the increased surface area and creation of well-connected internal pore structures will result in the improvement of glucose transport and electron transfer from GOx to the carbon electrode.

Once the enzymatic anode was fabricated, it was placed into a miniature BFC that was developed by adopting the design of stackable PEM fuel cells (Figure 2.2A). The smallest version of the miniature BFC is shown in Figure 2.2B, which is roughly the size of a U.S. penny. A Nafion® membrane was used to conduct protons from the anode to the cathode, while a Pt catalyst layer was applied to the one side of membrane to form the cathode electrode. Our BFC design incorporates an air-breathing cathode structure that allows oxygen to be supplied from ambient air. The reaction scheme at each electrode is highlighted in Figure 2.2C, whereby the overall function of the cell is to convert glucose

and O_2 to gluconolactone and H_2O. The enzyme anode serves as the electron source, while the cathode electrode serves as the electron drain to sink electrons and form H_2O.

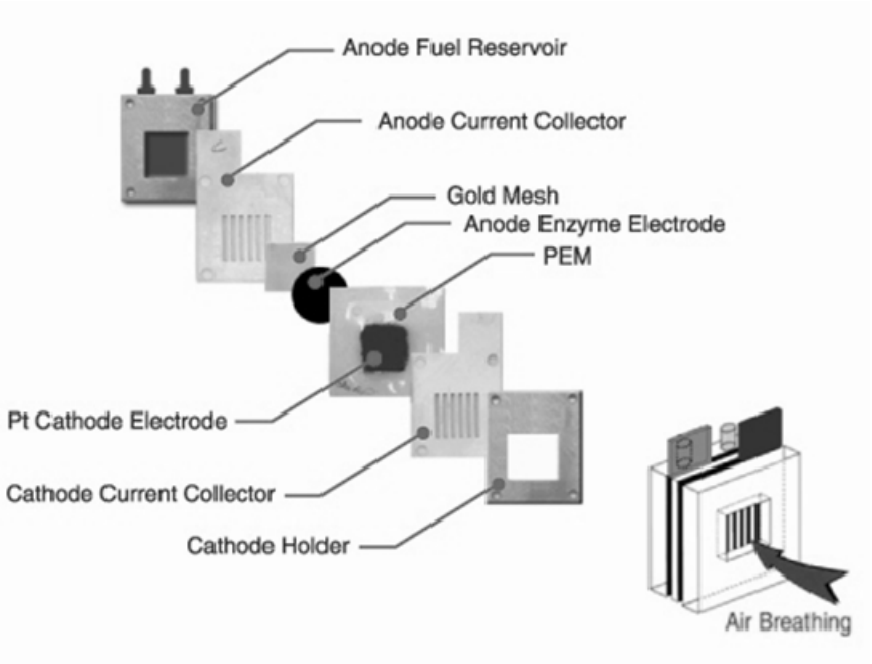

Figure 2.2. A) Assembly schematic of enzymatic miniature BFC consisting of an enzymatic anode and an air-breathing Pt cathode. B) Picture of a miniature BFC (1.3 cm^3 in total volume) on a U.S. penny.

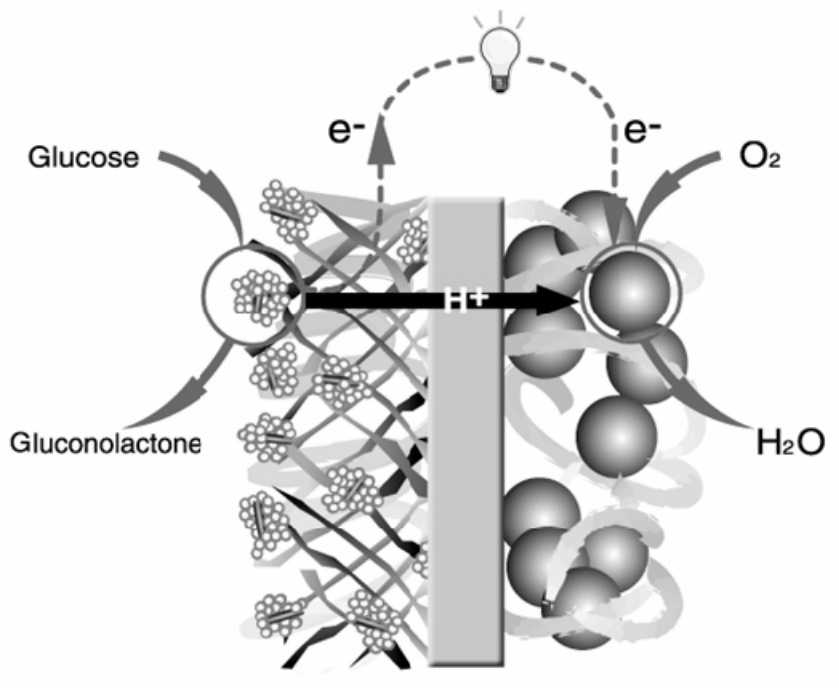

Figure 2.2. C) Reaction schemes at the anode and cathode of the BFC.

Figure 2.3A shows the effect of different buffers on the performance of the BFC by using two different buffer salts, sodium phosphate (Na-PBS) and ammonium phosphate (NH_4-PBS), together with an unbuffered run. The concentration of each buffer was fixed at 100 mM and the pH was adjusted to 7.0. In the initial stage of operation, the use of buffered solutions resulted in a much higher initial current and power densities than when the cell was operated with no buffer. However, the BFC runs performed with buffered solutions also showed a rapid performance drop within the initial 3 hours of operation, followed by a slow decay. On the other hand, the BFC operated with no buffer showed relatively stable performance. Table 2.1 summarizes the performance results of BFC with different buffers and no buffer after operating the cell for 0.2, 3 and 10 hours.

