GENERATING ELECTRICITY WITHIN THE PHYSIOLOGICAL ENVIRONMENT FOR LOW POWER IMPLANTABLE MEDICAL DEVICE APPLICATIONS: TOWARDS THE DEVELOPMENT OF IN-VIVO BIOFUEL CELL TECHNOLOGIES

by

Gusphyl Antonio Justin

BS, BA, University of Pittsburgh, 2001

MS, University of Pittsburgh, 2004

Submitted to the Graduate Faculty of

the School of Engineering in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2007

UNIVERSITY OF PITTSBURGH

SCHOOL OF ENGINEERING

This dissertation was presented

by

Gusphyl Antonio Justin

It was defended on

June 4th, 2007

and approved by

Robert Sclabassi, Professor, Department of Neurological Surgery

Mingui Sun, Professor, Department of Neurological Surgery

Harvey Borovetz, Professor, Bioengineering Department

Xinyan Tracy Cui, Assistant Professor, Bioengineering Department

Yingze Zhang, Associate Professor, Department of Pulmonary, Allergy and Critical Care Medicine

David Waldeck, Professor, Chemistry Department

Dissertation Director: Robert Sclabassi, Professor, Department of Neurological Surgery

GENERATING ELECTRICITY WITHIN THE PHYSIOLOGICAL ENVIRONMENT FOR LOW POWER IMPLANTABLE MEDICAL DEVICE APPLICATIONS: TOWARDS THE DEVELOPMENT OF IN-VIVO BIOFUEL CELL TECHNOLOGIES

Gusphyl A. Justin, PhD

University of Pittsburgh, 2007

Electrochemical studies were performed to explore electron transfer (ET) between human white blood cells (WBC) and carbon fiber electrodes (CFE). Currently, an active area of research involves encouraging ET between microbes and various electrodes in a biofuel cell (BFC). ET between microbes and electrodes are thought to occur i) directly through plasma membrane-bound electron transport chain proteins; and/or ii) indirectly through the release of metabolic products or biomolecules near the electrode surface. An important motivation of this research is the need for alternative long lasting power sources for implantable diagnostic and therapeutic devices. A particular interest is reducing the size and weight of implantable devices. Currently employed internal batteries largely contribute to both. BFCs are promising prospects as they couple the oxidation of a biofuel (such as glucose) to the reduction of molecular oxygen to water. Both glucose and oxygen are abundantly present within our body's cells and tissues. The goal of this project is to explore the feasibility of utilizing WBCs (a human cell model) to generate electricity by fostering direct or indirect ET between these cells - or more specifically, between the metabolic processes of these cells - and the anode of a BFC. ET from the metabolic processes of whole cells to electrodes had, to the best of our knowledge, only previously been demonstrated for microbes. The electrochemical activities of WBCs isolated from whole human blood by red blood cell (RBC) lysis, peripheral blood mononuclear cells (PBMCs) isolated on a Ficoll-Paque gradient, as well as cells from a BLCL cell line and two leukemia cell lines (K562

and Jurkat) were all investigated by incorporation of the cells into the anode compartment of a proton exchange membrane fuel cell (PEMFC). Cyclic voltammetry was employed as an electrochemical technique to investigate the ET ability of the cells, as it can reveal both thermodynamic and kinetic information regarding oxidation-reduction processes at the CFE surface. The results of our studies demonstrate that upon activation, biochemical species, such as serotonin, are released by PBMCs, which may become irreversibly oxidized at the electrode surface.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I did not complete this journey alone. There were many persons along the way who were instrumental in my success. These persons provided me with tremendous support, with persistent encouragement and with unconditional love. I cannot emphasize enough how essential my family and close friends have been to my progress over the years. They have helped me to maintain my faith, to overcome many rough moments and seemingly insurmountable obstacles and to remain focused on all that I have always wanted to achieve. There were many points during the course of my graduate studies where doubt began to creep in and times that I really felt that I was incapable of finishing this degree. It was very hard to individually distinguish between my moments of frustration and the reality of my accomplishments. However, with the incessant encouragement and love that I received daily from my wife, Sarah, and the battery of loving support and reminders of God's presence that I constantly received from my parents, Augustus and Phyllis Justin, those doubts and frustrations quickly vanished and were replaced with a sense of determination that I fostered and adhered to until now. I would like to take this opportunity to express my sincerest and most profound gratitude to Sarah and to my parents and to let them know that I would not be where I am today without them.

There are many other people who have been an integral part of my formation as a researcher and academic. My advisors, Dr. Robert Sclabassi and Dr. Mingui Sun offered me a unique opportunity and invaluable experiences that not many other people were willing to give me. Dr. Borovetz, the current chairman of our bioengineering department, also provided me with the opportunity to pursue my studies in this department in spite of my prior minimal engineering background. These are three people who I look up to greatly and I will always respect and value

their advice. I also cannot neglect to thank the many past and current members of the Laboratory for Computational Neuroscience and the Neural Tissue/Electrode Interfaces and Neural Tissue Engineering Laboratory of the University of Pittsburgh for their friendship and their willingness to assist me along the way. Thank you Qiang, Prophete, Rafael, Robyn, Miguel, Brian, Paul, Steve, Eli, Ola, Ning, Tyler, Tolga, Reecha, Wenyan, Jian, Erdrin, Bill, Wei, Nick. I should also thank all my committee members, including Dr. David Waldeck, Dr. Yingze Zhang and Dr. Xinyan Tracy Cui for their support, assistance and guidance, as well as Dr. Charles Bradberry for his helpful input and for allowing me to use his resources. I must also thank the high school and undergraduate summer interns who worked so hard to help me gather information for my research, including Mr. Jackson Sando, Ms. Molly Snyderman and Mr. David Hirsch.

From hundreds of miles away, on a small Caribbean island called St. Lucia, there are numerous people who I know have been thinking about me and praying for me over the past several years. Although I rarely had the opportunity to directly speak and interact with these persons, I am fully aware of their caring thoughts; and for that I am truly grateful to the residents of the Adelaide's and Frances Home for the Aged and to the members of the Friends of the Elderly who placed so much confidence in me and in my abilities.

At this point, I would also like to acknowledge that this research was supported in part by the National Institutes of Health grant No. R01EB002099, US Army SBIR W81XWH-05-C-0047, and Computational Diagnostics, Inc (Pittsburgh, PA).

1.0 INTRODUCTION

Energy delivery to implantable medical devices has for many years been faced with a myriad of hurdles. The primary problem is that once the device is implanted within the body, it becomes a difficult task to make any necessary adjustments to the device, such as replacing its power source. A list of implantable devices for electrical stimulation and their operating parameters is shown in Table 1 [1]. One of the most reliable power sources for any electronic device is the lithium ion battery that must be replaced once its fuel content has been expended. Despite the fact that significant improvements have been made and continue to be made in extending the lifetime of these batteries, replacement at some point in time would still become necessary. Replacing the internal battery of an implanted device is largely undesirable, particularly from the patient's perspective, since this inevitably requires surgical intervention. Rechargeable batteries have also been pursued, which would require the patient to be largely responsible for the recharging process. Recharging would be achieved through various methods of transcutaneous power delivery such as radiofrequency or optical methods. However, in placing this responsibility in the hands of the patient, compliance becomes a major problem [2].

The primary objective of this study is to investigate the concept of developing an implantable power supply which mimics the body's ability to metabolize organic substrates. The goal would be to develop a device that would be capable of transducing the biochemical energy

stores within the body into electrical energy. Previous research in this area was met with failure mostly related to biocompatibility issues, encapsulation of the device and decline in performance with time [3]. At that time, little was known about the reactions taking place at the electrode surface. There were also problems related to inefficiency of the electrode reactions due to mixed reaction kinetics [3] with the insertion of the anode and cathode into a homogenous environment. The hope is that the present work, which focuses on biofuel cell technologies, will further revitalize an interest in using these technologies to power implantable devices, particularly devices that require lower power densities as we move into an era of micro- and nano-technologies.

Table 1: Clinical stimulation parameters for some implantable medical devices [1].

APPLICATION	***METHOD OF STIMULATION DELIVERY***	***WAVEFORM/DURATION OF STIMULATION***	***CURRENT/VOLTAGE REQUIREMENTS***
Cardiac Pacing	Electrodes in contact with the heart. Electrode impedances between 250 and 1000 Ω.	Capacitor discharge pulse between 0.1 and 2ms duration, with charge balancing phase.	0.1 to 8V peak
Pain Relief	Electrodes in contact with spinal cord. Can also be in contact with the targeted peripheral nerve associated with the pain.	Either monophasic or biphasic pulses of approx. 210 μs duration. Delivered at frequencies between 30 and 80 Hz.	0.1 to 12 V peak
Deep Brain Stimulation	Electrodes implanted in the brain at regions responsible for the control of movement. Electrode impedances: 600 to 2000 Ω.	60 to 450 μs charge-balanced capacitor discharge pulses delivered at 2 to 185 Hz.	0.1 to 10.5 V

2.0 BACKGROUND

2.1 CHALLENGES IN ENERGY DELIVERY TO IMPLANTABLE DEVICES

In order to effectively tackle the problem of energy delivery to implantable devices, it is important to consider two facets of the problem: 1) the power source (an energy transduction device that converts energy from one form, such as chemical, to a more useful form, such as electricity); and 2) the transmission modality (method of transferring energy from an external power source to the target device). Several types of power sources have been investigated for use with implantable devices, including batteries [4, 5], nuclear/thermoelectric batteries [6, 7], piezoelectric devices [8, 9], and biological/biochemical fuel cells [3, 10]. In instances where the power source is located extracorporeally, transcutaneous energy transmission modalities including radiofrequency links/magnetic transcutaneous coupling [11], optical methods [5], ultrasound [12], and volume conduction [13, 14] have been proposed.

Each method has its own drawbacks, usually with respect to insufficient power levels (optical, ultrasound), decline in performance with time (batteries, implantable biochemical fuel cells), and intractability for patient use and maintenance (magnetic transcutaneous inductive coupling devices). As an illustrative example of progress in the development of implantable power sources, consider the cardiac pacemaker. The pacemaker may be considered one of the

most successful implantable devices ever built. Substantial effort has been placed into the design of the device, including the development of its power source. It was noted in one early journal article that by 1970, the average pacemaker was explanted in less than 2 years, 80% of those explanted having experienced some form of battery failure [15]. Zinc-mercury, nuclear and lithium batteries have all been used, but today lithium ion batteries are most commonly employed. With the lithium ion batteries, a service life of more than 10 years can now be expected [16]. Taking the above example of the cardiac pacemaker, it is crucial - both to the success of the device and the health of the patient - to develop a continuous, efficient, and low-maintenance energy supply with sufficiently high power output and longterm electrical stability for implantable devices. The power source should pose minimal threat to the health of the patient, and should not compromise the functionality of the device. With the current trend of improvements in biomaterials and biocompatibility, biosensor and biofuel cell technologies, and with the miniaturization of devices (micro- and nano-technologies) that demonstrate ever decreasing power requirements, biological power sources hold some promise for future implantable medical device applications. The utilization of biochemical energy resources in the body will become particularly important as we look toward miniature devices, where a lithium ion battery would become impractical due to constraints on size and weight.

2.2 POWER SOURCES

2.2.1 Internal Batteries

The main disadvantage of current internal batteries is that the duration of the power supply is often limited by the battery capacity, that is, limited by the quantity or concentration of the available reactants [4]. The energy generated by the battery is based on electrochemical reactions, which eventually go to completion after some time. Once the potential and current levels have diminished to a point where the battery is unusable, replacement of the battery becomes necessary. When speaking about a device that is implanted within the human body, the issue of replacement can be rather complicated. Surgical intervention is the only option to replace an internal battery that has either failed or reached the end of its lifetime. Multiple surgical interventions are costly, can be painful and are truly inconvenient for any patient. In the case of a deep brain stimulator, an average battery life of 4-5 years can be expected for typical settings, which can easily drop to 1-2 years if programming of the device is suboptimal [17]. Although the procedure for replacing the pulse generator is relatively simple, that is, an outpatient surgery with a small incision in close proximity to the device, there could be the detrimental effects of scarring and infection. In addition, the procedure itself is rather costly, in the vicinity of $10,000 [17].

In order to ensure that the length of time between surgeries is maximized, battery designers and developers have worked to increase the capacity and life expectancy of the batteries, without compromising weight and size. The smaller and lighter the battery, obviously the better. Internal batteries can be bulky, reflecting the fact that they must carry their energy

stores. Lithium batteries are most commonly used today and have had tremendous success, largely due to the fact that they are lighter and have a greater energy and power capacity than other batteries, such as the zinc-mercury batteries that were previously used. A lithium battery develops a voltage of 2.8V, which can be stepped up to 5V or higher using circuitry [16]. This was a great improvement over its predecessor, the zinc-mercury battery, which had an operational voltage of 1.35V. Depending on the pacemaker, 3 to 5 zinc-mercury batteries were needed. In addition to the drawback with respect to size, these batteries were often prone to failure within a very short time period, normally within two years [15]. The invention and application of lithium ion batteries greatly impacted the implantable medical device field. The lifetime of cardiac pacemakers was significantly increased, no gas was evolved by the battery (electrochemical cell could be hermetically sealed) unlike its predecessor, and it had an electric charge capacity of 4.14Ah, with a service life of more than 10 years [16]. By 1980, they powered over 95% of the world's pacemakers [15].

2.2.2 Nuclear Batteries

In the 1970s, the use of plutonium 238 appeared to be promising as a power source, particularly for cardiac pacemakers [7]. High velocity alpha particles emitted by plutonium during nuclear decay would bombard the container's wall generating heat. The heat would traverse the wall and subsequently be converted to electrical energy by thermopiles. The half-life of plutonium (Pu) is 87 years [16]. It was, therefore, capable of providing an almost limitless power supply to implantable electronic devices, such as the cardiac pacemaker. Nuclear batteries were introduced into cardiac pacemakers, which were then implanted into patients. Due to regulatory problems

concerning the control of the nuclear fuel, however, the total number of nuclear pacemakers never exceeded 1% of total pacemakers implanted annually [2]. Although safety really was not the primary issue, there was still some concern about the toxicity of the fuel (one microgram of plutonium 238 in the blood stream could be fatal). The real concern, however, was the exceedingly long half-life of the radioisotope, which made it difficult for its universal acceptance. Questions arose regarding the best way to keep track of the nuclear powered pacemakers. A lost nuclear powered pacemaker can still pose a threat to future generations hundreds of years later. From a purely technological perspective, the Pu 238 nuclear pacemaker was shown to be the most reliable pacing system every built. Between 1970 and 1975, nearly 1400 nuclear pacemakers were implanted without a single battery failure [6].

2.2.3 Piezoelectric Devices

Piezoelectric power sources convert kinetic or mechanical energy into electrical energy [8, 9, 18]. These often, however, do not produce sufficiently large current or power densities for implantable medical device applications. Recently, however, researchers at the Georgia Institute of Technology (Atlanta, GA) developed a prototype nanometer scale generator that produces continuous direct current by harvesting energy from environmental sources such as ultrasound, mechanical vibrations and blood flow [18]. The device was based on vertically aligned zinc oxide nanowires that generate small electrical charges when bent. This piezoelectric device was particularly attractive because zinc oxide has been deemed non-toxic and biocompatible. However, from a technical standpoint, it is difficult to identify a suitable mechanical energy source within the body.