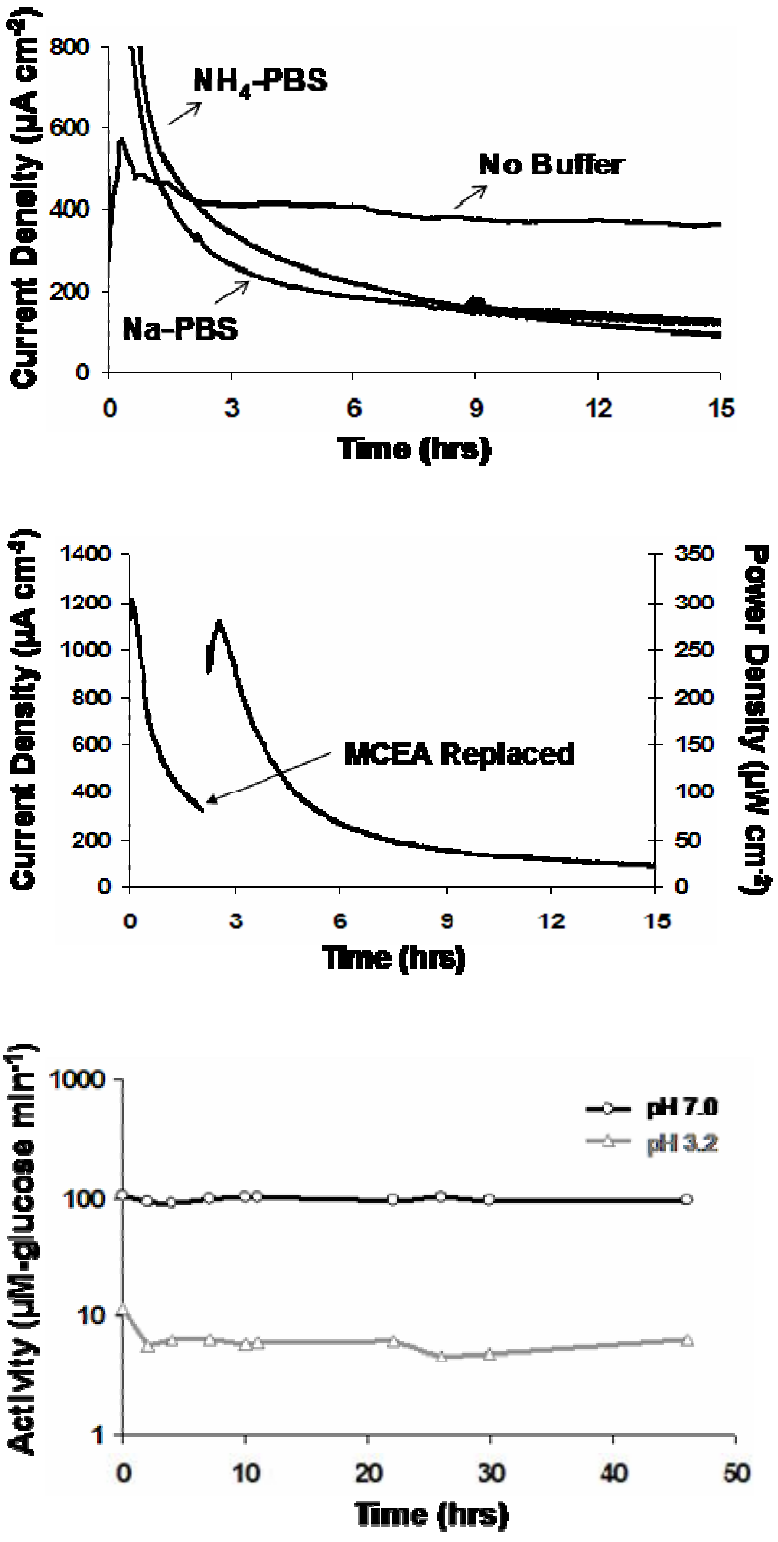

Figure 2.3. A) BFC performance operating at 0.25 V in different buffering conditions: i) no buffer, ii) 100 mM Na-PBS at pH 7.0, and iii) 100 mM NH_4-PBS at pH 7.0. B) Effect of MCEA replacement on BFC performance having operated for 2 hours. C) Activity of free GOx at pH 7.0 and 3.2.

Table 2.1. Effect of buffering conditions on the power densities of miniature BFC.

Buffer Salts	Power Density (mW cm^{-2})		
	0.2 hours	3 hours	10 hours
No Buffer	116.7	102.6	92.8
Sodium PBS	370.7	66.4	37.3
Ammonium PBS	329.7	85.7	35.4

Several possible causes can be proposed to explain the cell's performance drop with buffers: (1) degradation of the GOx enzymes, (2) fuel (substrate) depletion, (3) deactivation of the BQ, and (4) deactivation of the MCEA. To test which one of these possible causes is mostly responsible, either: the anode electrode was replaced with a new one, the glucose/BQ solution within the cell's reservoir was replenished with fresh solution, or the MCEA was replaced with a new one, all after running the BFC for 2 hours. It was found that none of these changes significantly influenced the cell performance (data not shown) except for replacement of the MCEA with a new one. Figure 2.3B shows a typical potentiostatic measurement of the BFC operated with 100 mM Na-PBS buffer at pH 7.0. The initial current and power density were 1200 mA cm^{-2} and 300 mW cm^{-2}, respectively. However, as the operation time increased, the BFC performance decreased rapidly. When the used MCEA was replaced with a new one after running the BFC for 2 hours, the cell was able to regain most of its initial performance, but it started to drop again as the operation time increased (Figure 2.3B).

MCEA was constructed from Nafion® membrane by applying the Pt electrode as described in Section 2.3. The MCEA would be degraded over the operation time if either the Nafion® membrane is deactivated and/or the Pt cathode electrode is poisoned.

Nafion® membrane is a cation exchange membrane, which serves to transport protons from the anode to the cathode. However, it has long been known that other cations also have an affinity for the membrane [15, 16]. For example, the Na^+ from the Na-PBS buffer can compete with protons for anionic sulfonic sites within the membrane pores, impeding the passage of protons from the anode to the cathode and decreasing the cell performance. Since different cations have different membrane affinities, the deactivation rate of the membrane should vary depending on the type of buffer solutions that one uses. However, the rate of performance drop is similar with either Na-PBS or NH_4-PBS buffer in Figure 2.3A, suggesting that Na^+ and NH_4^+ may have a similar affinity to the membrane. The unbuffered run showed the most stable BFC performance as no cations were present (Figure 2.3A).

The Pt cathode electrode can also be poisoned over the operation time if either the anions/cations of buffers, glucose, or BQ are diffused from the anode to the cathode via the membrane. However, the anions cannot diffuse across the membrane because Nafion® membrane only permeates cations or neutral species [17]. Thus, the crossover of buffers' anions cannot be the source of the Pt cathode poisoning. In addition, both the glucose and the BQ cannot be the sources of the Pt cathode poisoning because the BFC run with no buffer shows stable performance (Figure 2.3A). If they poison the Pt cathode, the performance of the unbuffered run would be deactivated as well over the operation time like that of the buffered runs. The crossover of buffers' cations is the only remaining source for the Pt cathode poisoning. However, these cations (Na^+ and NH_4^+) are commonly used in electrochemical experiments and there are no reports suggesting that they poison Pt electrodes. Thus, the decreasing performance of BFC in the presence

of buffer salts can be explained not by the poisoning of Pt cathode, but only by the impeded proton transfer through Nafion® membrane due to the existence of cations.

In Figure 2.3A, the unbuffered run demonstrated the highest stability and its performance was greater than all buffered runs' after 3 hours of operation. It is important to point out that many BFC reports do not address the effect of long term operation on cell performance. However, in order to make the BFC more viable for real applications, one must understand its true performance over extended operating times and under practical operating conditions. Thus, the high stability of the unbuffered run is of significance to real applications because, while buffers may be present in an ideal laboratory setting, it is unlikely that they will be present in actual operating environments. However, the unbuffered run exhibited a lower initial performance, and one of the main reasons may be the low pH of local environment around the GOx. For example, the pH of a fresh (unused) 200 mM glucose / 10 mM BQ solution without buffer was found to be 3.48. When this solution was recycled through the cell during operation, the pH of recycled solution without buffer was measured to be around ~3.2 due to the presence of slightly acidic gluconolactone which is a byproduct of glucose oxidation. Figure 2.3C shows the activity and stability of free GOx at pH 7.0 and 3.2. The activity of free GOx at pH 3.2 was initially only 11.2% of its activity at pH 7.0, explaining the low initial performance of the unbuffered run in Figure 2.3A.

To characterize the BFC system its voltage-current characteristics were surveyed in unbuffered solution. Before collecting any data points from the BFC, the cell was first operated at 0.1 V for more than 24 hours to damp out the majority of transient behavior. The current densities at various cell potentials were then collected at 10 minute intervals

using potentiostatic control, as this was judged to be sufficiently long to reach pseudo steady state operation for acquiring each data point. The open circuit potential (OCP) was 0.33 V while a maximum power output of 120 mW cm^{-2} occurred at a cell potential of 0.1 V (Figure 2.4). It is relevant to note that while the OCP of the unbuffered cell was 0.33 V, the initial OCP of all buffered experiments was ~ 0.55 V (data not shown). In addition, if a polarization curve was generated under buffered conditions and at the beginning of the run (at 0 hours instead of at > 24 hours), the maximum power output would have occurred at 0.25 – 0.3 V and the current output from the cell would have appeared *much* higher. However, as this would not have represented the steady state behavior of the BFC, the authors have not included this misleading performance data in this paper.