2.2.4 Biological Power Sources

Biofuel and biogalvanic cells have been investigated in the past [3, 10]. In one study by Drake et al. (1970) [3], the authors were able to achieve an open circuit potential of 650mV, maximum power density of 3.5 μWcm^{-2} and maximum current density of 8.3 μAcm^{-2} from an *in-vivo* implanted biochemical fuel cell. There were significant problems, however, with the system. One problem was the fact that the electrochemical cells (EC) eventually appeared to become permanently electrically insulated from the tissue environment, thus becoming inoperative. Protein adsorption on the electrode surface and host fibrotic response and encapsulation were observed. The authors also noted that the near neutral pH and low buffering capacity of the interstitial fluid reduced the electrode performance, with polarization of the cathode occurring due to the formation of hydroxide ions. Electrode poisoning was also an issue due to parasitic reactions and increased polarization that occurred as a result of mixed reaction kinetics. It should be noted that the biochemical fuel cell must be placed within a very homogenous environment, where both electrodes are simultaneously exposed to the same reactants and conditions. The homogeneity of the tissue environment is reflected in decreased performance of the biochemical fuel cells *in-vivo* due to fuel crossover or non-specificity of the reactions taking place at the electrodes. An interest in the clinical application of biochemical or biogalvanic fuel cells quickly faded. In an attempt to solve the problems related to the implantation of biofuel cells into homogenous environments, the development of enzyme-based biofuel cells has been actively pursued [19-21]. With the immobilization of enzymes at the anode and cathode, electrode specific reactions are encouraged, thereby reducing the problems associated with mixed reaction

kinetics. A more in depth description and discussion of enzyme-based biofuel cells will be presented in a later section (Section 3.3).

2.3 TRANSCUTANEOUS ENERGY TRANSMISSION

Radiofrequency [11, 22, 23], ultrasound [12], and optical (infrared) methods [5] have all been studied as possible means of delivering power transcutaneously to implanted medical devices or to internal rechargeable batteries used to power such devices. Transcutaneous energy transmission obviates the need for wire leads that penetrate the skin, thus removing the possibility of infections associated with sites of wire entry through the skin. Despite the high efficiency associated with the commonly used RF transmission (up to 95% [11]) RF may be associated with unwanted heating and tissue damage [24]. Ultrasound and optical methods, though attractive as non-invasive energy transmission modalities, have the disadvantage of quickly becoming attenuated through the skin. In addition, optical methods such as infrared can also be associated with unwanted heating [25].

The use of transcutaneous energy transmission modalities for powering implantable devices never really gained as much momentum as would have been expected. They provide a relatively simple approach for dealing with power issues with respect to implantable devices, because they could easily be applied to the recharging of an implanted secondary battery. In the past, rechargeable batteries have been used to power cardiac pacemakers [26]. A nickel-cadmium battery was developed with a 120mAh capacity. Transcutaneous recharging using two-way telemetry (magnetic coupling) was a feature of the system and more than 6000 units were implanted between 1973 and 1978 [2]. However, the drawback was that the patient was responsible for ensuring that the unit was recharged, which led to compliance problems. Despite the fact that the system was technologically successful and reliable, the subsequent development of Li-ion batteries made it obsolete.

3.0 BIOFUEL CELLS: A REVIEW

This section provides a brief overview of biofuel cell technologies that have been developed over the past several years. Enzyme-based and microbial-based biofuel cells are highlighted and historical milestones described. Microbial fuel cells have recently attracted a lot of attention due to their potential applications as alternative renewable energy sources (producing electricity from waste water treatment [27]). The idea of using bacteria to convert chemical energy stored in waste water sewage into electrical energy to power our homes and businesses is an attractive concept. Progress in the area of enzyme-based biofuel cells have also been accelerated due to potential medical applications, particularly as self-powered biosensors. Such biofuel cells take a biomimetic approach to solving the energy delivery problem in the body, seeking to imitate the biochemical processes involved in glucose metabolism. The goal of this section is to provide a general introduction to biofuel cell technologies and to examine their potential applications in the field of medicine as *in-vivo* energy delivery devices.

3.1 INTRODUCTION TO BIOFUEL CELLS

With recent governmental initiatives to fund research in alternative energy sources, significant work has been performed in studying and developing biofuel cell technologies, particularly studies related to electricity generation from microbial fuel cells, as well as enzyme-based biofuel cells. There appears to be a renewed interest in this field, particularly in the area of microbial fuel cells (MFCs). The ability of microbes to metabolize a vast range of organic substrates as well as their evident ability to transfer high energy electrons derived from their metabolic processes to electrode surfaces have sparked an interest in utilizing microbes to generate electricity from organic fuels and even waste water effluent. The microbes would essentially serve as miniature bioreactors, breaking down organic substrates (such as glucose, lactate, acetate) into carbon dioxide, while liberating electrons derived from the catabolism of the fuels to an electrode in close proximity. This process of electron transfer between the cell and electrode, is perhaps the most important and challenging aspect in the development of efficient biofuel cell systems or any bioelectronic system. Much of the work involved in optimizing the current and power output of biofuel cells is associated with increasing the electron transfer efficiency, not only between whole cells and electrodes, but between enzymes and electrodes as well.

Biological fuel cells or biofuel cells (BFC) are energy transduction devices that convert the chemical energy stored in organic substrates into electrical energy. In order to access the energy from these substrates, enzymes as well as whole cells, particularly microbial organisms, have been used [27-40]. BFCs function by coupling two reactions, the oxidation of a biofuel (by microbes or enzymes) at the anode and the reduction of molecular oxygen to water at the

cathode. High energy electrons derived from the oxidation of glucose, for example, can be transferred from the metabolic processes of various types of bacteria, such as Escherichia Coli [27, 34, 35, 40], or from the redox centers of immobilized enzymes, such as glucose oxidase [19-21], to an electrode. The electrons travel from the anode to the cathode of the BFC, where they are finally accepted by molecular oxygen. Protons released at the anode during the oxidation of the glucose fuel traverse the electrolyte volume - and perhaps also a proton exchange membrane (PEM) separating the two electrodes in the case of MFCs - into the cathode compartment. The availability of electrons and protons at the cathode foster the reduction of molecular oxygen to water. For an efficient system, the only byproducts of a BFC should be carbon dioxide and water. This is an attractive feature of BFCs since the byproducts are non-toxic. BFCs would, therefore, be more environmentally friendly, compared to other electrochemical cells such as lithium-ion or zinc mercury batteries.

Fuel cells (FC), in general, are unique in that they are capable of producing electricity as long as there is a continuous supply of chemical reactants [41, 42]. Batteries, however, eventually lack sufficient quantities of reactants necessary to maintain the desired potential and power output [16]. The working voltage produced by a single cell normally lies between 0.5 and 0.8V [41]. For a hydrogen fuel cell (see Figure 1), the theoretical maximum potential is 1.23V, which corresponds to the standard reduction potentials for the reaction between hydrogen and oxygen to form water. They are generally much more efficient than other energy conversion devices, such as internal combustion engines, which rely on the expansion of gases to do work. The proton exchange membrane (PEM), often used to separate the anode from the cathode compartment in low temperature PEM fuel cells, comprises long chains of

polytetrafluoroethylene (PTFE), commonly known as Teflon, with the side chain ending with sulphonic acid (HSO_3) [41].

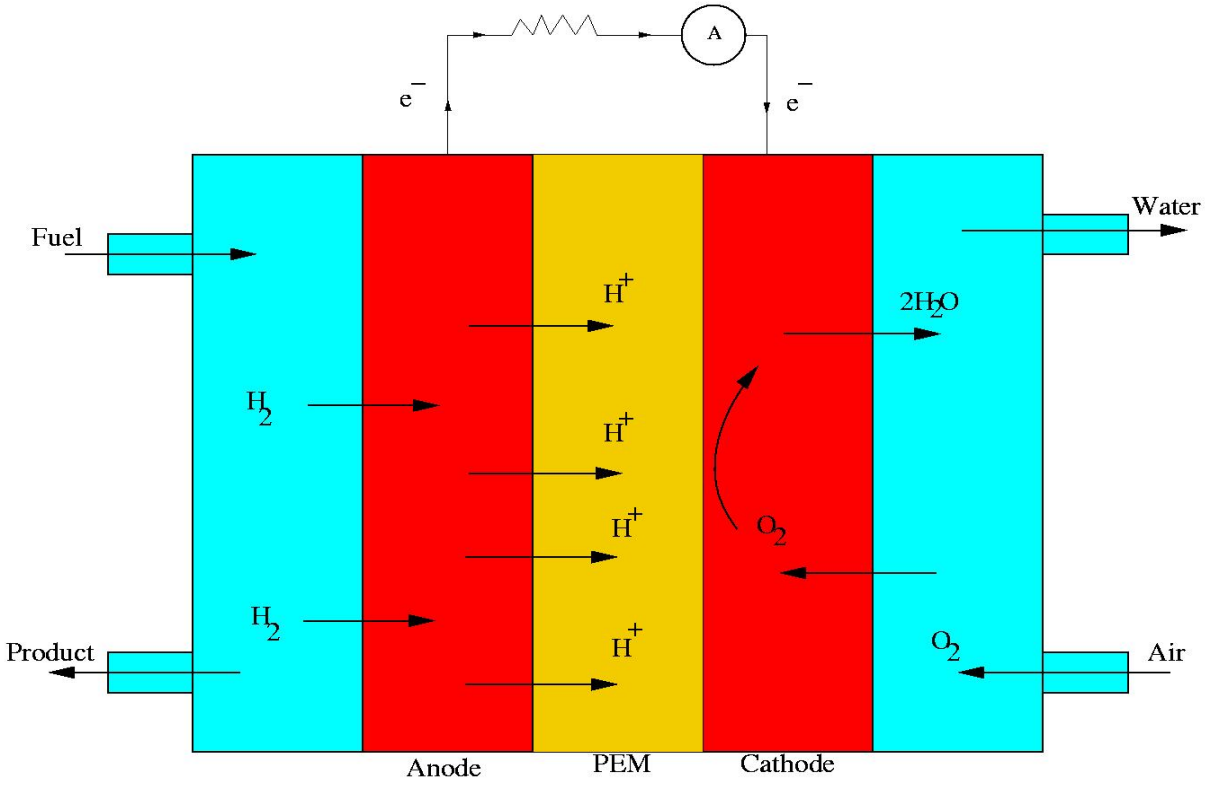


Figure 1: A Hydrogen Fuel Cell

3.2 MICROBIAL FUEL CELLS

Microbial Fuel Cells (MFC) employ bacteria such as Escherichia Coli [27, 34, 35, 40], Rhodoferax ferrireducens [31], Geobacter sulfurreducens [30], Saccharomyces Cerevisiae [43] among others to generate electricity by coupling electron transfer between the cells and the anode electrode to oxygen reduction at the cathode (Figure 2). A mixed bacterial population may also be used [29, 37, 38, 44, 45]. Artificial electron mediators were often introduced to the anode chamber in an effort to increase the efficiency of electron transfer. In several MFC studies, it was reported that electron transfer occurred only at very low efficiencies between the cells and electrodes [46]. One such mediator, methylene blue (labeled MB in Figure 2) has been used by researchers [43] to shuttle electrons between the metabolic processes of the micro-organisms and the anode. Other researchers have succeeded in immobilizing artificial electron mediators such as neutral red (NR) onto the electrode surface, leading to improved electron transfer efficiencies, and higher current and power densities [34]. In a study by Park et al. (2000), a MFC was designed with E. Coli as the bacteria and acetate as the organic substrate [34]. NR was immobilized using N,N'-dicyclohexylcarbodiimide, which can induce the formation of peptide bonds between the amine of NR and carboxy group of the graphite anode. The current density obtained from the MFC with immobilized NR was 3.1μAcm^{-2}, compared to a current density of 1.41μAcm^{-2} for the MFC with a native (unmodified) graphite anode. Apart from the immobilization of artificial electron mediators on the surface of graphite or other carbon-based electrodes, other electrode modification methods have been developed, such as the overlaying of conductive polymers [40] and the attachment of the transition metal Mn [35].

Table 2: Summary of some Microbial Fuel Cell studies.

provides a summary of previous MFC studies by various research groups. In an ideal system, the reactions that would be expected at the anode and cathode of a biofuel cell would be as follows:

Anode

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24e^- + 24H^+$$

Cathode

$$4Fe(CN)_6^{3-} + 4e^- \rightarrow 4Fe(CN)_6^{4-}$$
$$4Fe(CN)_6^{4-} + 4H^+ + O_2 \rightarrow 4Fe(CN)_6^{3-} + 2H_2O$$

The catalysis of glucose to carbon dioxide would be facilitated by the bacterial species or enzyme at the anode. Electron transfer would occur either through the release of metabolic species or through a direct electrical communication between the electron transport proteins in the cell membrane and the electrode. Artificial electron mediators, as described previously, would also act to shuttle electrons between the intracellular metabolic processes and the electrode surface. At the cathode, in order to increase the efficiency of electron transfer to oxygen, potassium ferricyanide is used. Ferricyanide has a very high electron affinity and can be coupled to oxygen reduction as it changes between its reduced state ($Fe(CN)_6^{4-}$) and its oxidized state ($Fe(CN)_6^{3-}$). Protons liberated during glucose catabolism traverse the electrolyte and/or PEM to take part in the oxygen reduction reaction at the cathode.

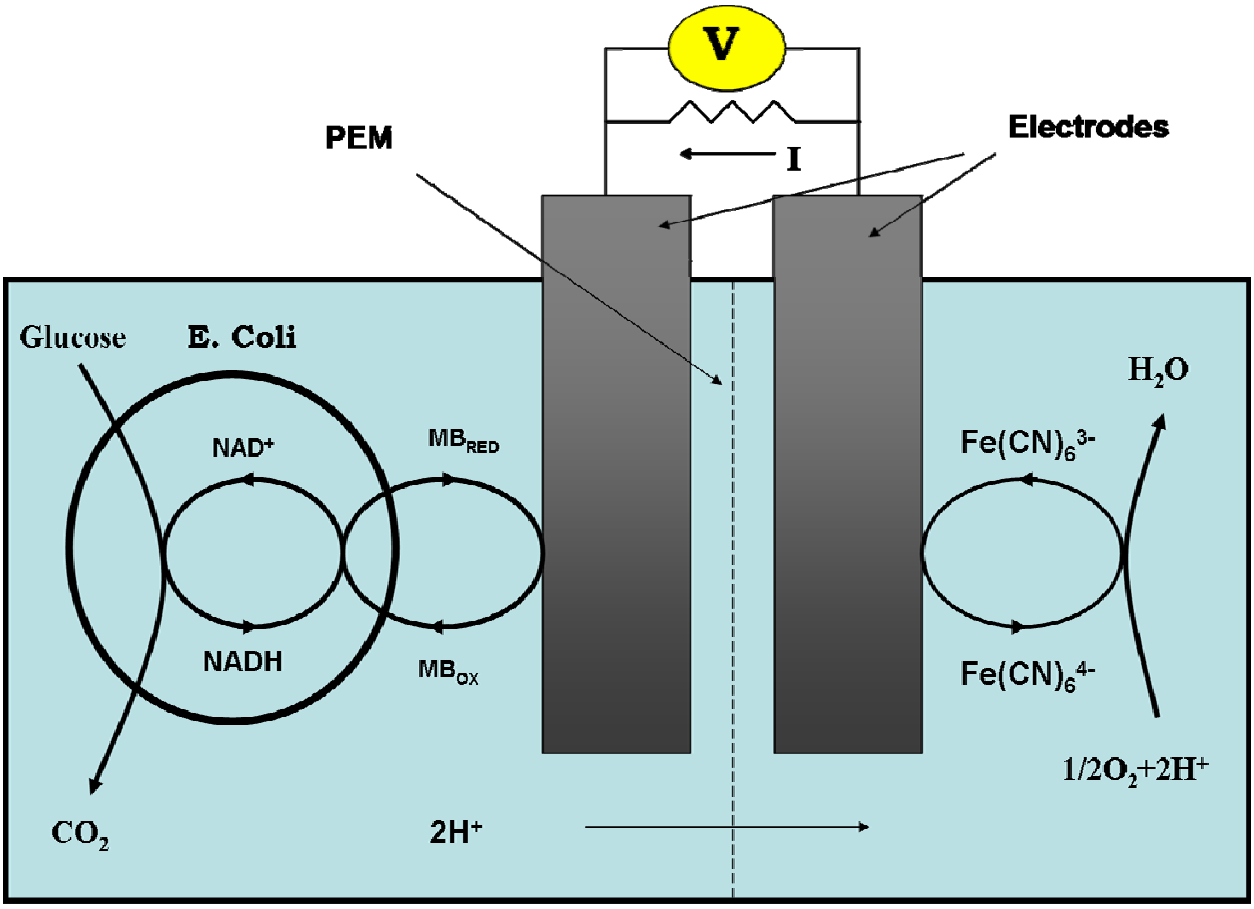


Figure 2: A Microbial Fuel Cell. Methylene blue (MB) is an electron mediator commonly used to increase the efficiency of electron transfer between the whole cells and the electrode surface.