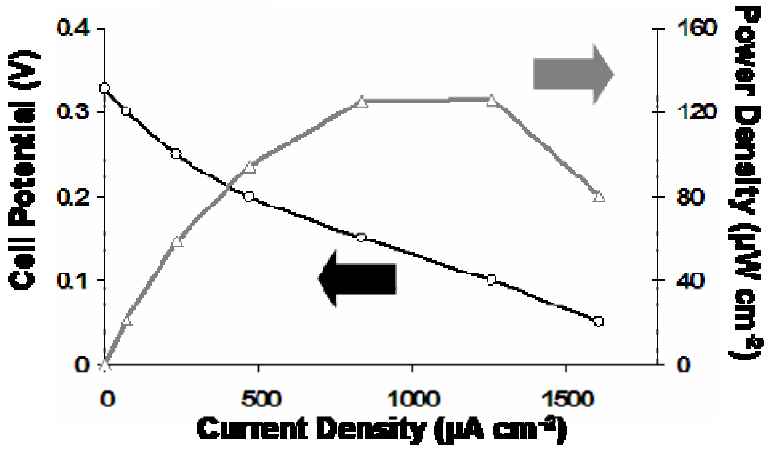

Figure 2.4. V-I and P-I curves indicating the behavior of the unbuffered working BFC.

While the activity and stability of CEC-GOx has been investigated using common laboratory assay techniques, as reported previously [12], its true long-term performance under continuous operation of a miniature BFC device had not been carried out

previously. In order to investigate the long-term performance of a miniature BFC with CEC-GOx, potentiostatic measurements were performed at 0.1 and 0.25 V (Figure 2.5). While some transient behavior was observed initially, the cell performance became stabilized after approximately 2 hours. Especially, even at a low cell potential of 0.1 V where a heavy load was applied to the BFC, the performance of our BFC was stable without showing any significant performance drop for more than 16 hours. Our BFC shows this great stability and robustness because the enzymes are immobilized in our BFC using our novel procedure of covalently linking enzyme clusters to CNTs.

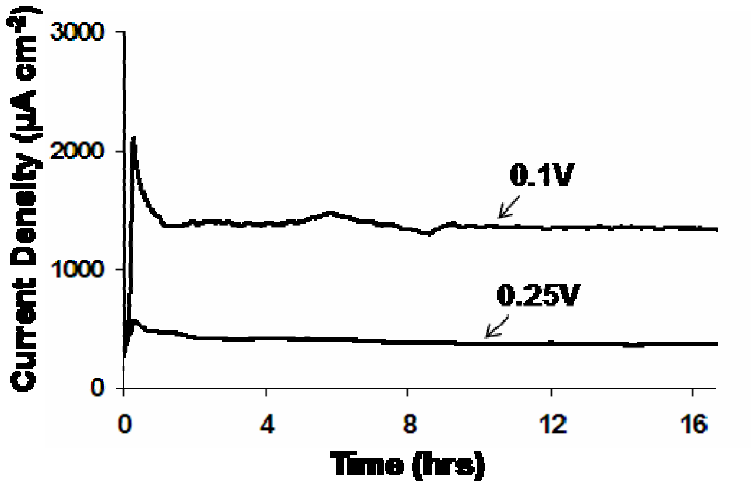

Figure 2.5. Stabilized performance of a miniature BFC at 0.1 and 0.25 V.

Finally, we investigated the effect of cell size on the performance of BFCs. While all results presented thus far were collected in a BFC with dimensions of 24x20x18 mm (8.6 cm^3) due to its ease of use, a few experiments were repeated in an even further miniaturized BFC with dimensions of 12x12x9 mm (1.3 cm^3). Interestingly, the use of this smaller version of a miniature BFC with the same size of enzymatic anode (0.332

cm^2) resulted in no appreciable differences in performance, suggesting that the quantity of immobilized GOx within the anode plays a key role in determining the cell performance. More importantly, the use of electrode with a larger surface area can improve the performance of BFCs due to the increased enzyme loading. This miniature BFC, therefore, provides an excellent format for the production of a working device of the size needed for several real applications and provides a template from which even smaller cells could be assembled.

2.6 Conclusions

We have developed a simple but readily-applicable design of miniature biofuel cells, which consists of an air-breathing cathode and an enzymatic anode with the hybrid materials of enzymes and CNTs. It has been demonstrated that the buffer solution plays an important role in determining the performance and stability of miniature BFCs. When the cell was buffered, the initial performance was very high, but its performance quickly dropped solely due to a deactivation of the proton exchange membrane. On the other hand, when the cell was unbuffered, the initial performance was lower than that of the buffered runs due to the low pH condition, but its performance was very stable for a long operation time. The improved lifetime of miniature BFCs based on the stabilized enzymes and unbuffered fuel solution will bring the BFC community one step closer to the realization of BFCs for practical uses. In particular, the use of miniature system and unbuffered fuel solution will be a benefit to practical applications.

2.7 Acknowledgements

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2.8 References

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CHAPTER THREE

OPTIMIZATION OF GLUCOSE AND MEDIATOR CONCENTRATION AND THE EFFECT OF ANODE ELECTRODE PREPARATION METHOD ON PERFORMANCE AND STABILITY

This chapter is an addendum to the previous chapter. As the previous chapter was prepared as a single journal publication, not all of the experimental data collected could be presented in the *Results and Discussion* section. Therefore, this chapter is written to highlight other meaningful experiments which were performed and to explore the significance of the results.

3.1 Results and Discussion

As discussed previously, the eventual goal of the biofuel cell (BFC) is to be able to use it in an *in vivo* locale or to produce electrical energy from naturally available fuels. It is therefore important to be able to predict how a working device will perform in a given environment and to have knowledge of what the optimum operating conditions are. This includes a determination of how glucose and mediator concentrations affect cell performance, what the expected power output will be depending on the enzyme immobilization method employed, and what the long term operational characteristics of the cell are.

To help to answer the first of these questions, the performance of the BFC reported in *Chapter 2* was determined as a function of glucose concentration. It is desirable to be able to predict how the glucose concentration will affect the cell's performance as potential *in vivo* hosts or naturally available fuels have varying sugar concentrations. For example, the typical sugar concentration in human blood is ~5.6 mM while the sugar concentration of plants and trees varies widely but can be on the order of a few hundred millimolar. However, we also wanted to ensure that the work performed in *Chapter 2* was done near the optimal glucose concentration.

To determine the effect of glucose concentration on the BFC's power output, glucose concentrations ranging from 1 mM to 1 M were fed to the cell. A potentiostatic measurement was used to fix the cell potential at 0.25 V. Data was subsequently collected after 10 hours of cell operation with 10 mM benzoquinone (BQ) in an unbuffered fuel solution feeding the cell at 68 μl min^{-1}. The results are shown in Figure

3.1. The results suggest that the optimum glucose concentration falls near 100 mM. A 200 mM glucose concentration was thus chosen for the entirety of the BFC research, as this concentration is near the optimum yet slightly higher to minimize substrate depletion effects.

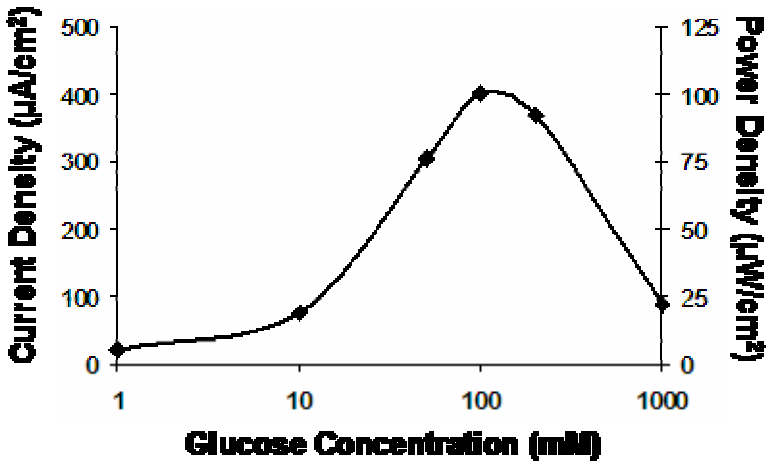

Figure 3.1. Effect of glucose concentration on BFC performance.