Table 2: Summary of some Microbial Fuel Cell studies.

Microbial Organism	Electron Mediator	Anode	Fuel	Current/Current Density	Voltage	Power Density	Reference
Rhodoferax ferrireducens	none	graphite rods	glucose	31mA/m2	265 mV		Chaudhuri et al (2003)
Rhodoferax ferrireducens	none	graphite felt	glucose	28mA/m2	620 mV		Chaudhuri et al (2003)
Rhodoferax ferrireducens	none	graphite foam	glucose	74mA/m2	445 mV		Chaudhuri et al (2003)
Mixed bacterial culture	none	graphite rods	glucose	30.9mA	664 mV	4.31W/m2	Rabaey et al (2004)
Mixed bacterial culture	none	plain graphite	glucose			3.6W/m2	Rabaey et al (2003)
Geobacter sulfurreducens	none	graphite	acetate	65mA/m2			Bond et al (2003)
Escherichia Coli K12	none	Pyrolytic carbon modified with Pt black and polyaniline	glucose	1.5mA/cm2	469mV	9mW	Schroeder et al (2004)

The applicability of MFCs has been limited due to a number of factors, including: 1) the frequent need to introduce artificial electron mediators at the anode; 2) the lack of long term stability of the electrodes (electrode fouling); and 3) incomplete oxidation of the biofuel used by the microbes [47]. The current output of the MFC may be limited by: 1) the rate of metabolism of the biofuel by the microbial population; 2) the number of electrons transferred (electron efficiency) and 3) the rate of electron transfer (which for many cases depends on the electron carrying ability of the electron mediators).

Microorganisms

Microorganisms used in MFCs may be divided primarily into three groups: 1) organisms that directly transfer electrons through membrane-bound proteins; 2) organisms that transfer electrons through the release of metabolic products; 3) organisms that require the intervention of artificial electron mediators to effectuate electron transfer. Earlier studies investigating the unique ability of microbes to effectuate electron transfer between their metabolic processes and electrodes indicated that this electron transfer ability could only occur at very low efficiencies [39]. As a result, artificial electron mediators were introduced that could penetrate the plasma membranes of the cells, to interact directly with their metabolic processes. The dual nature of the electron mediators (both hydrophobic and hydrophilic characteristics) permitted them to move effectively between the aqueous phase of the extracellular solution and the lipid phase of the cell membrane. Several electron mediators have been utilized with a diverse range of bacterial species. As interest in MFCs has grown, it has been found that certain bacteria are capable of effectuating direct electron transfer at much higher levels of efficiency than observed before [29, 30, 36, 37, 47]. One such type of bacteria is Geobacter sulfurreducens [30]. Members of the

Geobacteraceae family have been shown to be capable of transferring electrons to insoluble electron acceptors such as Fe (III) oxides. Bacteria belonging to this family are not known to produce any soluble electron mediators themselves [48], and therefore, it has been hypothesized that they are able to directly transfer electrons to an electrode surface. In a study by Bond et al., it was shown that 1) G. sulfurreducens can completely oxidize electron donors, using electrodes as the final electron acceptor; 2) electron transfer can occur to electrodes in the absence of electron mediators; 3) electron transfer was due to a population of microbes attached to the electrode that are capable of rates of electron transport similar to those observed for electron transport to Fe(III) citrate [30].

Electrode Design

Various types of electrodes have been designed in an attempt to maximize the current and power output available from MFCs. The difficulty that electrode modifications seek to overcome is the generally low efficiency in electron transfer that normally occurs between the redox active centers in the plasma membranes of the microbes and the electrode surface. The distance between the electrode surface and the redox active species in the cell membrane impacts greatly on electron tunneling between the donor and acceptor. As a result, researchers have experimented with novel ways by which improved electron transfer efficiencies can be achieved. In one study by Schroeder et al. (2003) [40], a novel electrode was described that was constructed from carbon cloth modified by application of a platinum black layer and covered by an electrocatalytic conductive polymer (polyaniline). With this novel electrode, as well as with the application of periodic regenerative potentials to the electrode surface, the researchers were able to acquire current densities as high as 1.5mAcm^{-2}, 9mW power output and a cell potential of

469mV from their MFC. The bacterial population used in the study consisted of Escherichia Coli K12. The authors hypothesized that the conductive polymers contributed extensively to the high current and power densities, by fulfilling a multitude of tasks including: i) functioning as a redox mediator, by interacting with the redox centers of the bacteria; ii) becoming involved in the oxidation of metabolites released by the bacteria; iii) serving as a barrier to large molecules that would otherwise contribute to electrode fouling.

Electron Mediators

The usual means of improving electron transfer efficiencies was through the use of artificial electron mediators, including methylene blue [43], neutral red [27, 34] and 2-hydroxy-1,4-naphthoquinone (HNQ) [28]. There are, however, problems associated with the use of such mediators. Roller et al. (1984) [39] outlined some conditions for efficient MFCs employing electron mediators:

1) the microorganism should be able to rapidly reduce the mediator
2) rapid oxidation of the mediator at the MFC electrode
3) the redox potential of the mediator should be highly negative, in order to achieve the maximum potential difference between anode and cathode, while still being able to couple with the microbial metabolism
4) the mediators should be both relatively stable and soluble in aqueous systems.

3.3 ENZYME-BASED BIOFUEL CELLS

Enzyme-based biofuel cells (enzBFC) have been studied extensively [19-21, 49], particularly for possible applications as *in-vivo* power sources. These BFCs involve the immobilization of specific enzymes onto various electrode (such as carbon fiber) surfaces (Figure 3). Glucose oxidase (GOx) is the primary enzyme employed at the anode. It catalyzes the oxidation of glucose to gluconolactone. At the cathode, either the enzymes laccase [21] or bilirubin oxidase (BOD) [19] have been used to facilitate the reduction of molecular oxygen to water. The enzymes are immobilized by polymer tethers that link the redox active sites of the enzymes to the electrode surface. Mano et al. (2003) developed a miniature compartment-less biofuel cell that was shown to be capable of generating small electrical currents within a living grape [19]. The polymer used for BOD wiring at the cathode was (PAA-PVI-[Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl]$^{+/2+}$) and that used for GOx wiring at the anode was (PVP-[Os(N.N'-dialkylated-2,2'-biimidazole)$_3$]$^{2+/3+}$). The authors reported that the miniature BFC was capable of generating a power output of 2.4 μW and 0.52 V potential.

Eliminating the proton exchange membrane

The direct wiring of enzymes to electrode surfaces has a number of advantages with respect to BFC systems, including improved efficiency in electron transfer and obviation of a proton exchange membrane to separate the anode and cathode compartments. Using dissolved electron mediators to shuttle electrons between the enzymes and the electrodes made the PEM necessary in order to prevent electroxidation of the anode mediator at the cathode and reduction of the cathode mediator at the anode [50-52]. The PEM was also necessary to prevent the occurrence of

fuel crossover, where electrons from the organic substrate would be transferred directly to oxygen, rather than to the electrode, the desired electron acceptor. In such an instance, there would be a very low incidence of electron transfer to the electrode, resulting in small current and power densities. Modification of the electrode surface with enzymes contributed to greater specificity in the reactions that occurred at each electrode. A summary of recent work performed in the field of enzyme-based biofuel cells can be found in Table 3.

Electrical communication between enzymes and electrodes

The lack of electrical communication between enzymes and electronic elements has been identified as a fundamental difficulty in the development of bioelectronic systems [53]. This challenge can be explained by: 1) Marcus theory; 2) superexchange charge transfer theory; and 3) superior tunneling paths in proteins. In order to overcome this difficulty, a number of methods have been employed to improve the efficiency of electron transfer: 1) structural engineering of proteins with electron relays; 2) immobilization of redox enzymes in conductive polymers or redox active polymers; and 3) steric alignment of proteins on electron relays associated with electrodes [53].

.

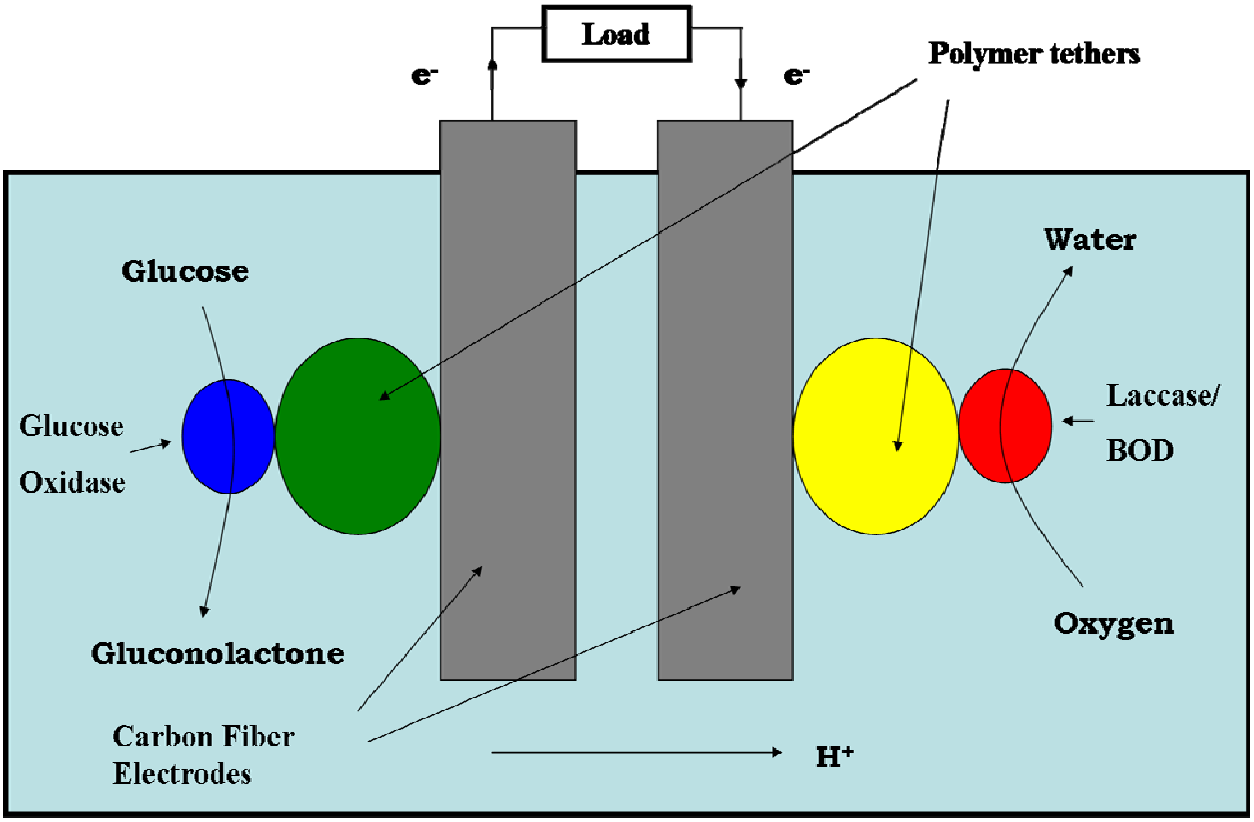


Figure 3: An Enzyme-based Biofuel Cell

Table 3: Summary of some research studies on Enzyme-based Biofuel Cells.

Anode	*Cathode*	*Fuel*	*Voltage*	*Power*	*Reference*
Glucose oxidase from Aspergillus niger with polymer (polymer I) on carbon fibers. The polymer establishes an electrical comection between the reaction center of glucose oxidase to the carbon fiber.	Bilirubin oxidase from Trachyderma tsunodae and copolymer of polyacrylamide and poly(N-vinylimidazole) complexed with [Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl]$^{+/2+}$ II (on carbon fiber).	glucose	0.52 V	1.9 µW (4.3 µW/mm^2	Mano,Mao,Heller(2002)
Glucose oxidase from Aspergillus niger electrically wired by polymer I on carbon fiber.	Bilirubin oxidase from Trachyderma tsunodae wired by polyer II (on carbon fiber).	glucose (implantation in a grape	0.52 V	2.4 µW/mm^2	Mano,Mao,Heller(2003)
Glucose oxidase wired by polymer II on carbon fiber.	Copper enzyme laccase wired with redox polymer I (on carbon fiber).	glucose	0.88 V	350 µW/cm^2 (pH 5)	Soukharev,Mano,Heller (2004)
Copper(II)-poly(acrylic acid) film covalently linked to pyrroloquinoline quinone (PQQ) and flavin adenine dinucleotide (FAD) cofactor. Apo-glucose oxidase linked to FAD sites.	Copper(II)-poly(acrylic acid) film with covalently attached cytochrome c (Cyt c) which is further linked to cytochrome oxidase.	glucose	120 mV	4.3 µW (loading resistance of 1kΩ)	Katz, Willner (2003)
Glucose oxidase linked to redox polymer	Laccase from Coriolus hirsutus linked to redox polymer	glucose	0.4 V	137µW/cm^2 (37°C)	Chen et al (2001)

4.0 NADPH OXIDASE

Electron transport through NADPH oxidase

Electron transport is generally not observed across the plasma membrane of eukaryotic cells. However, recent data demonstrates that such electron currents do exist in white blood cells [54-57]. Schrenzel et al. (1998) demonstrated that small currents can be measured across the plasma membrane of human eosinophil granulocytes [57]. The experiments were performed based on the hypothesis that the membrane associated enzyme NADPH oxidase, found in blood phagocytes, generates reactive oxygen species (ROS) through electron transfer from NADPH to extracellular oxygen (Figure 4). The magnitudes of the currents recorded across the cellular membranes were on the order of 10 to 20 pA per cell. NADPH oxidase is a complex of several proteins that associate with each other when activated [54, 58, 59]. Activation *in vitro* was achieved by application of phorbol-12-myristate-13-acetate (PMA) [55-57]. PMA most likely activates NADPH oxidase through the protein kinase C (PKC) pathway [58]. NADPH oxidase has also been found in microglia [60], vascular smooth muscle [61], endothelial cells [62] and hematopoietic stem cells [63]. The presence of an electron transport chain in the plasma membranes of white blood cells and glia means that it may be possible for electrons to be ”hijacked” and diverted to a nearby electrode, such as was observed in the case of microbial fuel cells. Interactions between the cells and the electrode surface become important. The surface of

the electrodes may be modified to improve these interactions, and facilitate the electron transfer process. Electron mediators such as methylene blue and neutral red may not be ideal due to the potential for toxicity with time.

Structure of NADPH Oxidase

NADPH oxidase consists of five primary components, namely p40PHOX, p47PHOX, p67PHOX, p22PHOX, gp91PHOX (PHOX for PHagocyte OXidase). Three of the five components - p40PHOX, p47PHOX and p67PHOX - exist as a complex in the cytosol of resting cells. The other two are primarily located in the membranes of secretory vesicles and specific granules. These two components occur as a heterodimeric flavohemoprotein known as cytochrome b558 [64]. A sixth cytosolic component has also been identified, namely Rac, which belongs to the Rho-subfamily of small GTPases [58]. Upon activation, the components of NADPH oxidase are transferred to the plasma membrane of the cell through vesicular fusion. Reactive oxygen species produced by activated NADPH oxidase of phagocytic cells play a key role in immune responses to pathogens. Life-threatening conditions, such as chronic granulomatous disease (CGD) result from genetic defects that target NADPH oxidase, compromising its function and ability to produce ROS. However, uncontrolled production of ROS has been associated with tissue damage and several diseases.

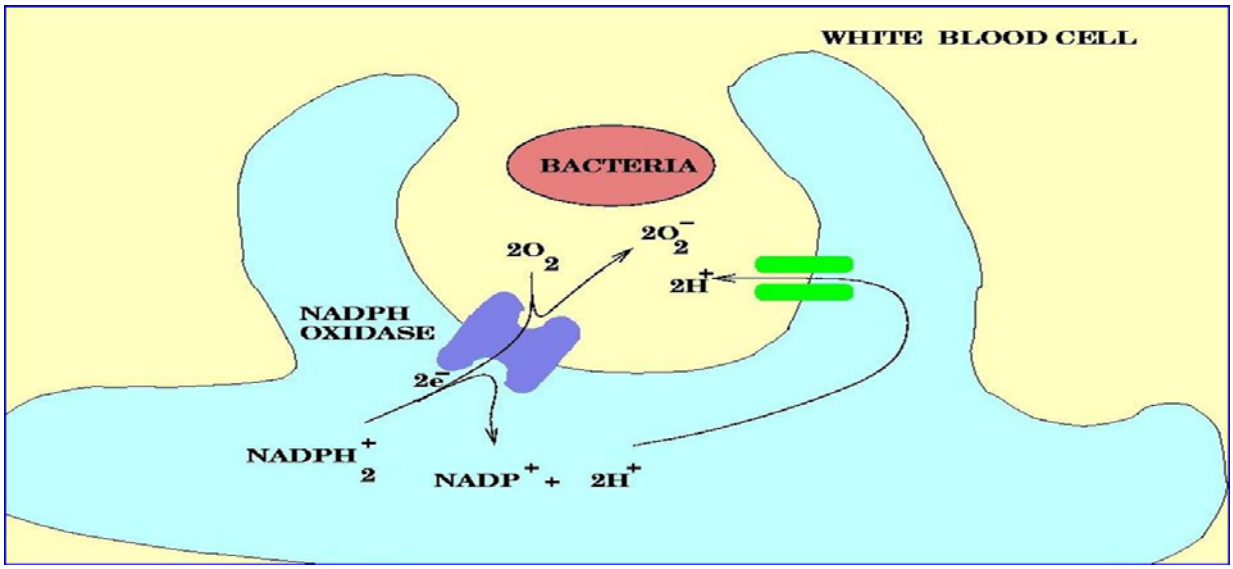


Figure 4: White blood cell NADPH oxidase activity during respiratory burst.

NADPH Oxidase and Disease

Several studies have linked NADPH oxidase to various diseases and disease states including Parkinson's [60], Alzheimer's [65], oxidative damage resulting from epileptic seizures [66] and hypoxic pulmonary vasoconstriction [67]. The pentose phosphate pathway (PPP) and NADPH oxidase activity are normally very tightly regulated to ensure that there is minimal or no damage resulting from the overproduction of reactive oxygen species (ROS) in the physiological environment. In certain circumstances, however, this regulated balance in ROS can be compromised, and the NADPH oxidase complex can become overactive, either leading to or exacerbating pathological conditions, such as those previously described.

Electrical communication between NADPH oxidase and electrodes?

In order for ROS to form, high energy electrons from the PPP of glucose metabolism must be transferred to oxygen. In this case, it might be possible to channel some of those electrons to an electrode and through an electric circuit (Figure 5), powering implanted devices. The most fundamental problem to tackle is, therefore, promoting the transfer of metabolically derived electrons to interfacing, biocompatible, implanted electrodes. For certain neurological disorders where NADPH oxidase has been shown to be overactive, electrons could be harnessed from the metabolism of the associated cells to power a drug delivery device. This device would have special target drugs that can then be used to suppress an epileptic seizure or slow the progress of neurodegenerative disorders including Parkinson's and Alzheimer's disease.

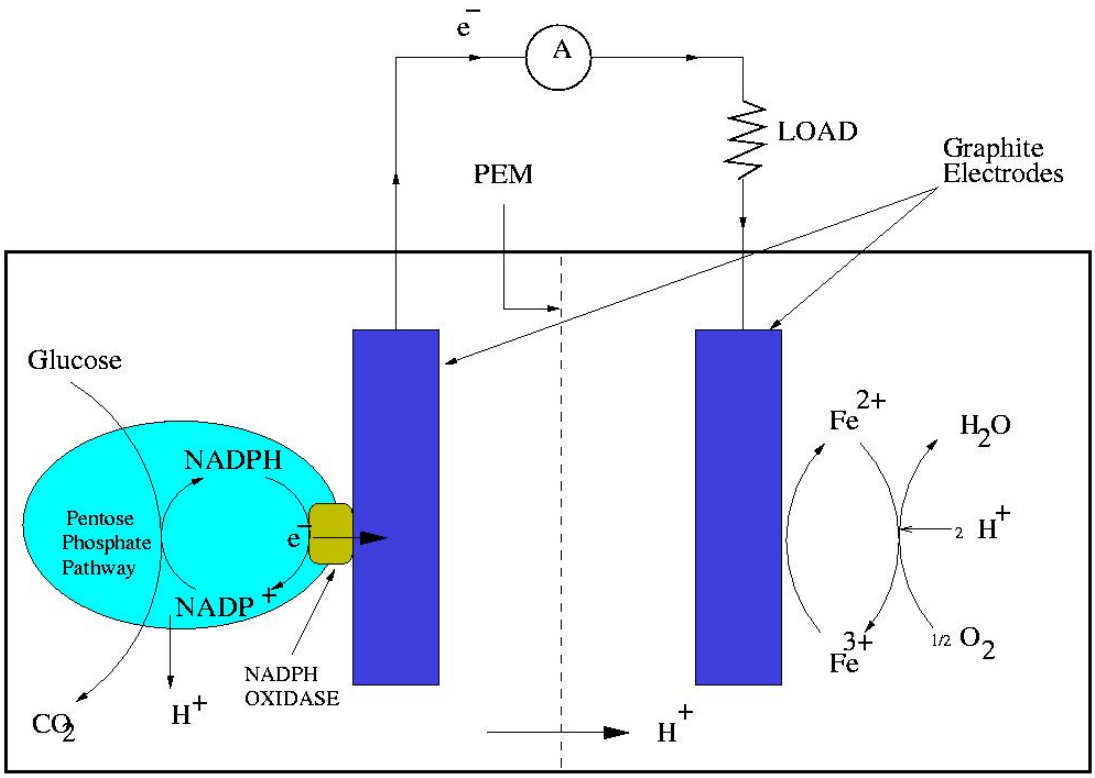


Figure 5: A theoretical eukaryotic cell-based biofuel cell that relies on electronic coupling between NADPH oxidase and a carbon-based electrode.

5.0 LEUKOCYTE-BASED BIOFUEL CELL STUDY

5.1 ABSTRACT

The electrochemical activity of human leukocytes (white blood cells) was investigated by measuring the current output and open circuit potential produced from a proton exchange membrane fuel cell (PEMFC) bioreactor with the cells of interest at the anode. Human polymorphonuclear leukocytes (PML) and peripheral blood mononuclear cells (PBMC) were isolated from whole human blood, suspended in phosphate buffered saline (PBS) solution and introduced to the anode compartment of a proton exchange membrane fuel cell (PEMFC). The leukocyte-based PEMFC was allowed to discharge its current across a 100 ohm resistor. Measurements of current and open circuit potential were made over time. Small currents densities on the order of microamps and open circuit potentials between 100 and 500mV were obtained from the leukocyte-based BFCs. In the absence of cells, small currents were also observed indicating the presence of small background reactions. The results of this study suggest that white blood cells are electrochemically active and contribute to the observed currents from the BFCs. The results of this study, however, also emphasize the complexity of electrochemical systems, particularly those involving biological organisms.

Keywords: proton exchange membrane, biofuel cell, PML, PBMC, K562

5.2 INTRODUCTION

There has been a lot of work previously on developing microbial fuel cell (MFC) technologies [28-30, 34, 35, 37, 38, 40, 43, 47, 68]. In these studies, it was repeatedly demonstrated that many types of bacteria can be encouraged to transfer metabolically derived electrons to an electrode. Some bacteria have revealed their ability to self-mediate electron transfer without any external intervention [29, 30, 36, 37, 47], while other types of bacteria demonstrated a higher electron transfer capacity only with the introduction of certain artificial electron mediators or electron carriers that shuttle electrons between the metabolic processes of these organisms and the electrode surface [28, 34, 35]. With various electrode modifications, including immobilization of artificial electron mediators [34, 35] and the placing of electrically conducting polymers [40] onto the electrode surface, higher current and power densities continue to be achieved.

The ability of certain bacteria to transfer electrons directly to metal or carbon-based electrodes has been attributed largely to electron transport chain proteins that reside in their cell membranes [29, 30]. Bacteria do not have organelles such as mitochondria and, therefore, metabolism occurs in the cytosol [69]. Geobacter sulfurreducens is a metal reducing bacteria that has been shown to attach to electrode surfaces and are capable of directly mediating electron transfer to the electrodes [30]. These bacteria among others belonging to the Geobacteraceae family, including D. acetoxidans and G. metallireducens, demonstrated an ability to use electrodes as electron acceptors for the oxidation of organic compounds [29]. A membrane-bound Fe(III) reductase complex (comprising an 89 kDa c-type cytochrome) used by G. sulfurreducens to reduce Fe(III) oxides [70, 71] is a possible pathway for the electrons to reach the electrode surfaces to which the bacteria are attached [30].

Electron transport, pervasive in the plasma membranes of microbes, is generally not observed across the extracellular membranes of eukaryotic cells. The presence of an electron transport chain in the extracellular membranes of microbes provides a greater likelihood of electron tunneling occurring between the membrane-bound species and the electrode surface. An electron transport system within the cell, such as in the case of the mitochondrial electron transport chain, would be effectively isolated from the electrode surface due the presence of the extracellular membrane. In eukaryotic cells, electron transport is a process that is normally relegated to the membranes of intracellular mitochondria. Recently, however, a number of research articles have been published demonstrating that such electron currents do in fact also exist among certain white blood cells. Schrenzel et al. (1998) showed that small currents can be measured across the plasma membranes of human eosinophil granulocytes [57]. The experiments were performed based on the hypothesis that the membrane associated enzyme NADPH oxidase, found in blood phagocytes (e.g. neutrophils and eosinophils), generates superoxide through electron transfer from NADPH to extracellular oxygen. The magnitudes of the currents recorded across the extracellular membranes were on the order of 10 to 20 pA per cell in these studies.

The purpose of this study is to investigate whether electron transfer can occur between the metabolic processes of human white blood cells and the carbon fiber electrodes of biological fuel cells. The hope is to encourage electron transfer between NADPH oxidase and the electrodes. From a much broader perspective, the ultimate goal of this project is to develop a BFC that may be used within the physiological environment to provide energy to low-power implantable medical devices, such as miniature drug delivery devices or biosensors. It is expected that with further advances in micro- and nano-technologies that lead to dramatic reductions in the size of electronic circuits, energy requirements will decrease for implantable

devices. In addition, the development of novel biomaterials for improved biocompatibility will mean that new electrodes can be designed to facilitate specific electrochemical interactions while reducing inflammatory responses. With such advancements, it will become possible to design an implantable BFC that can transduce the biochemical energy of cells into electrical energy. Our research is currently focusing on two main areas: i) *in-vitro* models to demonstrate that electron transfer can be facilitated between human cells and electrodes; and ii) exploring possible means of increasing current and power densities from the BFC.

5.3 EXPERIMENT I: MATERIALS AND METHODS

5.3.1 White blood cell isolation

Human white blood cells, specifically polymorphonuclear leukocytes (PML) and peripheral blood mononuclear cells (PBMC), were isolated from 10ml of whole human blood using a standard Ficoll-Paque^{TM} gradient. PMLs include neutrophil, basophil and eosinophil granulocytes, while PBMCs include the B and T lymphocytes as well as monocytes. This study received prior approval from the Institutional Review Board (IRB) of the University of Pittsburgh. The isolation process involved the very careful addition of whole human blood to an equivalent volume of the Ficoll solution initially placed in a 50ml tube. Very little disruption of the interface between the Ficoll solution and the blood volume should occur, and two distinct layers should be perceived without significant mixing at the interface. The two-layer mixture was then centrifuged at 2000rpm for 20 minutes, after which four clearly defined layers could be perceived: i) erythrocytes or red blood cells at the bottom; ii) clear Ficoll-Paque solution, iii) thin layer of PBMCs; iv) blood plasma at the top (Figure 6). The PBMCs were easily removed by aspiration using a pipette. The PMLs, however, are more difficult to extract using the Ficoll-Paque method of isolation. A very thin, almost unperceivable layer of PMLs normally exists just above the erythrocyte layer at the bottom of the gradient. The cells from this region were carefully aspirated using a pipette and washed at least twice by centrifugation in 50ml 1X phosphate-buffered saline (PBS, pH 7.4, Invitrogen, Carlsbad, CA) solution at 1700rpm for 15 minutes. After washing, the cells were finally resuspended in 10ml PBS at a cell density on the

order of 10^6 cells/ml. The final cell density was determined using a hematocytometer and light microscope.

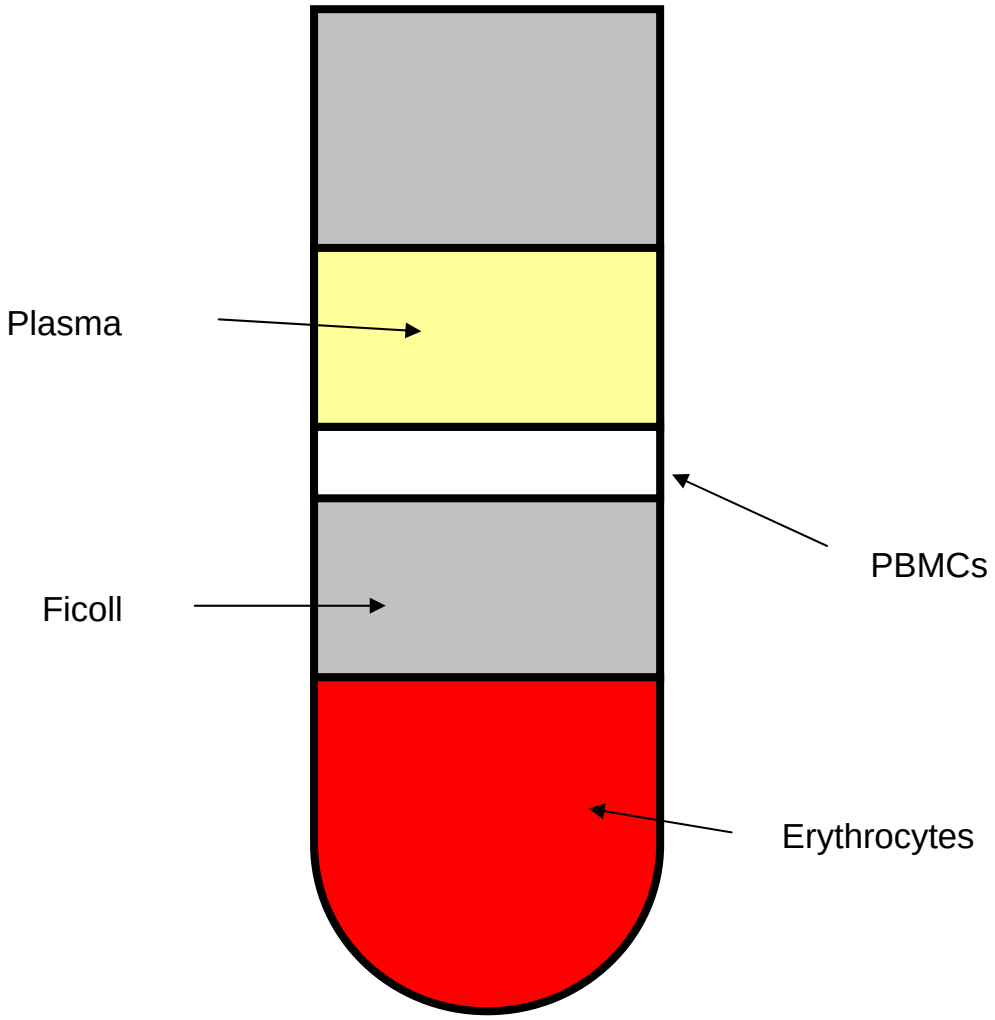


Figure 6: **Ficoll-Paque gradient for the isolation of human white blood cells from whole human blood.**

5.3.2 Biofuel cell Bioreactor Preparation

The cell suspension was introduced into the anode compartment of a biofuel cell bioreactor (National Center of Biotechnology Education (NCBE), University of Reading, U.K.). The cathode solution was made up from 5 mL of 0.1 M potassium ferricyanide and 5 mL PBS (pH 7.4) to a final volume of 10mL and ferricyanide solution concentration of 0.05M. The anode and cathode compartments were separated by a Nafion-117 proton exchange membrane (PEM, Sigma-Aldrich, St. Louis, MO). Each compartment had a total working volume of 10ml. Carbon fiber sheets (NCBE, University of Reading, U.K.) were used to construct the anode and cathode electrodes. The carbon sheets were used due to their high specific surface area. SEM scans of the carbon fiber sheets reveal a highly interwoven matrix of cylindrical tubes (Figure 7). The dimensions of the electrodes were approximately 2cm x 2cm. The biofuel cell was connected to a 100 Ω resistor, across which measurements of electric potential were made using a standard multimeter. A switch was used to make measurements of both the open and close circuit potentials across the resistor (Figure 8).