When the glucose concentration was lower than 100 mM, it is believed that the power output density was limited by glucose transport deficiencies. However, the cause of the decrease in power output densities when the glucose concentration was above 100 mM is not yet clear. However, glucose oxidase (GOx) shows no substrate inhibition effect except at very low oxygen concentrations ($< 2 \times 10^{-5}$ M), indicating that a cause other than substrate inhibition is responsible for the decrease in power output at high glucose concentrations [1].

In order to allow the BFC to be operated at its optimum mediator concentration for the work presented throughout this study, the effect of the mediator concentration was

also studied in addition to glucose concentration. The effect of the mediating benzoquinone (BQ) concentration was also explored so that its effect on BFC systems could be determined over a wide range of concentrations. Therefore, fuel solutions with BQ concentrations ranging from 0 to 50 mM were fed to the cell. Data was collected after 10 hours of potentiostatic operation at 0.25 V while feeding an unbuffered 200 mM glucose solution to the cell at 68 μl min^{-1}. The results are illustrated in Figure 3.2 and suggest that the optimum BQ concentration falls near 10 mM. A BQ concentration of 10 mM was thus used for all work presented herein.

At concentrations lower than 10 mM the rationale for the decreased performance is clear; there is not enough of the mediator present to efficiently shuttle electrons (mass transport issue). However, at higher concentrations the explanation for BQ's inhibitory effect on cell performance is a little more obscure. One possibility is that, in an unbuffered environment, BQ lowers the pH of the fuel solution, and, as shown in Figure 2.3C of the previous chapter, the activity of GOx is extremely sensitive to pH. The pH's of various concentrations of BQ solutions are given in Table 3.1. As can be seen, pH decreases with increasing BQ concentration, albeit slightly.

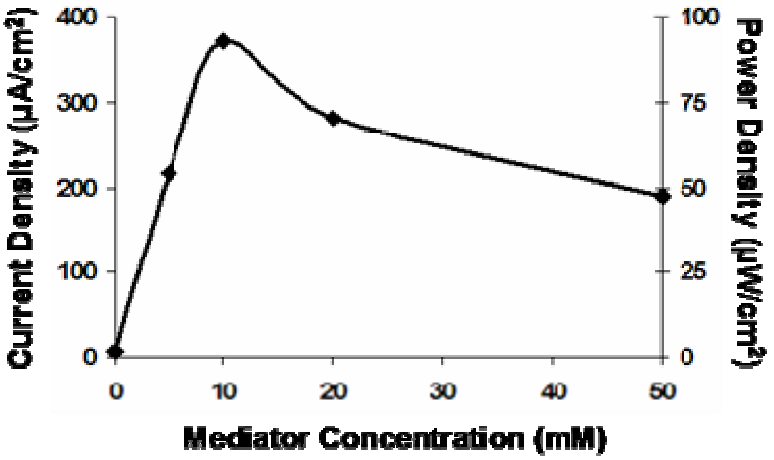

Figure 3.2. Effect of mediator concentration on BFC performance.

Table 3.1. pH of solutions prepared with differing concentrations of glucose and BQ.

[BQ (mM)]	[Glucose (mM)]	pH
0	200	5.37
5	200	3.64
10	200	3.48
20	200	3.43
50	200	3.39
10	0	3.85
Nanopure Water		5.86
Lab Tap Water		7.59

To further characterize and optimize the system, an assortment of immobilization techniques and carbon supports were used to prepare the anode electrodes, as described in the *anode electrode preparation* section of the previous chapter and in *Appendix B*. The first goal was to see how the performance and stability of anodes prepared by our novel covalent enzyme cluster (CEC) enzyme immobilization method compared with that of anodes prepared by conventional techniques. The second goal was to establish what

performance difference existed when either carbon felt (CF) or carbon paper (CP) materials were used to support the immobilized enzymes on CNTs.

While the activity and stability of these samples has been investigated using common laboratory assay techniques [2], their true performance in a working BFC device needed to be sought out to evaluate the differently prepared anodes' performance. Potentiostatic measurements were performed at 0.18 V while feeding the cell 200 mM glucose and 10 mM BQ in an unbuffered solution at 68 μl min^{-1}. The results are presented in Figure 3.3 and Table 3.2. The CEC-GOx (crosslinked enzyme clusters – glucose oxidase) electrodes exhibited far greater performance than the CA- and EC-GOx (covalently attached and enzyme coated) electrodes. This is because the enzyme loading on the CEC-GOx samples was greater than that of the other samples due to our novel procedure of covalently linking enzyme aggregates to carbon nanotubes (CNTs). Interestingly, however, the nature of the carbon support made little difference on power output even though the total enzyme content of the CF electrodes was theoretically much greater, and there was no correlation found between the 190 and 370 μm thick CP electrodes. This suggests that either only the surfaces of the carbon supports were coated with the enzyme-linked CNTs or that there is a mass transport issue within the dense matrix of the carbon supports. The stability of the CEC-GOx samples was also much greater than other samples indicating that the covalently linked enzyme clusters are robust. The CEC-GOx electrodes showed little performance drop over the 15 hour trial.

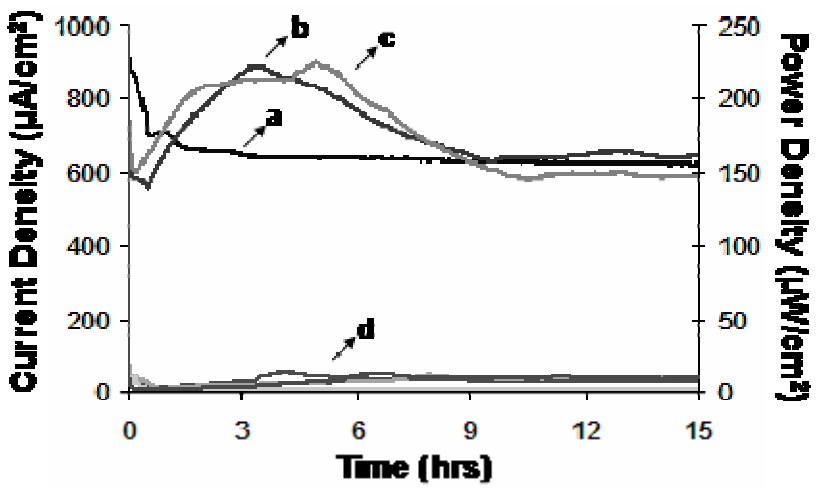

Figure 3.3. Effect of differently prepared anode electrodes on fuel cell performance. Electrode: (a) CEC-GOx on CF, (b) CEC-GOx on 370 µm CP, (c) CEC-GOx on 190 µm CP, (d) EC- and CA-GOx on 190 and 370 µm CP.

Table 3.2. Effect of anode electrode nature on fuel cell performance after 10 hours.

Anode Electrode	**PD (µW/cm^2) at 10hrs**
CEC, CF	113.3
CEC, 370µm CP	114.9
CEC, 190µm CP	106.8
EC, 370µm CP	6.3
EC, 190µm CP	7.1
CA, 370µm CP	1.9
CA, 190µm Cp	7.5

To help better predict what the long term stability of the CEC-GOx on CP electrodes might be in real applications, a 25 day stability experiment was performed. A 370 µm CP CEC-GOx electrode was selected and run in the BFC at 0.18 V while feeding an unbuffered 200 mM glucose and 10 mM BQ solution to cell at 68 µl min^{-1}. The results of the 25 day experiment are illustrated in Figure 3.4 and showed an average daily power drop of only 3% over the lifetime of the experiment. We theorize that the multitude of covalent bonds linking each enzyme to its neighboring enzymes reduces the enzyme's denaturation rate by holding the GOx in its native conformation.

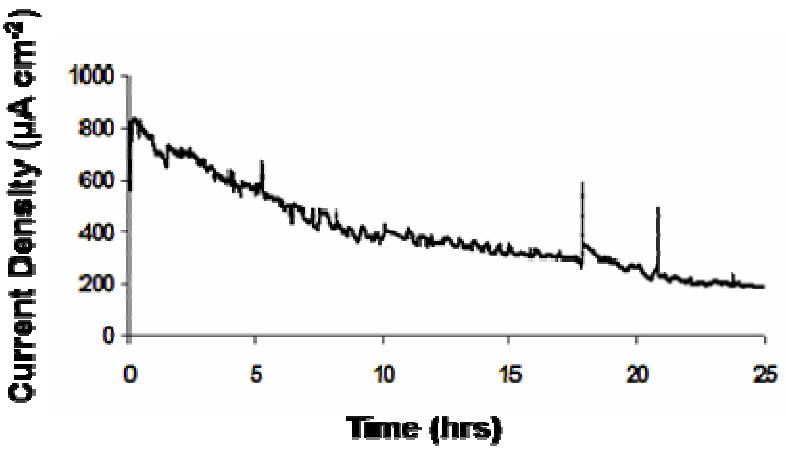

Figure 3.4. Life test of CEC-GOx at 0.18V.

The knowledge gained through these experiments of how glucose concentration, enzyme immobilization technique, and supporting material effect cell power output density and stability, and the knowledge gained relating to the cell's long term stability, aid in the assessment of how a BFC of this nature might operate in real working environments. We were able to demonstrate that an optimum glucose and BQ concentration exist and that the optimum glucose concentration falls near 100 mM and that the optimum BQ concentration is around 10 mM. We were also able to demonstrate that our novel CEC enzyme immobilization technique serves to increase the power output density more than an order of magnitude over those enzymes immobilized by conventional covalent attachment and enzyme coasting methods. Finally we were able to demonstrate that the average daily power loss of our CEC-GOx anodes in a working BFC was an unprecedentedly low 3%, opening new doors for BFC applications which require good long term stability.

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CHAPTER FOUR

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

The two largest obstacles to creating biofuel cells (BFCs) which will be of commercial value are increasing the power output densities of the cells and increasing the working lifetimes of the cells. Depending upon the extent to which these problems are able to be overcome, BFCs may eventually be used not only to power small autonomous sensing and/or transmitting devices but eventually to power a number of larger devices which can be used either *in situ* in humans or to produce power from any number of other naturally available fuel sources, such as waste water or flora.

As a starting point from which these problems can begin to be resolved, we have proposed a universal platform from which BFCs can be constructed. The design was kept very simple and was modeled after conventional stackable proton exchange membrane (PEM) fuel cells. The design consisted only of anode and cathode current collectors, a membrane electrode assembly (MEA), and a fuel cell housing which contained a fuel storage reservoir. This design utilized an air breathing Pt cathode, such that the power output from and stability of the BFC would both be limited by the enzymatic anode, allowing the anode to be studied independently. The design offered incredible flexibility allowing flow and no flow fuel delivery systems to be used, allowing enzymatic anodes of various areas and thicknesses to be used, and allowing for any combination or concentration of fuels, buffers, and mediators to be employed. The

purposed platform also allows the size of the entire BFC system to be scaled to virtually any size, as demonstrated by the various systems we constructed, one with a working area of 4 cm^2 and one with an area of 1 cm^2, smaller than a US penny.

Through the characterization of our BFC system we demonstrated that many parameters exist which must be optimized to achieve maximum performance. Through the optimization of these parameters we also gained insight into the science behind how various parameters effect BFC performance and were able to prove, or at least speculate, on the reasons why. For example, we were able to demonstrate that the nature of the buffer used to control the pH of the fuel solution had an impact of the lifetime and performance of the BFC. We were able to demonstrate that this effect is due to the fact that the cations of the buffers had an affinity for the proton conducting sulfonic sites of the Nafion membrane, leading to an increased resistance through the membrane depending upon the nature of the cations. We were also able to demonstrate that both an optimal glucose and mediator concentration exist, around 100 mM and 10 mM respectively. We speculated that at too low of a glucose or mediator concentration the performance of the BFC was limited by glucose's mass transport while at too high of glucose or mediator concentration the enzymes became inhibited. The low performance at high mediator concentrations may also have been due to the fact that the mediator lowered the pH of the unbuffered fuel solution, however, further investigation is necessary.

Most significant of the findings presented throughout this work is the improvement made to existing BFC technology through the introduction of our novel crosslinked enzyme cluster immobilization method. We found that when the enzymes

were precipitated out of solution and were subsequently linked together to form covalently linked enzyme aggregates, not only could the quantity of immobilized enzyme housed within the BFC be greatly increased, but the stability of the enzymes could also be increased. We demonstrated that the crosslinked enzyme cluster immobilization procedure produced anode electrodes with more than an order of magnitude increase in power output density compared to conventional methods such as covalent attachment and enzyme coating methods. Furthermore, we demonstrated that the CEC-GOx (covalent enzyme clusters – glucose oxidase) electrode could be operated for longer than 25 days with an average daily power decrease of only 3%, a length of time longer than ever reported previously, to the author's knowledge.

4.2 Future Work

The viability and value of the biofuel cell concept will depend upon the development of even more robust BFC systems in the near future. Systems will need to be able to deliver even greater power output densities and have an even longer operational lifetime before their full potential will be reached. Currently, the major causes of these deficiencies arise from poor electron transfer mechanisms and the presence of a membrane to contain the fuel / mediator solution. Therefore, it is our intent to further improve upon BFC designs and enzyme immobilization techniques to solve these problems.

As discussed previously, if a buffer is used in conjunction with a BFC system which incorporates a membrane, the buffer will greatly increase the proton transfer resistance through the membrane over time, eventually killing the BFC. Therefore, for systems which utilize a membrane, it is currently optimal to operate the system unbuffered. However, the enzyme activity is greatly pH dependent such that at pHs away from neutrality the power output from the BFC system is greatly diminished. Therefore, it would greatly benefit the BFC technology if we could eliminate the membrane. However, the membrane is a necessary component of the system for two reasons: 1) it contains the fuel solution so that the fuel does not react at the cathode, and 2) it contains the mediator so that the mediator is not oxidized at the cathode.

The first of these needs for a membrane can be easily circumvented. By using enzymes to catalyze the cathode reaction in addition to the anode reaction, instead of using precious metal catalysts, the fuel is unable to react at the cathode due to the

cathodic enzyme's selectivity. Therefore, the only remaining challenge to eliminating the membrane is the need to contain the diffusive redox mediator so that it cannot oxidize at the cathode. A logical approach is to use a mediator which is either non-diffusive (immobilized) or to not use a mediator at all, instead relying on direct electron transfer (DET). It is therefore our intent to explore a number of purposed techniques which rely on non-diffusive mediators or mechanisms which can achieve DET. While many mechanisms have been purposed, none have yet shown great promise. However, this is a necessary step to improving BFC technology. We therefore plan to explore enzyme and DET or immobilized mediator methods side-by-side to study which mechanism shows the most potential for further use, what the shortcomings of each method are, and to suggest and best ways in which these methods might be improved upon. Figure 4.1 illustrates our three-pronged approach to tackling this future research.