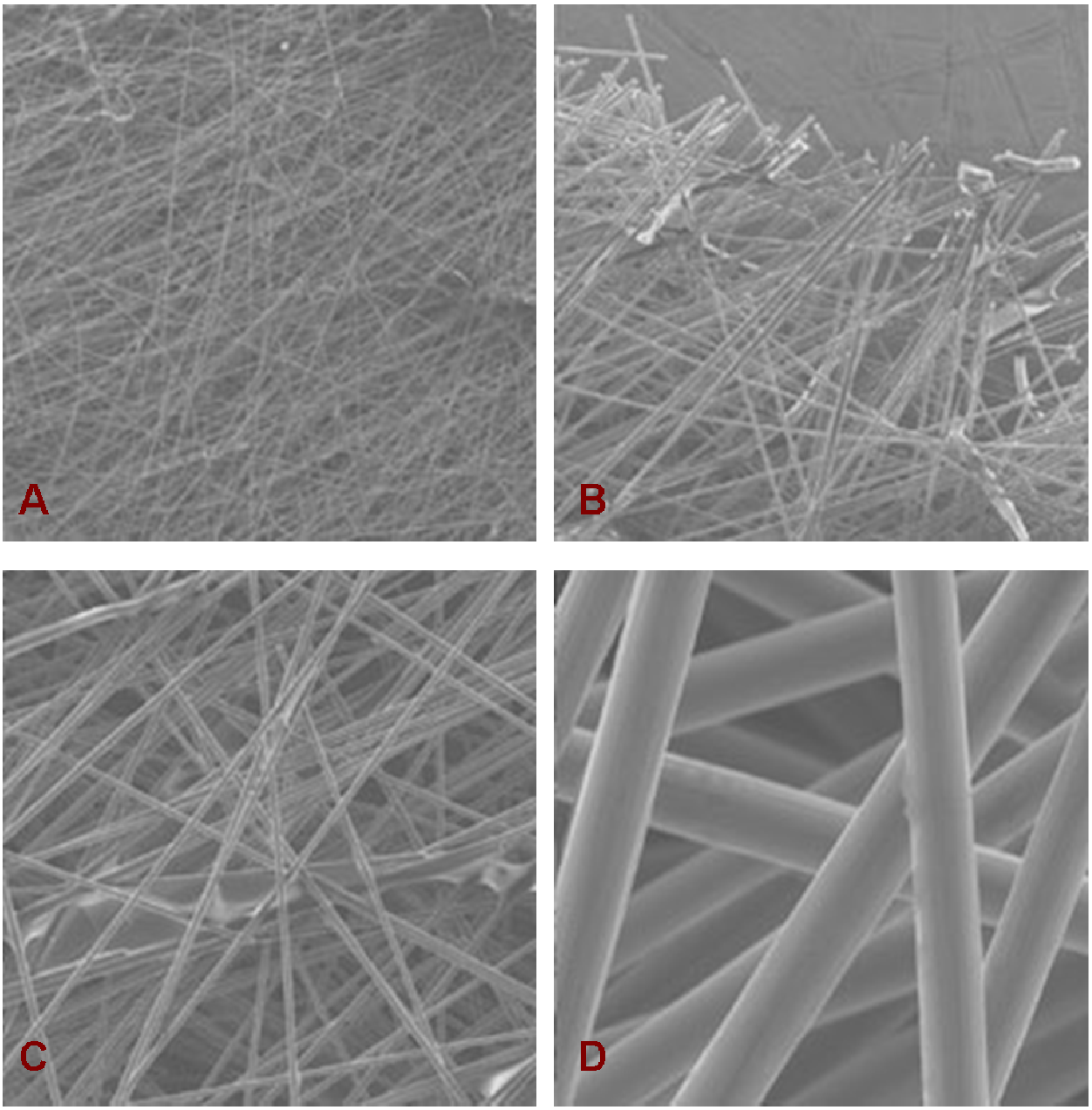


Figure 7: **SEM scans of carbon fiber electrodes at A) 25X, B) 100X, C) 150X and D) 1500X.**

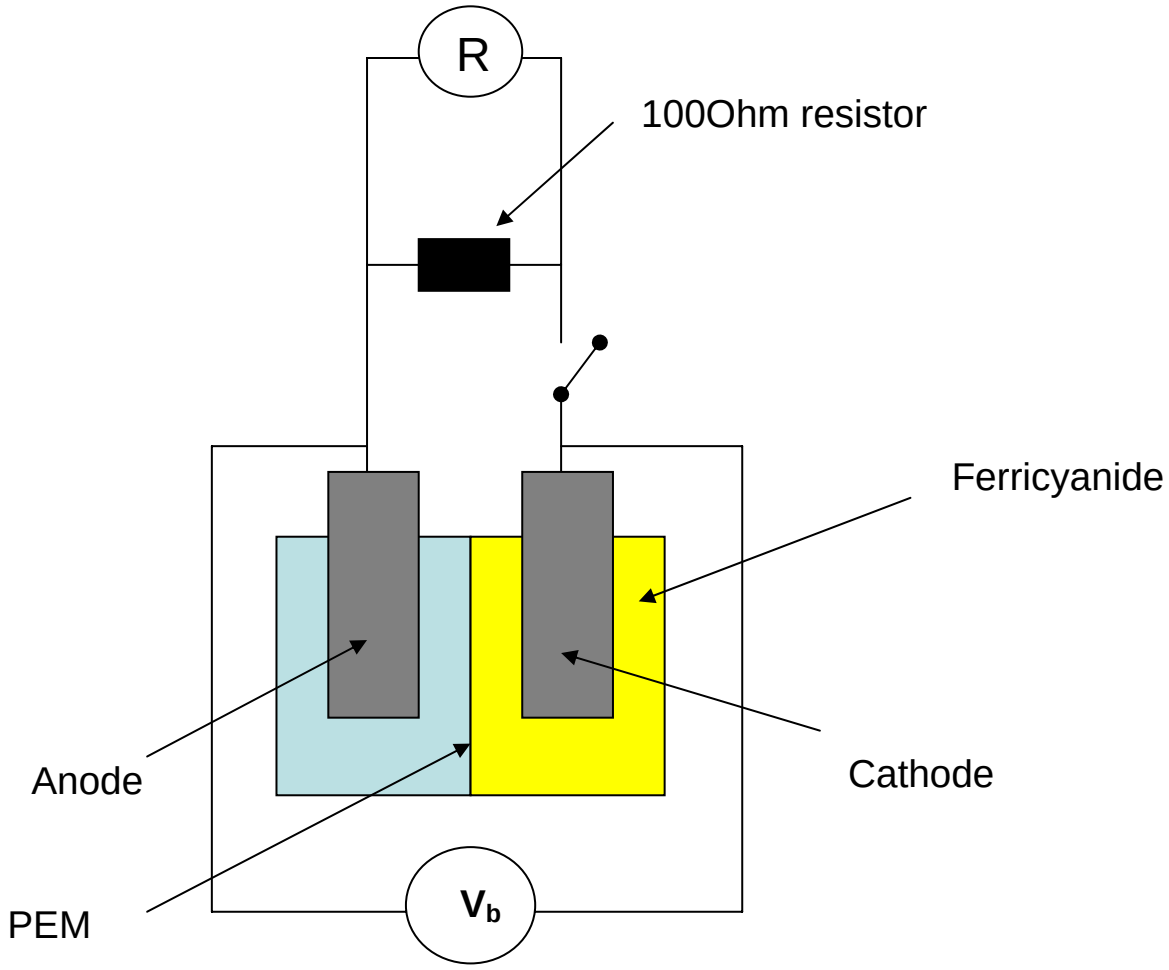


Figure 8: Circuit setup for measurement of electrical parameters (open circuit potential and current).

5.3.3 Cell activation and chemical manipulation

In order to achieve cell (and NADPH oxidase) activation, 10ul each of phorbol-12-myristate-13-acetate (PMA, 5μg/ml) and calcium ionomycin (500μg/ml) were added to the 10mL cell suspension to reach final concentrations of 5ng/ml and 500ng/ml, respectively. Based on the theory that NADPH oxidase can mediate electron transfer to an electrode, two hypotheses for the mechanism involved in the electron transfer process were developed: 1) direct electron transfer occurs (through electron tunneling) between the electron donor site of the NADPH oxidase complex (cytochrome b558) and the electrode; 2) indirect electron transfer occurs as a result of the oxidation of the reactive oxygen species (ROS) released by the cells during the simulated respiratory burst at the electrode surface. The effect of a non-ionic detergent, Igepal (Sigma Aldrich, St. Louis, MO), was investigated. If any observed currents are mediated by NADPH oxidase and electron transfer occurs through either of the proposed mechanisms, then the introduction of the detergent would effectively extinguish the currents by disrupting the lipid membrane and consequently the enzyme complex.

5.4 EXPERIMENT I: RESULTS AND DISCUSSION

BFC Currents and Open Circuit Potentials

Small electrical currents on the order of 0.1 to 0.2mA were observed for the two leukocyte-based biofuel cells containing PBMCs in one and PMLs in the other (Figure 9). The currents from the PML-based BFC increased from an initial average current of 0.17 mA to 0.19 mA by the end of the recording period, while for the PBMCs the average current increased from 0.12 to 0.16 mA. As described previously, current values were determined by measuring the electric potential across a 100 Ω (+/- 5%) resistor using a BK Tookit 2707A multimeter (accuracy +/- 0.05% + 1 digit). The current measured was associated with a systematic error of +/- 6%. Currents measured for the individual PML and PBMC samples are shown in Figure 10 and Figure 11, respectively. A clear difference in the behavior of the current output over time can be discerned between the PMLs (Figure 10) and the PBMCs (Figure 11). In one experiment, the current from the PBMC-based BFC was much lower than expected, starting at 0.031 mA, and then increasing dramatically to a final current of 0.18 mA (PBMC Sample I). In another instance, there was an unexpected decrease in the current output of the PBMC-based BFC and then a subsequent increase in the current (PBMC Sample II). PBMC sample III had slightly larger currents than the first two samples; however, there was also some variation in the current output, with a slight dip in the current measurement at about 25 minutes. Compared to the PMLs, the current output of the PBMCs proved to be very unstable, with dramatic changes observed during small perturbations of the fuel cell apparatus. Such dramatic changes during perturbation of the BFC apparatus were not observed in the case of the PMLs. One explanation for the instability in the current output could be postulated to be a faulty connection between the BFC and the electronic

circuit used for the measurement of current and potential. The instability in the current output could be blamed on faulty connections between the BFC and the electronic circuit used for the measurement of current and potential.

The open circuit potentials recorded from the BFCs also proved to be rather unstable for both the PMLs and PBMCs, normally starting at an initial value of about 500mV and decreasing to final potentials of about 300mV by the end of the recording period (results not shown). The fact that an increase in current was observed with time for both the PMLs and PBMCs in spite of a decreasing open circuit potential was a confusing result. Due to polarization effects and increases in internal resistance normally associated with electrochemical systems, a decreasing open circuit potential would normally be associated with a decreasing current. In these studies, however, this was not the case.

Background reactions and currents

A weak correlation between current and cell density was shown to exist for both the PML and PBMC-based BFCs (Figure 12 and Figure 13). For the PBMCs (Figure 13), the average current for Sample 1 was much lower than would be expected for the corresponding cell density when compared to Samples 2 and 3. There are a number of reasons that could explain this observation. The lack of good cell-electrode electrical interactions, cell inactivity or low cell activity, and unusually high internal resistance due to extensive electrode fouling and PEM obstruction could all be possible explanations for the lower than expected current. The lack of a stronger relationship between cell density and the average current may also suggest that other factors apart from the cells themselves are contributing in whole or in part to the observed currents.

The issue of possible background currents occurring within the BFC system as a result of non-cellular interactions was also supported by the observance of significant currents in the absence of cells at the BFC anode (Figure 14, Figure 15, Figure 16). These currents were normally smaller in magnitude than the currents observed in the presence of the cells, particularly during the initial measurement period (Figure 16). The currents increased gradually with time. There was also an unusual instance where the magnitude of the current measured for one of the controls was on the same order of magnitude as that obtained from the experiments with the white blood cells (Figure 15 and Figure 16). The profile of the current in this particular instance; however, was slightly different from that obtained with the cells. The current in this instance appeared to increase gradually from its initial value and began to decrease after achieving a peak current value of 0.14 mA. This dome-shaped profile was not characteristic of any of the experimental cases involving either the PBMCs or PMLs.

The addition of PMA and ionomycin was often associated with an increase in the current output of the BFC, indicating that both activating agents do have some electroactive capacity, a fact that further confounds the experimental results and contributes to the complexity of the bio-electrochemical system. The electrochemical activity of all chemical agents used must be taken into consideration in further experimental designs.

Electricity generating ability of white blood cells

Currents from the BFC containing cells were generally higher than currents from those without cells at the anode (Figure 16), strongly suggesting that the white blood cells are contributing to the observed currents. However, the exact mechanism by which the cells transfer electrons to the electrode as well as the extent to which they contribute to the observed currents is not

immediately apparent. There is the possibility that electron transfer might occur via the reduction of superoxide radicals released by NADPH oxidase at the anode. NADPH oxidase might also be capable of directly mediating electron transfer to the electrodes without the intervention of chemical mediators. Such a direct electron transfer to plain carbon fiber electrodes, however, is not likely due to the environmental conditions (an aqueous, oxygenated anode compartment) which are unfavorable for electron tunneling between the redox active centers and the electrode surface. When the non-ionic detergent, Igepal^{TM}, was added to the cell suspension comprising the PMLs, a decrease in current was observed. In spite of the decrease, however, the current generated by the BFC was not completely extinguished (see Figure 16) and a subsequent increase in the current, was observed. This increase, however, should not be surprising. Disruption of the cell membrane would inevitably lead to expulsion of the intracellular contents of the white blood cells. A large number of the biochemical species that would be expelled are electrochemically active, such as the numerous coenzymes involved in the various cellular metabolic pathways. These species could all interact electrochemically with the electrode surface.

Impurity of PMLs

Polymorphonuclear leukocytes include neutrophils, basophils and eosinophils. In this study, an attempt was made to isolate these cells from whole blood on a Ficoll gradient. The cells were aspirated using a pipette from the region just above the red blood cell layer. Although a large proportion of these cells may actually be PMLs, many of the cells isolated may also belong to the PBMC type, including the B and T lymphocytes and monocytes. The purity of the PML samples is, therefore, questionable with respect to these experiments and consequently, it is impossible to

attribute the currents observed entirely to the PMLs. The currents observed from the PML-based BFCs may be the result of contributions from the PMLs as well as the PBMCs.