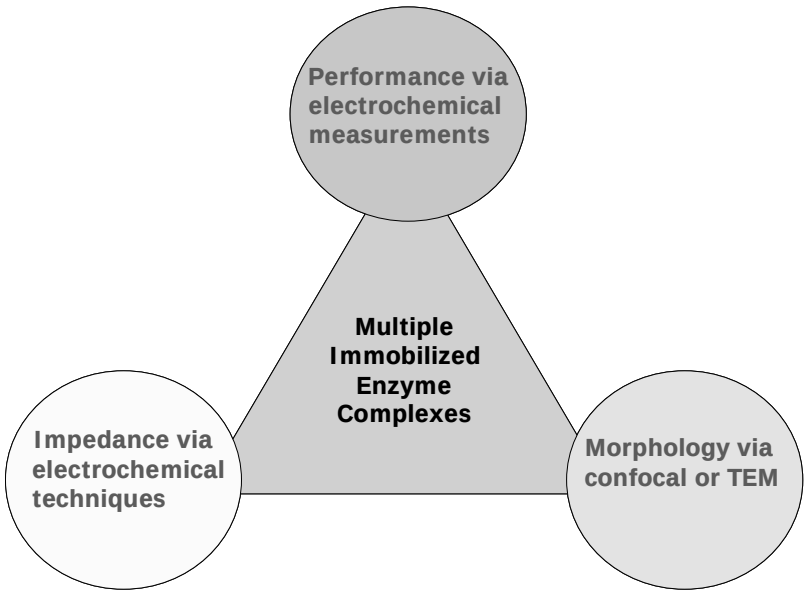

Figure 4.1. Three-pronged immobilized enzyme complex characterization procedure.

The three-pronged approach has the advantage of providing us with information about the performance of the complexes, morphology of the complexes, and where the bottlenecks lie for each of the complexes tested. For example, the potentiometric experiments performed in an electrochemical cell will provide information about the onset potential of the complexes (the potential at which current first is able to be generated), the activity of the complexes (how much current they can produce as a function of applied potential), and about the stability of the complexes (current output as a function of time at a fixed potential). Confocal or TEM imaging of the tagged enzymes incorporated into the complexes will provide us with information on the quantity of enzymes immobilized, the structure and morphology of the immobilized enzymes, and the porosity of the complexes. Finally, impedance analysis performed in an electrochemical cell will indicate what the major resistances to efficient power output arise from. For example, impedance analysis can be used to compare fuel mass transfer resistance, mediator mass transfer resistances, enzyme kinetics, and electron transfer resistances, allowing their relative magnitudes to be weighed thus pinpointing the bottlenecks of each complex.

Once the relative advantages of various immobilization methods and electron transfer mechanisms are known, we can proceed toward developing further improved enzymatic anode and cathode materials. One of the main objectives of this three-pronged research will be to develop efficient DET mechanisms. For example, we believe that if we can immobilize enzymes in such a way that carbon nanotubes (CNTs) are within electron tunneling distance from the enzyme's active centers then we will be able to conduct electrons without the use of either diffusive or immobilized mediators. This

concept is illustrated in Figure 4.2 where glucose oxidase has chosen as the model enzyme.

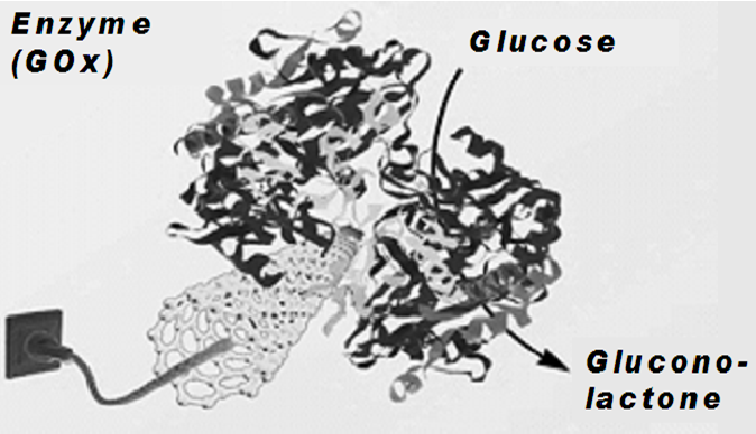

Figure 4.2. Direct enzyme electron transfer mechanism via carbon nanotubes.

If DET can be achieved, it will open up new possibilities for BFC systems. For example, the now necessary membrane component of BFC systems can be eliminated as there will no longer be a need for containing the mediator within the systems. The elimination of the membrane, and thus the fuel cell body whose only function is to hold the membrane, will allow us to build even smaller, even MEMS scale, BFC devices. The achievement of DET may also serve to increase the power output density and working lifetime of BFC systems. The use of a mediator reduces the theoretical open cell voltage of BFC as electrons need to first be transferred from the enzyme to the mediator and then from the mediator to the electrode (electrons flow down a potential gradient), and thus the elimination of the mediator may increase the performance of BFCs. Also, electron transport via mediators is currently the largest bottleneck to achieving high cell power output densities. If a DET mechanism is employed, the electron transfer kinetics should

be greatly improved. Furthermore, without the need for a proton conducting membrane, proton transfer resistances should also decrease. Finally, without the need for a membrane, buffers can be utilized to fix the pH within BFC systems at a neutral value, thus greatly increasing the enzyme's activity and also serving to allow the enzymes to operate in a more stable environment.

We have, however, also developed an alternative future research plan in the event that DET cannot be realized. If DET proves itself to be too complicated or impossible then BFCs will remain dependent upon mediators and thus upon membranes for the case of diffusive mediators (diffusive mediator have shown much more promise thus far than immobilized mediators). In order to retain the power output and stability advantages that buffered conditions offer while still utilizing a membrane, we will seek to develop alternative, non-polymer based membrane alternatives. For example, we believe that micro-porous silicon glass, similar to that which is currently used in pH meters and various other ion selective electrodes, might offer similar (or improved) proton transport characteristics and fuel retention characteristics to Nafion membrane, while having the advantage of not being impaired by buffer cations.

APPENDIX A

BIOFUEL CELL TESTING VIA POTENTIOMETRIC MEASUREMENTS

Equipment: Solartron Analytical, 1287 Potentiostat

Software: Scribner Associates, Inc., CorrWare for Windows

Before potentiometric measurements can be performed on a BFC system, the potentiostat must be connected to the fuel cell. The working electrode and second reference electrode terminals (labeled WE and RE2) are connected to the BFC's anode and the counter electrode and first reference electrode terminals (labeled CE and RE1) are connected to the BFC's Cathode. An assembly diagram is offered in Figure A.1. Just before the BFC is connected, or just after the BFC is connected, the potentiostat can be turned on by flipping the power switch located in the back of the machine.

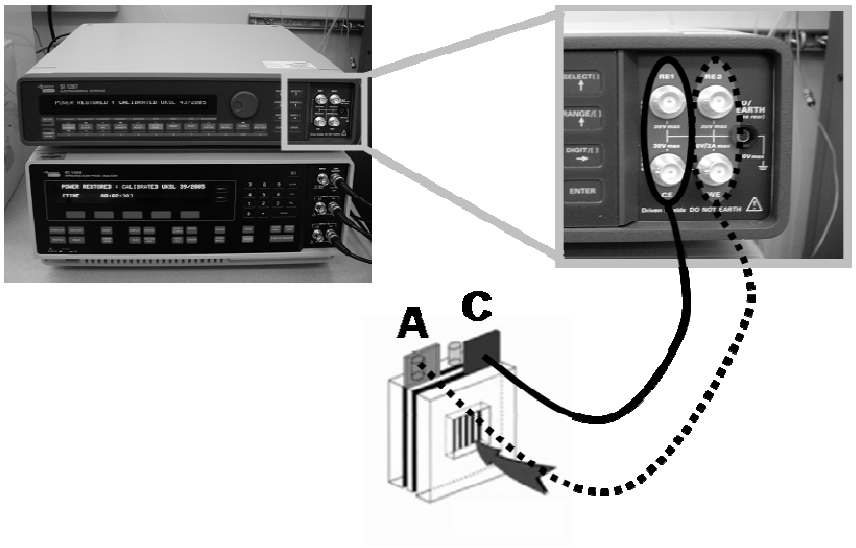

Figure A.1. Potentiostat / BFC hookup diagram.