Possible sources of background currents

The observation of currents in the absence of the white blood cells as well as the very weak correlation between cell density and current are both indicators that there are background reactions unrelated to the cells that are present within the electrochemical system. The decrease in the current produced from the PML-based BFC upon introduction of the Igepal detergent also cannot be confidently taken as the consequence of an electrical pathway disruption between cell membrane proteins (of NADPH oxidase) and the carbon fiber electrode surface (Figure 17). A much simpler explanation may be presented, such as the formation of an Igepal overlay on the electrode surface that impedes the kinetics of the unknown reaction.

Closer inspection of the bioreactor system and the electrode-circuit connections revealed the formation of a bluish-green substance at the interface between the carbon fiber electrodes and the electrical circuit. It was also noted that the highly absorbent carbon fiber anode and cathode electrodes were thoroughly soaked with their respective compartment solutions. It was hypothesized that significant background currents may have occurred as a result of chemical reactions at the carbon fiber electrode-circuit interface. The alligator clips used to attach the carbon fiber electrodes to the resistor had undergone some corrosion, which likely resulted in an electrochemical interaction when contact with the compartment solutions were made. The experiment was, therefore, repeated with small strips of stainless steel inserted between the alligator clips and carbon fiber electrode connections. Stainless steel was selected due to the fact that it is both easily available and relatively inert. In Figure 14 and Figure 15, one would

immediately notice that the magnitude and profile of the current obtained for the control experiment when stainless steel was used to protect the interface differed greatly from the previous control experiments with PBS at the anode.

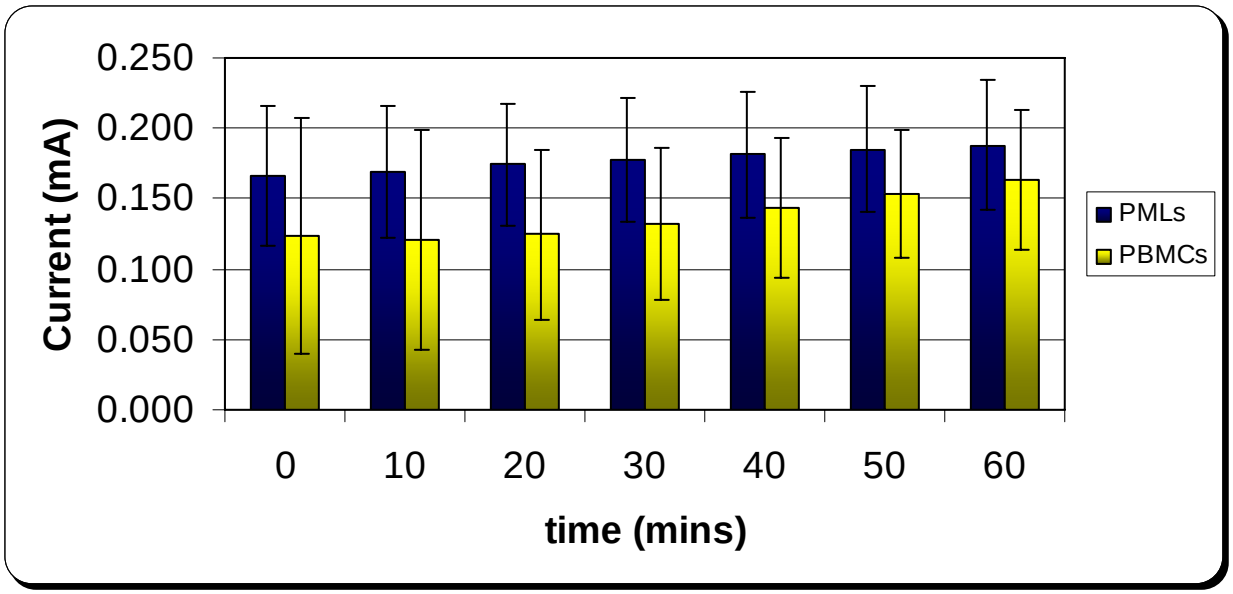


Figure 9: **Average currents (n=3) for 1) PML-based BFC and 2) PBMC-based BFC.**

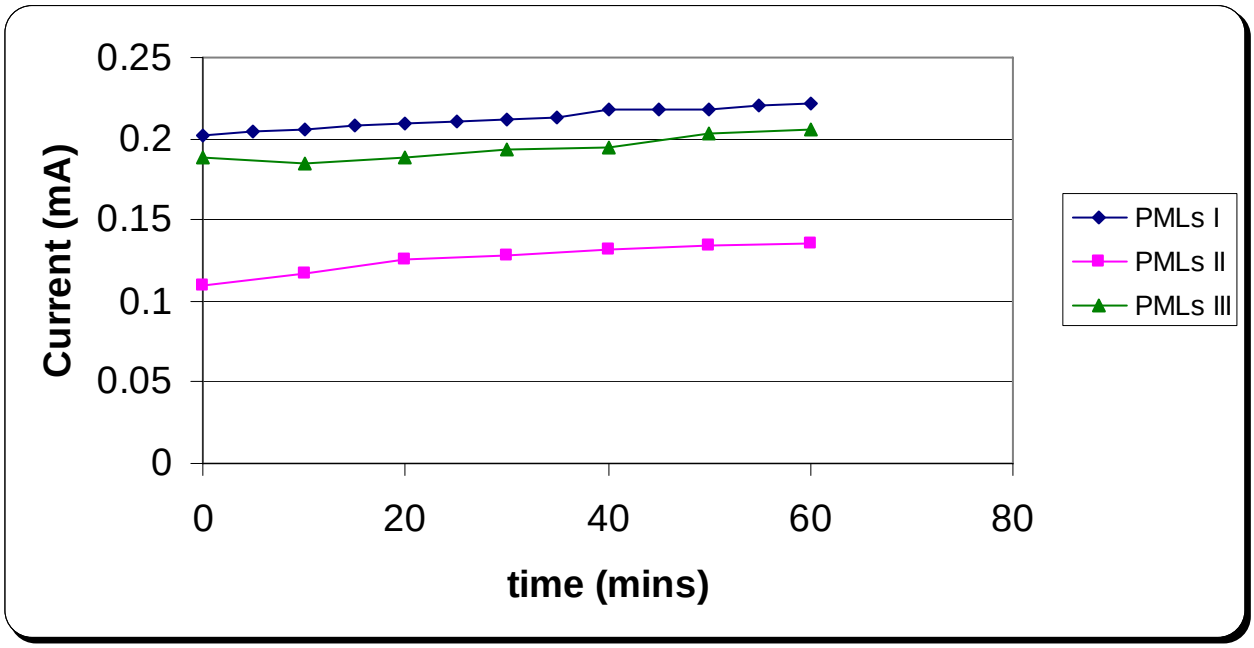


Figure 10: **Current output from a BFC containing human PMLs. A gradual increase in current was observed over the one hour period of measurement for all samples (current measurement accuracy +/- 6%).**

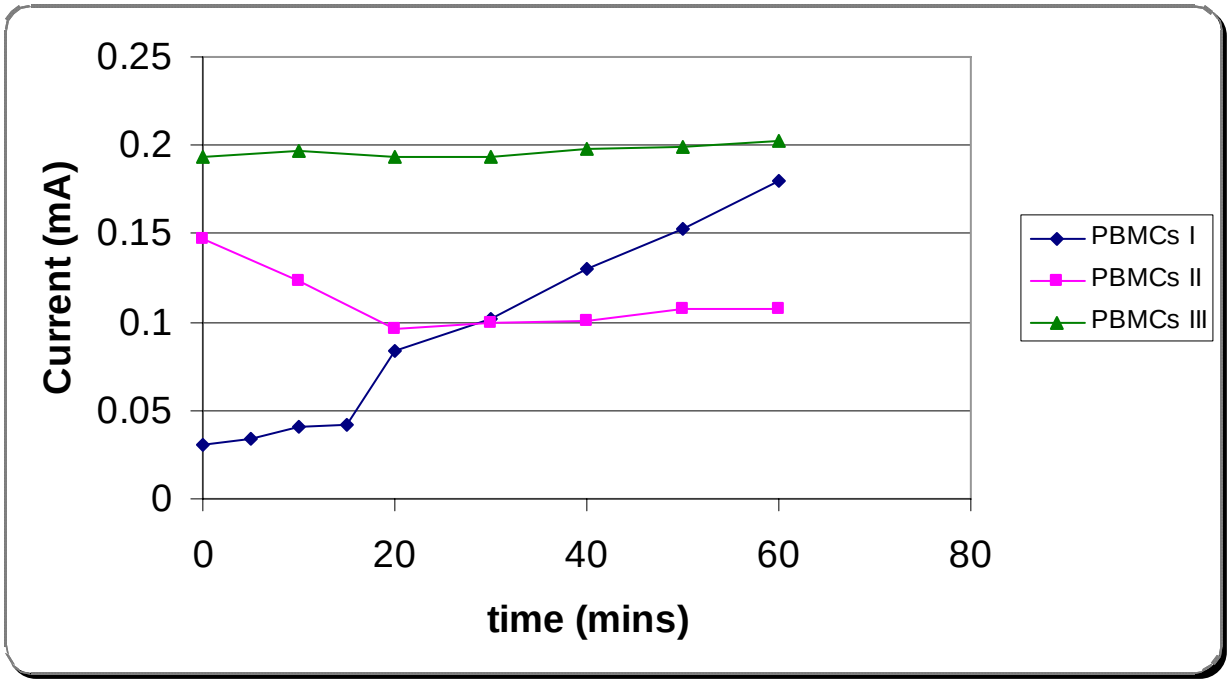


Figure 11: **Current output from a BFC containing human PBMCs. The current from these BFCs were generally unstable and for two of the samples showed large variations in their current as a function of time (current measurement accuracy +/- 6%).**

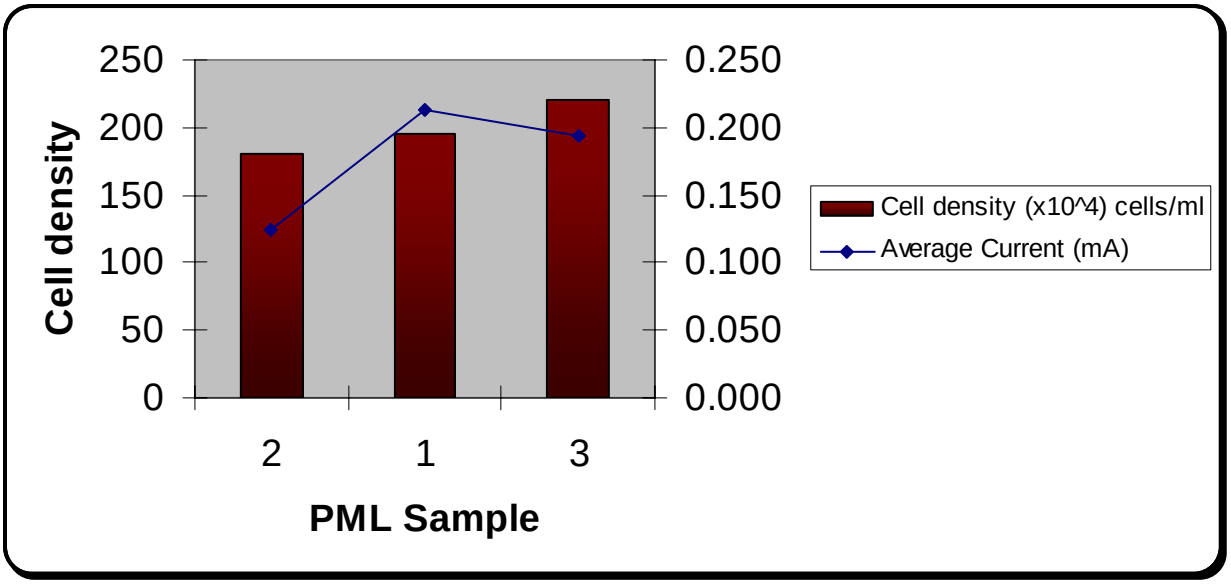


Figure 12: **Relationship between cell density and average current. Very little correlation is observed between the two parameters, indicating that other factors are involved in the generation of the observed currents that are separate from the cells.**

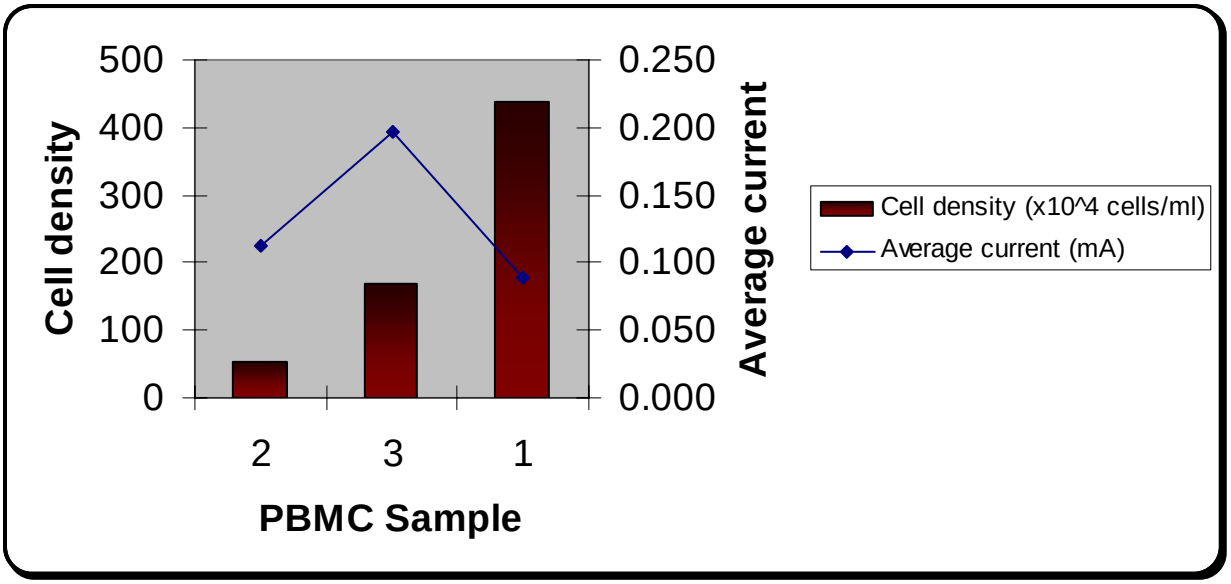


Figure 13: **A relationship between the average current and PBMC cell density was investigated. However, no correlation was observed. These results strongly suggest that there may be additional factors (reactions) responsible for the observed currents.**