To open the software which operates the potentiostat, the icon which says "CorrWare" needs to be clicked or the software can be opened by going to "Programs" and then selecting "CorrWare". Once the program is open, click the "Experiments" tab and then select "Insert New Experiment" on the drop-down menu. At this point a new window will appear with a list of experiments: open circuit, potentiostatic, potentiodynamic, cyclic voltammogram, potential square-wave, potential stair-step, potential scan/hold, galvanostatic, galvanodynamic, galvanic square-wave, galnavic cycle, E & I noise, scanner, impedance, run external utility, polarization resistance. The type of experiment which is to be performed can now be selected. A detailed description of each type of experiment can be found in the CorrWare software user manual.

Once the desired experiment is selected, the experiment will appear in the "Experiment" window. To change the operating parameters, the desired experiment can be double-clicked to bring up a new window specific to the type of experiment. Once the parameters are set, the experiment can be begun by clicking on the "Run Selected" icon. When the experiment is complete, a new window will appear asking the operator how they would like to save the data from the experiment.

As a side note, the CorrWare software considers current generated by the fuel cell (electrons flowing from the working electrode toward the counter electrode) as positive current, as one would expect. However, if the counter electrode has a more positive potential than the working electrode, the software considers the potential negative, the opposite convention of most voltmeters. This means that when the BFC is connected as described above, the CorrWare software will register a negative potential. Therefore, when the experimental parameters are set using the software, one must always be aware

of the convention used by the software. The reason for this seemingly confusing convention is that the software is designed for corrosion and electrochemical measurements where power needs to be applied to the system under study, as opposed to fuel cell research where energy comes from the system under study. Therefore, the potential the software reads is the *applied* potential, which is negative when the fuel cell is the system under study.

APPENDIX B

ENZYMATIC ANODE PREPARATION PROCEDURE

B.1 Carbon Nanotube Acid Functionalization

Reagents:

Carbon Nanotubes: Multiwalled, Purity 95%, Diameter 30±15 nm, Length 1~5 μm (Nanolab, Nanolab #PD30L15)

Filter Paper: Mixed cellulose esters, 0.8 μm cutoff (Pall Life Sciences, VWR #28148-325)

Nitric Acid: Reagent grade, ~15.8 M (Generic)

Sulfuric Acid: Reagent grade, ~18 M (Generic)

Procedure:

- Weigh 0.25g carbon nanotubes (CNTs)
- Combine 6.25 ml nitric acid and 18.75 ml sulfuric acid in a 50 ml bottle
- Allow temperature to return to room temperature
- Add CNTs to acid solution while mixing with magnetic stir bar
- Shake upright at 100 rpm for 24 hours on an orbital shaker
- Sonicate until CNT suspension is homogeneous using an ultrasonic water bath[1]
- Add 225 ml of Nanopure water to a 250 ml bottle
- Slowly add CNT suspension while stirring with a magnetic stir bar
- Vacuum filter the suspension

- Rinse through with additional Nanopure water until all acid is removed, as indicated by pH measurements
- Dry filter paper with CNTs for at least 12 hours at 80 ^{o}C
- Remove CNTs from filter paper and place into a mortar
- Grind CNT aggregates to a fine powder

B.2 Carbon Support Acid Functionalization

Reagents:

CF (Carbon felt): 3.18 mm thick (Alfa Aesar, Alfa Aesar #43199)

CP (Carbon Toray Paper): 190 and 370 μm thick (Fuel Cell Store, Fuel Cell Store #591037 & #591637)

Nitric Acid: Reagent grade, ~15.8 M (Generic)

Sulfuric Acid: Reagent grade, ~18 M (Generic)

Procedure:

- Add nitric acid to sulfuric acid in a ratio of 1:3 in a bottle
- Allow temperature to return to room temperature
- Cut CF or CP into 2x2 cm squares using an exacto knife
- Add CF or CP to acid solution
- Shake upright at 100 rpm for 24 hours on an orbital shaker
- Remove CF or CP with forceps and place into 500 ml of Nanopure
- Shake upright at 100 rpm for 15 minutes on an orbital shaker
- Repeat last two steps until pH reaches 6.0
- Dry CF or CP in hood
- If 0.332 cm^2 electrodes are desired, use a standard hole punch to create disks

B.3 Immobilization of Glucose Oxidase on Carbon Nanotubes

Reagents:

Ammonium sulfate: Molecular biology grade (Sigma, Sigma #A4418)

BupH MES (2-(morpholino)ethanesulfonic acid): Buffered saline packs, 0.1 M, 0.9% NaCl (Pierce, Pierce #28390)

EDC (*N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride): Protein sequencing grade (Sigma, Sigma #E6383)

GA (Glutaraldehyde): 8% aqueous, Grade I (Sigma, Sigma #G7526)

GOx (Glucose oxidase): Type VII from Aspergillus niger, Lyophilized, Min 60% protein (Sigma, Sigma #G2133)

Hydrochloric acid (HCl): Reagent grade (Generic)

NHS (N-hydroxysulfosuccinimide sodium salt): HPLC grade (Fluka, Sigma #56485)

Sodium hydroxide (NaOH): Reagent grade (Generic)

Sodium phosphate dibasic: ReagentPlus® grade (Sigma, Sigma #S0876)

Sodium phosphate monobasic: ReagentPlus® grade (Sigma, Sigma #S0751)

Tris base (2-Amino-2-(hydroxymethyl)-1,3-propanediol): Purity > 99% (Sigma, Sigma #T1378)

Procedure:

- Prepare the following **stock** solutions (these can be kept at 4 ^{o}C for months):
    - 100 mM phosphate buffer solution (PBS) of pH 7.0

      This is prepared by combining mono- and di-basic sodium phosphate.
    - 100 mM MES buffer of pH 6.5

      Adjust pH with HCl and NaOH
    - 100 mM Tris buffer of pH 7.2

      Adjust pH with HCl and NaOH
- Prepare the following solutions **just before use**:
    - 10 ml of 50 mg ml^{-1} (434 mM) NHS in Nanopure
    - 5 ml of 10 mg ml^{-1} (53.2 mM) EDC in Nanopure
- Combine 50 mg acid-treated CNTs with 25 ml Nanopure in a 50 ml bottle and sonicate in ultrasonic bath until homogeneous[1]
- Add 10 ml MES buffer, 10 ml NHS, and 5 ml EDC solution in this order
- Shake at a 45^{o} incline at 250 rpm for 30 minutes using orbital shaker
- Sonicate until homogeneous in ultrasonic bath[1]
- Shake at a 45^{o} incline at 250 rpm for 30 minutes using orbital shaker
- Centrifuge for 15 minutes at ~ 950 x g[2]
- Remove supernatant with pipette being careful not to remove any CNTs
- Wash with 100 mM MES buffer twice
- Finally, resuspend CNTs in 100 mM MES buffer to a concentration of 1 mg ml^{-1}

At this point, the suspention can be kept at 4 ^{o}C for 1 – 2 days.