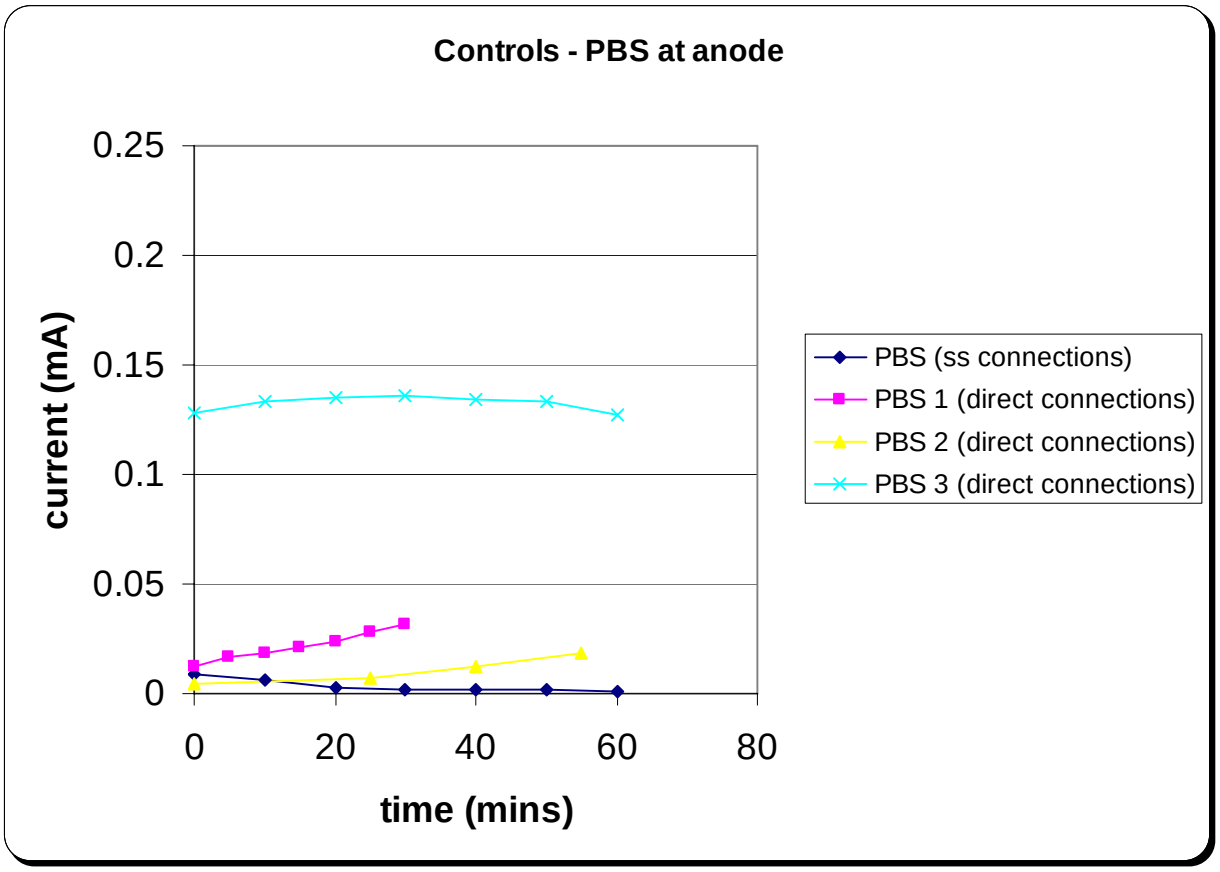


Figure 14: **Controls: Plain PBS was introduced into the anode compartment of the BFC, while a ferricyanide solution made up in PBS was introduced into the cathode solution. Despite the absence of cells in the solution, currents of significant magnitude were observed. Upon using stainless steel to ensure that the anode and cathode solutions did not come into contact with the alligator clips, the current from the BFC decreased dramatically and resulted in a current output that decreased monotonically with time.**

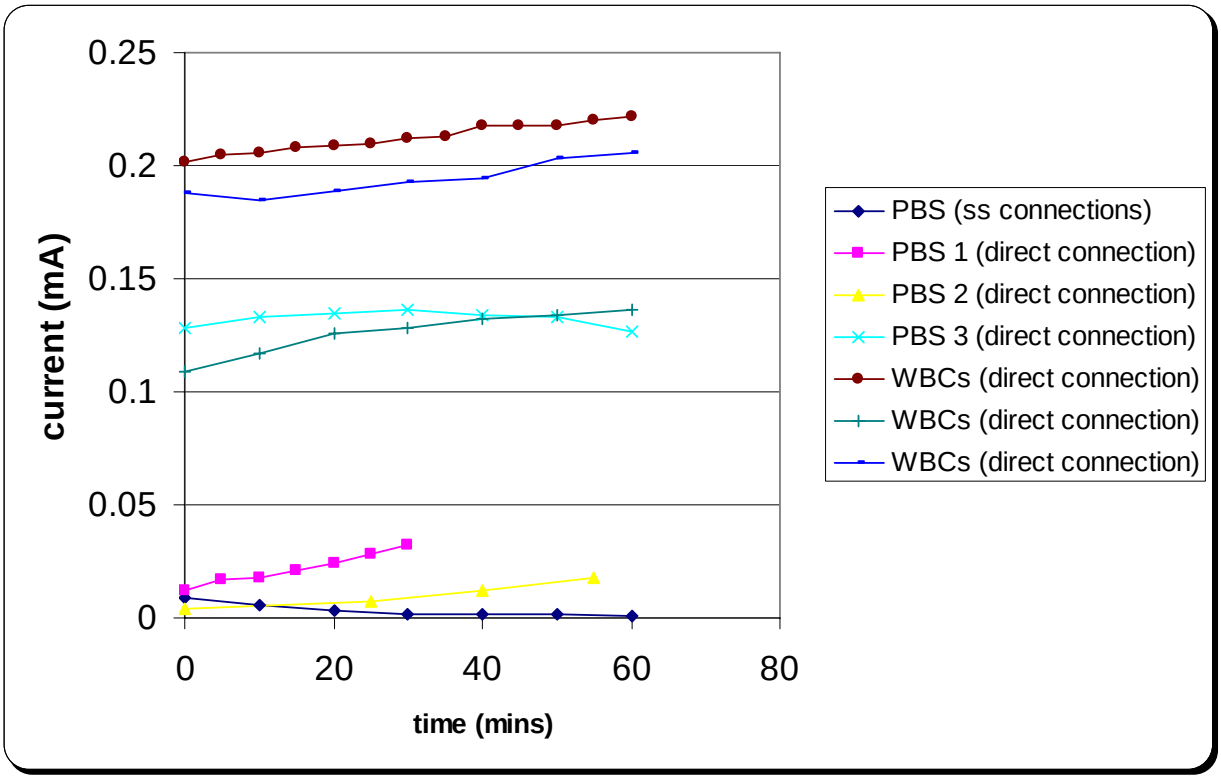


Figure 15: **Overall results of experiments and controls. Strips of stainless steel (ss) were used to bridge the connection between the carbon fiber electrodes and the alligator clips connected to the 100 Ohm resistor. A "direct connection" signifies that the carbon fiber electrodes were directly in contact with the alligator clips.**

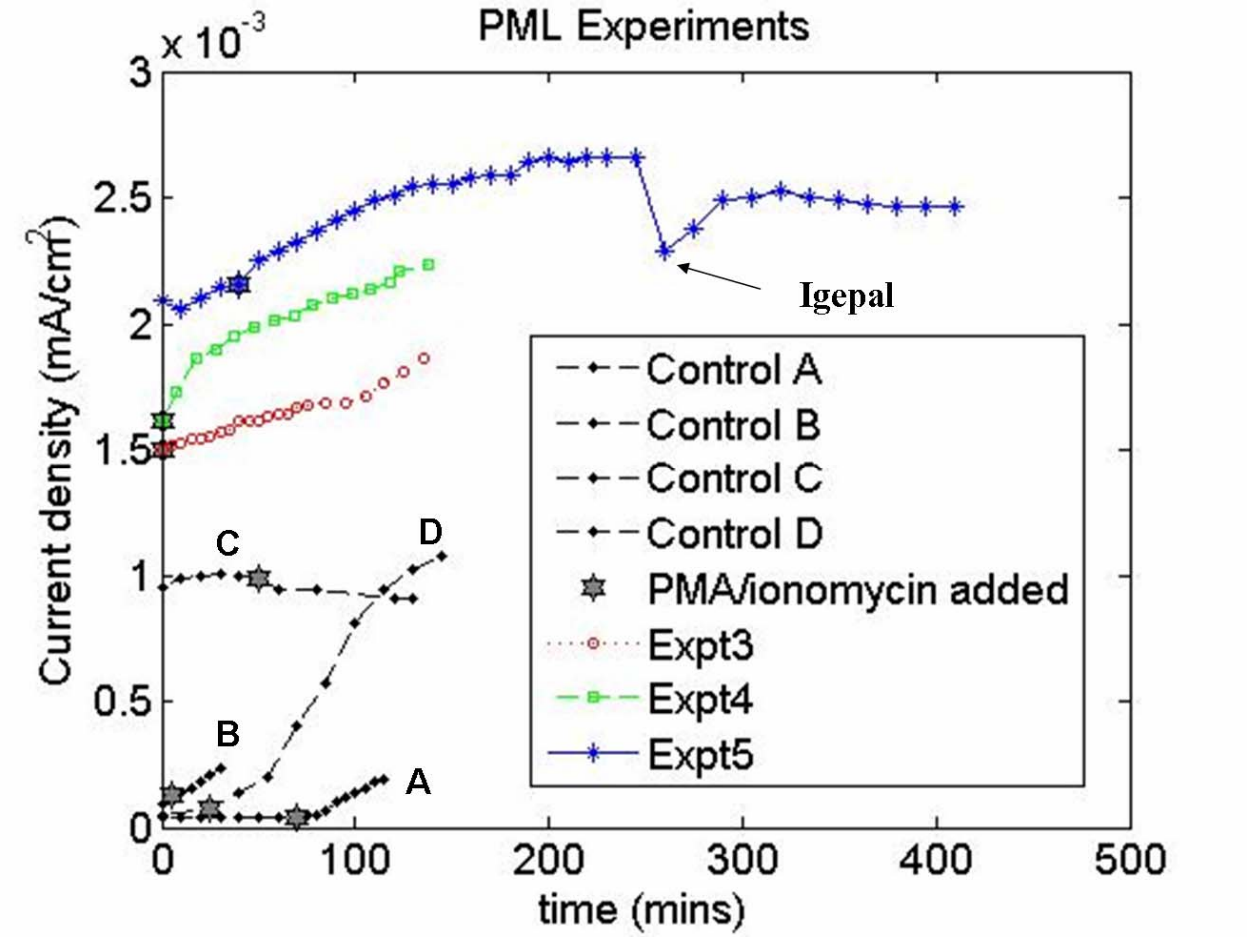


Figure 16: **Current output from three PML-based BFCs and four control experiments. Introduction of Igepal to the anode compartment containing the PMLs resulted in a slight decrease in current. The current was not completely extinguished and subsequently began to increase. In the case of the control experiments (no cells), currents of variable magnitude were observed. Noticeable was the fact that addition of PMA and ionomycin to the anode compartments of the bioreactors without cells resulted in significant increases in current, with the exception of one case.**

5.5 EXPERIMENT II: MATERIALS AND METHODS

5.5.1 White blood cell isolation

Human white blood cells (WBC) were isolated from approximately 10mL of whole human blood. The total WBC population was isolated using a red blood cell (RBC) lysis technique. This required preparation of a 3:1 ratio of RBC lysis solution from a Puregene® kit (Gentra, Minneapolis, MN) solution to whole blood, which was allowed to sit for approximately 10 minutes and then centrifuged at 2000 rpm for 10 minutes. The supernatant containing the lysed red blood cells was removed and the remaining WBC pellets subsequently washed multiple times (at least three times) in 1X PBS. After washing, the cells were finally resuspended in 15ml PBS. For specific isolation of PBMCs, a Ficoll-Paque^{TM} density gradient was used as described previously. The cells were washed twice in PBS (pH 7.4) by centrifugation at 1600 rpm for 15 minutes and were finally resuspended in 15mL PBS. Cells from a K562 (monocyte progenitor cells) cell line were also cultured over a period of one week in RPMI with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). The cells were extracted from the culture media by centrifugation (1700 rpm for 15 mins), washed twice and finally resuspension in 1X PBS. A light microscope and hematocytometer were used to determine the final cell densities in PBS.

5.5.2 Measurement of open circuit potential and current

Multimeters were used to measure the open-circuit potential, Voc, as well as the potential across a 100Ω (+/- 5%) resistor, Vr, during current flow through the BFC. Current, I (μA), and current density, J (μAcm^{-2}), were found using Ohm's law. The surface area of the electrodes was taken as the *geometric* surface area and *not* the actual surface area. Woven carbon fiber electrodes (8.0 cm^2 geometric surface area) were used for both the anode and cathode electrodes. The cell suspensions were confined to the anode compartment, while the cathode compartment contained a 50mM potassium ferricyanide ($K_3[Fe(CN)_6]$) solution made up in 1X PBS (pH 7.4). The anode and cathode electrodes were separated by a proton exchange membrane (PEM). WBC activation was achieved with 5ng/ml phorbol-12-myristate-13-acetate (PMA) and 500ng/ml calcium ionomycin (final concentrations in solution). The components used to construct the BFC, including the walls, chamber and carbon fiber electrodes, were purchased from the National Center of Biotechnology Education (NCBE), University of Reading (United Kingdom). The Nafion-117 proton exchange membrane was purchased from Sigma-Aldrich Co (St. Louis, MO). Strips of stainless steel were used as an interfacial connector between the carbon fiber electrodes and the alligator clips with leads to the resistor, in order to prevent the anode and cathode solutions from reacting with elements within the clips.

5.6 EXPERIMENT II: RESULTS AND DISCUSSION

BFC Currents and Open Circuit Potentials

In a preliminary experiment, where the current output for a BFC containing WBCs (isolated by RBC lysis) was measured (Figure 18), current densities (J) between 1.0 and 1.6 μAcm^{-2} (between 8 and 13 μA) were measured for PMA activated cells and current densities between 1.1 and 2.6 μAcm^{-2} (9 and 21 μA) were measured for non-activated cells (current density error +/- 5%). Open circuit potentials (Voc) ranged between 202mV and 285mV for the activated cells, while the range was between 180mV and 350mV for the non-activated cells (electric potential error +/- 0.5%). In a second experiment, where the current output of PBMCs (isolated on Ficoll-Paque gradient) in a BFC were measured (Figure 19), current densities between 0.1 and 0.5 μAcm^{-2} (1 and 4μA) were obtained for the PMA activated cells. For non-activated cells, the current densities recorded ranged between 0.4 and 0.6 μAcm^{-2} (3 and 5 μA), which were slightly higher than the previous case (activated cells). The error associated with the current measurements was +/- 5%. Open circuit potentials for the PBMCs ranged between 62 and 159mV for the activated cells and between 180 and 350mV for the non-activated cells (error +/- 0.5 %). Voc and J values for the WBCs were higher than control values, i.e. compared to anode solutions of (1) plain PBS and (2) PBS with activating agents PMA and ionomycin (Figure 21). In the absence of white blood cells, small currents could still be detected when the anode compartment contained a solution of PBS, PMA and ionomycin (Figure 21).

PMA and ionomycin electrochemical activity

The fact that currents were observed with PMA and ionomycin dissolved in PBS is an indication of their electrochemical activity. Cyclic voltammetry of a PBS solution with dissolved PMA and ionomycin, at similar concentrations to that used in this experiment, resulted in no observable oxidation or reduction peaks (results discussed in Section 6.0). The electrochemical activity of PBS, PMA and ionomycin leading to currents in the bioreactor system should be associated with oxidation peaks in the cyclic voltammograms. The absence of any observable peaks may be a consequence of the very low concentrations of the reactants, which fall outside the detection limits of cyclic voltammetry. A PEMFC containing plain PBS only at the anode did not produce any observable currents or open circuit potentials (Figure 21).

Relationship between Voc and J

WBC activation with PMA was associated with high correlation coefficient values (R^2 values) for Voc plotted against J in contrast to non-activated cells (Table 4). The same was true for the PBMCs. A high correlation coefficient (close to 1.00) may be an indication of a dominant reaction that is fully responsible for driving the observed currents. The relationship between Voc and J for the activated WBCs was strongly linear and was not observed for any other situation.

Decreasing open circuit potentials

In Figure 18 and Figure 19, the open circuit potential noticeably decreases over time. The same was also true for the closed circuit current. The decreasing potential may be a consequence of the method employed in acquiring the data. Measurements of open circuit potential and the closed circuit potential across the resistor (used to calculate current from Ohm's law) were made every

10 minutes. After the circuit is closed to measure current, upon reverting to an open circuit, the reactants at the anode and cathode would require some period of time to achieve equilibrium. Achieving this equilibrium state may take more than 10 minutes. Therefore, the open circuit potential measured at each time interval was actually lower than the true value. The current values may also be lower than their true values due to the rapid polarization that would occur upon closing the circuit. The effects of polarization may have been reduced by using a much higher external resistor.