- Prepare 25 ml of 1mg ml^{-1} GOx in PBS
- Sonicate CNT suspension until homogeneous in an ultrasonic bath[1]
- Add 15 ml of CNT suspension to three 50 ml centrifuge tubes (15 ml per tube)
- Add 7.5 ml of GOx solution to each tube
- Vortex tubes
- Shake at 45^{o} incline for 1 hour at 200 rpm
- Prepare 20 ml of 0.55 g ml^{-1} ammonium sulfate in 100 mM PBS
- For covalent enzyme clusters (CEC): Add 15 ml of ammonium sulfate solution

  For enzyme coating (EC): Add 15 ml of 100 mM PBS

  For covalent attachment (CA): Add 15 ml of 100 mM PBS
- Vortex tubes and sonicate in an ultrasonic bath until homogeneous[1]
- Shake at 45^{o} incline for 30 minutes at 200 rpm
- For CEC: Adjust GA concentration to 0.5vol%

  For EC: Adjust GA concentration to 0.5vol%

  For CA: Add same amount of 100 mM PBS as volume added to other tubes
- Sonicate until homogeneous in ultrasonic bath[1]
- Shake at 45^{o} incline for 30 minutes at 200 rpm
- Incubate at least 12 hours at 4 ^{o}C
- Centrifuge for 15 minutes at ~ 950 x g and remove supernatant[2]
- Wash with 100 mM tris buffer and decant supernatant
- Add 15 ml tris buffer to each tube
- Shake at 45^{o} incline for 30 minutes at 200 rpm

- Centrifuge for 15 minutes at ~ 950 x g and remove supernatant[2]
- Wash four times with 30 ml of 100 mM PBS per tube and decant supernatant[3]
- Resuspend by addition of 7.5 ml of 100 mM PBS per tube (2 mg ml^{-1} CNTs)
- Check activities by method outlined in the *Enzymatic Assay of Glucose Oxidase* section of *Appendix B*
- Suspensions can be kept at 4 ^{o}C for 1 – 2 days

B.4 Enzymatic Assay of Glucose Oxidase

Reagents:

D-(+)-glucose: SigmaUltra grade (Sigma, Sigma #G7528)

Sigma technical support indicates that α : β is 1 : 2.

GOx (Glucose oxidase): Type VII from Aspergillus niger, Lyophilized, Min 60% protein (Sigma, Sigma #G2133)

ODS (o-Dianisidine dihydrochloride): 10 mg substrate tablets (Sigma, Sigma #D9154)

POD (Peroxidase): Type II from Horseradish, Lyophilized (Sigma, Sigma #P8250)

Sodium phosphate dibasic: ReagentPlus® grade (Sigma, Sigma #S0876)

Sodium phosphate monobasic: ReagentPlus® grade (Sigma, Sigma #S0751)

Procedure:

- Prepare the following **stock** solution (this can be kept at 4 ^{o}C for months):
  - 100 mM phosphate buffer solution (PBS) of pH 7.0

    This is prepared by combining mono- and di-basic sodium phosphate.

- Prepare the following solutions **just before use**:
    - Dissolve one 10 mg ODS tablet in 1.52 ml of Nanopure. Vortex. Then dilute 0.15 ml of this solution to 15 ml with 100 mM PBS (0.21 mM ODS in PBS). Vortex. Protect ODS from light by wrapping vial in aluminum foil.
    - Dissolve 0.75 g β-D-glucose into 5 ml Nanopure (150 mg ml^{-1} or 832.5 mM). Vortex.
    - Combine 12 ml of 0.21 mM ODS solution with 2.5 ml of 832.5 mM glucose solution (forms 0.17 mM ODS, 143.6 mM glucose solution). Vortex. Protect ODS from light by wrapping vial in aluminum foil.
    - Prepare ~ 0.5 ml of 3.797 mg ml^{-1} POD in Nanopure. Vortex.
    - Prepare ~ 0.5 ml of 0.05 mg ml^{-1} of CNTs (as described in the *Immobilization of Glucose Oxidase on Carbon Nanotubes* section of *Appendix B*) in 100 mM PBS. Sonicate until homogeneous in ultrasonic bath.[1]
- Setup the spectrophotometer to measure absorbance at a wavelength of 500 nm. Calibrate the sepectrophotometer by running a cuvette with 100 mM PBS as a blank.
- Into a cuvette place 980 µl of the ODS / glucose solution prepared above.[4]
- Add 10 µl of the POD solution prepared above to the cuvette and mix with a pipette tip.

    Make sure no air bubbles are present.

- Make sure CNT suspension prepared above is homogeneous by sonicating in an ultrasonic bath.[1]
- Add 10 μl of the CNT suspension to the cuvette and quickly mix with the pipette tip while being careful not to create air bubbles. Simultaneously while the CNT suspension is added, start the spectrophotometer.
- Once the absorbance has been measured as a function of time at 500 nm for about 3 minutes, the test is complete. The activity of the GOx-CNT suspensions is proportional to the rate of change in absorbance. The rate of change used in this determination should be the **initial** rate.

B.5 Immobilization of Glucose Oxidase / Carbon Nanotube Complexes on Carbon Supports

Reagents:

Nafion perfluorinated ion-exchange resin: 5wt% in lower aliphatic alcohols and water (Aldrich, Sigma #527084)

Sodium phosphate dibasic: ReagentPlus® grade (Sigma, Sigma #S0876)

Sodium phosphate monobasic: ReagentPlus® grade (Sigma, Sigma #S0751)

Procedure:

- Prepare the following **stock** solution (this can be kept at 4 ^{o}C for months):
  - 100 mM phosphate buffer solution (PBS) of pH 7.0

    This is prepared by combining mono- and di-basic sodium phosphate.
- Vortex GOx-CNT suspensions (see *Immobilization of Glucose Oxidase on Carbon Nanotubes* section of *Appendix B*)
- Sonicate GOx-CNT suspensions until homogeneous in an ultrasonic bath[1]
- Pipette 1 ml of GOx-CNT (2 mg ml^{-1} CNTs) suspensions into small centrifuge tubes
- Centrifuge tubes for 5 minutes at 12,100 x g[2]
- Remove 100 µl of supernatant from each tube
- Add 100 µl of 5% Nafion solution to each tube

- Sonicate GOx-CNT suspensions until homogeneous in an ultrasonic bath[1]
- Add five CP 0.332 cm^2 electrode disks to each tube
- Leave CP disks immersed for 10 minutes, gently shaking every 2 minutes[5]
- Remove CP disks (electrodes) from solution with forceps and place them on the edge of a Petri dish
- Allow electrodes to dry for 1 hour under hood
- Place the electrodes into 100 mM PBS and store at 4 ^oC

B.6 Footnotes

[1] It is important that the ultrasonic bath be kept at or below room temperature. The bath temperature increases as the bath is operated so it is important that a means of cooling is available. The reason it is not favorable to sonicate in warm bath water is that the enzymes are susceptible to denaturation. Also, the bath has more "power" the cooler the water is. Never sonicate solutions containing enzymes for any longer than necessary to reach homogeneity as the ultrasonic waves can also denature the enzymes.

[2] A G-force value is indicated for each centrifugation step. G-force values can be converted to revolutions per minute (RPM) or vice versa by the following equation: $g = (1.118 \times 10^{-5}) R S^2$. The letter "g" indicates G-force, "R" is the rotor radius, and "S" is the speed of the centrifuge expressed in RPM.

[3] To "wash" means the following: 1) Add the same amount of, or more, buffer to the tube than the amount of supernatant removed from the tube. 2) Sonicate until homogeneous with ultrasonic bath. 3) Shake for 5 minutes horizontally at 200 rpm with an orbital shaker. 4) Repeat centrifuge step (centrifuge for 15 minutes at ~ 950 x g) and remove supernatant with a pipette.

[4] Make sure all reagents are at room temperature. Enzymes' activities are a function of temperature and therefore it is important to consistently perform the activity measurements at room temperature.

[5] It is important to be very gentle while shaking the electrodes. Carbon paper is brittle and thus the edges of the electrode disk can be damaged in violent shaking is employed.