Evidence supporting electrochemical activity of WBCs

In Figure 20, J values measured for PBMCs *only* (BFC I) and WBCs (BFC II) are plotted against time. As mentioned previously, the current output for the WBCs was higher than that of the PBMCs. By replacing the original WBC cell suspension with PBS containing PMA and ionomycin, the current output of BFC II decreased significantly. No significant change in current was observed when the PBMCs were replaced in BFC I. One possible explanation that could be put forward for the relatively larger currents and potentials observed with WBCs at the anode compared to other preparations may be that residual substances from the isolation process could have contaminated the anode solution and the electrodes, leading to some electrochemical activity. In an attempt to disprove this explanation, samples of the PBS supernatant from washing the cells were saved and later introduced at the anode of the PEMFC. By acquiring the supernatant from the first wash, it would be highly likely that any residual substances would remain dissolved in the PBS at a much higher concentration than would be found in the final cell suspension. This was done for both the WBCs and PBMCs. The results of this experiment can be found in Figure 22 and Figure 23. In both cases (WBCs and PBMCs) there is a clear increase in

potential and current when the supernatant is replaced by the cell suspension. In order to ensure that the increase is not due to changing pH, measurements of the acidity of the cell suspensions were recorded after PMA activation for at least 30 minutes. The pH of the suspension remained at around 7.5, which is approximately the same pH as the original PBS solution. Therefore, there is not a significant enough change in the concentration of protons in solution to warrant a dramatic change in the pH of the buffer and thus an increase in the rate of reaction (resulting in increased current) or a change in the reduction potential, $E^{0'}$.

Investigation of the electrochemical activity of K562 cells

The current output and open circuit potential of a PEMFC containing K562 cells were also investigated (Figure 24). The currents in the presence of a K562 cell suspension proved to be rather small (average current density of 0.4 μAcm^{-2}) despite the relatively large open circuit potentials recorded. In addition, the correlation coefficient (expressed as its square value, R^2) between Voc and J was smaller than that determined for the WBCs.

Identifying cell types responsible for the currents

In Figure 25, the average currents and average open circuit potentials were plotted with respect to the specific cell populations at the anode, as well as with respect to the cell density in the PBS suspension. The highest average currents were associated with the white blood cell suspension (regardless of whether they were stimulated or not). Within this white blood cell population, it is expected that the following specific cell types would be found: neutrophils, basophils, eosinophils, monocytes, PBMCs. Since the PBMCs appear to be associated with very small currents, it is not likely that these currents contribute significantly to those observed for the total

WBC population. The results imply that within the general WBC population, certain cells or perhaps even the molecules released by certain cells are electrochemically active at the electrode surface. Although the exact source of the currents observed is not clear, they may be attributed to the oxidation of electrochemically active species that are either released by the cells into the extracellular environment (such as serotonin, cytokines) or by redox active species that are present in the cell membrane (such as flavohemoproteins of the NADPH oxidase complex or coenzyme A). It has proven difficult, however, to ascertain the exact identity of the species being oxidized at the anode. In order to be able to produce a current from the BFC, protons must also be released by the cells into the extracellular space. The increase in proton concentration in the anode compartment is important to create a diffusion gradient, whereby the protons can traverse the PEM to react with molecular oxygen to form water at the cathode. Placing either PBS solution or deionized water in both the anode and cathode compartments did not produce any detectable currents and were associated with open circuit potentials that were less than 20mV.

Average open circuit potentials and average current densities (n=3) were compared for the PEM fuel cell containing i) activated WBCs and ii) PBS with PMA and ionomycin at the anode (Figure 26 and Figure 27). The magnitude of the values for both average Voc (Figure 26) and average J (Figure 27) were higher for the WBC suspension than for a solution of PBS, PMA and ionomycin at the anode. For both the WBC suspension and PBS solution, large variations in Voc were observed across the three samples, leading to rather large standard deviations. These large standard deviations were a stark contrast to those observed for the average current density, particularly at later time points. Toward the end of the 60 minute period of measurement, there was a noticeable decrease in the standard deviation associated with the average current densities. The fact that Voc and J are generally larger for the WBC suspension than for the PBS solution

suggests that the cells are largely responsible for the observed currents. There is an evident contribution from reactions associated with PMA and ionomycin at the anode. Identification of the specific chemical agent, PMA or ionomycin that is actually electrochemically active was not determined. The fact that higher overall current densities are produced by the total WBC population compared to the isolated PBMCs strongly supports our theory that polymorphonuclear leukocytes (PML) – to which neutrophils, eosinophils and basophils belong – are the primary contributors to the observed currents. The PBMCs have much less of a respiratory burst response and have a very low density of NADPH oxidase in the cell membranes compared to PMLs. This is true for most, if not all, PBMCs (including monocytes). A low respiratory burst would correspond to very little proton release by the cells, which directly impacts the observable currents since proton translocation across the PEM now becomes a limiting factor.

Polarization effects

Regardless of how significant the open circuit potential of the PEM fuel cell may be, there will be very limited current and consequently low power generation as a result of the lack of a sufficient proton gradient between the anode and cathode compartments. A low proton gradient would therefore translate into a very high internal resistance, Rm (resistance of the PEM), and as a result a significant ohmic overpotential (polarization). Potential losses are associated with charge transfer at the electrode surface (activation polarization), diffusion effects (concentration polarization), and the resistance of the PEM (ohmic polarization). The large voltage losses associated with the BFC is clearly observed once the circuit is closed. There is an almost

immediate and rapid drop in potential from the initial open circuit potential values - on the order of hundreds of millivolts - to only tens of millivolts.

Implications of study with respect to the development of an in vivo BFC power supply

In this study, we proposed the development of a novel biological fuel cell that can utilize the body's own resources to generate electricity, through specific electrochemical interactions between cells and electrodes in close proximity. The motivation of this study was to develop a BFC that can be used to power implantable medical devices, including micro- and nano-biosensors for either therapeutic or physiological monitoring purposes. The study sought to demonstrate that electron transfer between human white blood cells and an interfacing electrode can occur through any or all of three possible mechanisms: 1) direct electron transfer through membrane bound redox species (such as the flavocytochrome of NADPH oxidase); 2) indirect electron transfer through exocytosed non-metabolic biochemical species; and 3) indirect electron transfer through exocytosed metabolically relevant biochemical species.

A significant hurdle at this point is the necessity of the proton exchange membrane (PEM) to separate the anode and cathode compartments. A PEM would make it difficult to incorporate a biofuel cell into the human body. However, a solution might already exist. In articles by Mano et al. [19, 20, 49], an enzymatic biofuel cell was described that facilitated the reduction of molecular oxygen to water at the cathode through an enzyme that was immobilized at the electrode surface. Glucose oxidase was immobilized at the anode to facilitate the oxidation of glucose to gluconolactone. The enzymatic biofuel cell was able to generate potentials as high as 800mV, which is several times larger than what we were able to produce in our own studies (200mV). Our biological fuel cell, however, may have the advantage of

simplification compared to the enzymatic biofuel cell previously described, in that it would not be necessary to modify the anode electrode. An 800mV potential may also not be necessary for certain applications.

This research started with the goal of using NADPH oxidase as a mediator of electrons between the biological cells and the anode electrode of a biofuel cell. However, over the course of this research project, it was found that such a direct electron transfer is very difficult to accomplish. Previous studies performed by other researchers on microbial fuel cells suggested that under normal conditions, it is very difficult to achieve direct electron transfer between the cell membrane components of the microbes and an interfacing electrode. An important reason for this is the fact that the redox active groups are generally embedded within integral membrane proteins, resulting in a significant energy barrier that increases with increasing distance between electron donor and receptor pairs. Electron tunneling between the membrane components and the electrode surface is further discouraged because of the thermodynamically unfavorable conditions surrounding the movement of free electrons through an aqueous solution. In order to overcome this challenge, many microbial fuel cell researchers employed electron mediators that were capable of shuttling electrons between the membrane-bound redox active components of the microbes and the electrode. The use of these electron mediators significantly increased the efficiency in electron transfer and thus the current output of the microbial fuel cells.

Difficulties associated with electron transfer between NADPH oxidase and an electrode

In our own study, the fact that we are dealing with eukaryotic cells makes the situation a bit complicated for *in-vitro* analysis in a biofuel cell setup. White blood cells, like all other cells in the human body, require oxygen in order to metabolize glucose to produce ATP. A problem with

many variations of biofuel cells, particularly those dependent on whole cells, is the fact that oxygen is under most circumstances the preferential electron acceptor for the metabolic processes that occur in both prokaryotes and eukaryotes. It is the final electron acceptor at the plasma membrane for most prokaryotes and within the mitochondrial membrane of eukaryotes. Oxygen is also the final electron acceptor of the activated NADPH oxidase complex. In the case of microbial organisms, eliminating oxygen from the anode compartment is not a major issue and is not detrimental to the microbes, since electrons can be diverted to the anode electrode, through an electronic circuit and finally oxygen at the cathode. There are usually no other metabolic processes within the microbes that would be dependent on the presence of oxygen. When dealing with human white blood cells, however, it is not possible to eliminate oxygen completely from the anode compartment due to the fact that oxidative metabolism in the mitochondria requires oxygen in order for the cell to survive. The presence of oxygen around the cell would also mean that electrons available in the NADPH oxidase complex would be preferentially transferred to any oxygen molecule in close proximity. Very few or no electrons would be transferred to an unmodified carbon felt electrode. Dissolved oxygen in the surrounding extracellular fluid would ultimately succeed in grasping up the electrons before they could ever be transferred to the electrode surface. It is possible, however, as demonstrated in the BFC results presented previously, that there is some mechanism of electron transfer that occurs upon the stimulation of the white blood cells. One possibility is that free radicals, such as reactive oxygen species (superoxide and peroxide), released by the NADPH oxidase complex, could undergo irreversible oxidation at the anode electrode. The simultaneous ejection of protons from the cell to compensate for the electron transfer would facilitate the generation of electrical currents as the build up in the proton concentration would lead to a diffusion gradient that drives

the movement of protons from the anode to the cathode chamber, where they finally combine with oxygen to form water. The observation that currents are produced for unstimulated cells suggests that there may be either other mechanisms of electron transfer or that some contamination is occurring. One other possibility for an electron transfer mechanism is oxidation of coenzyme A (CoA) present in the cell membrane [72, 73].

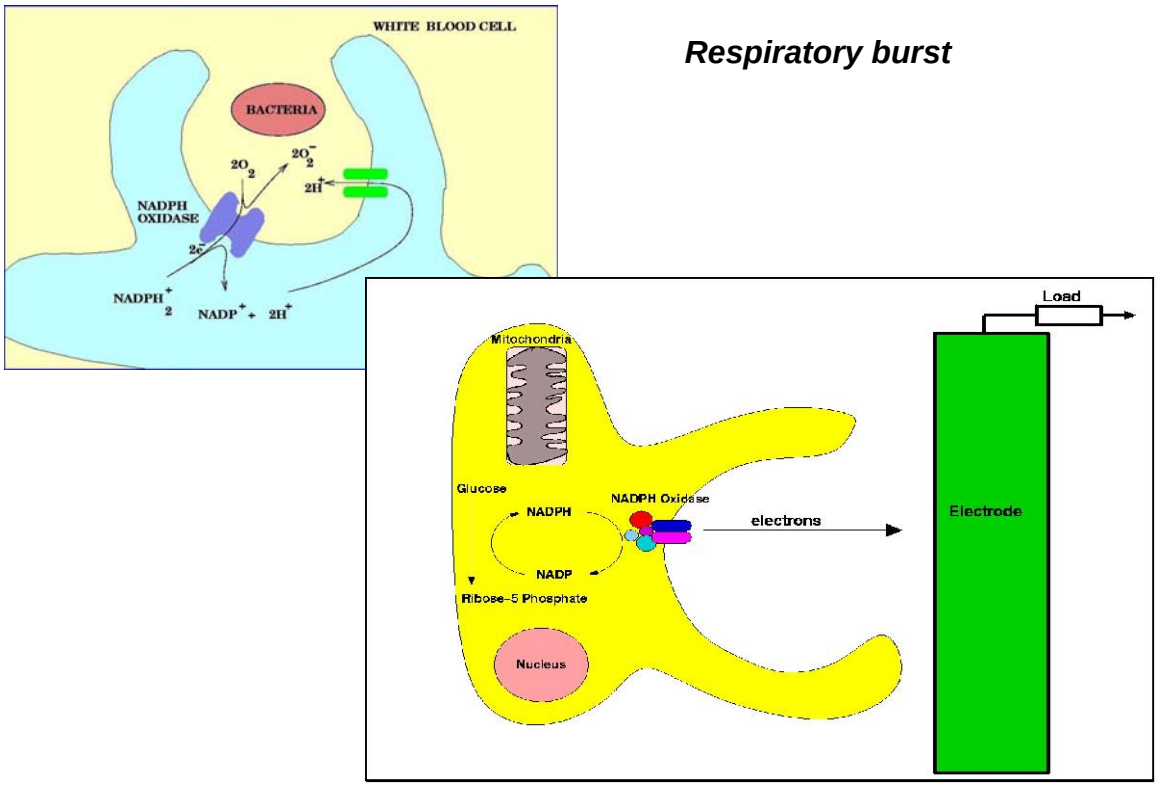


Figure 17: NADPH oxidase and respiratory burst in phagocytic white blood cells.

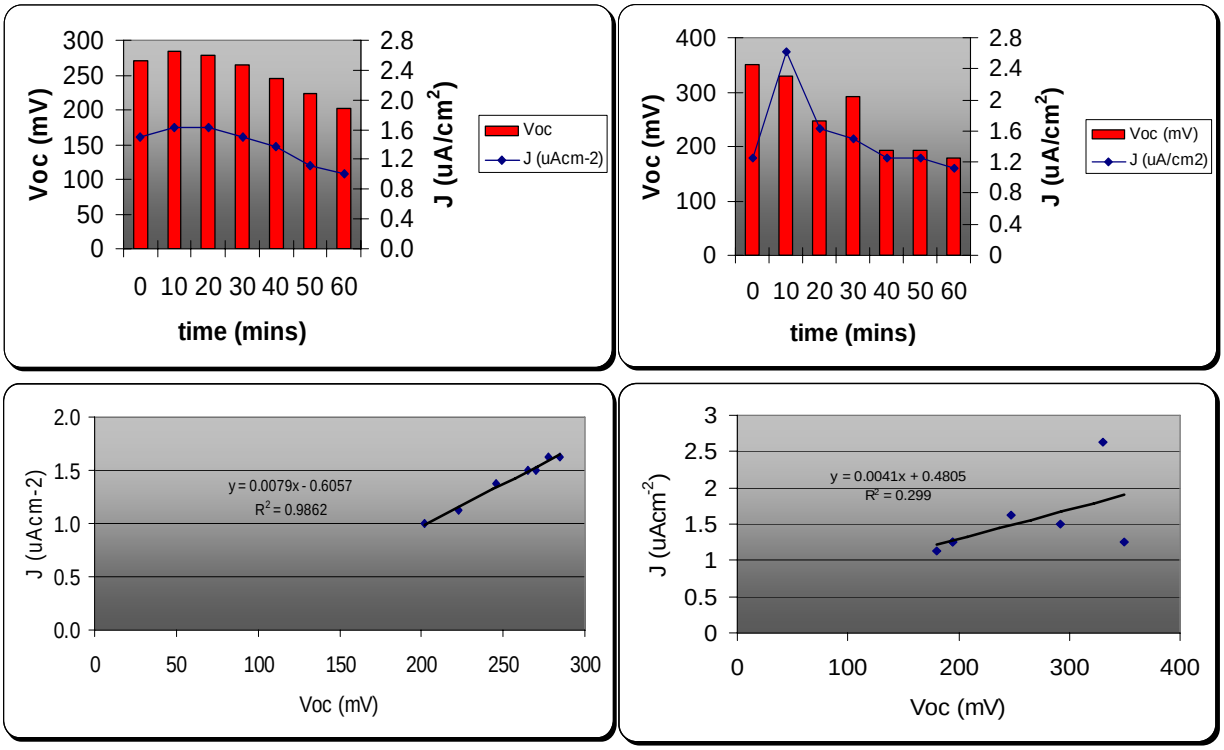


Figure 18: **Stimulated (left panels) and non-stimulated (right panels) white blood cells (isolated using RBC lysis technique).**

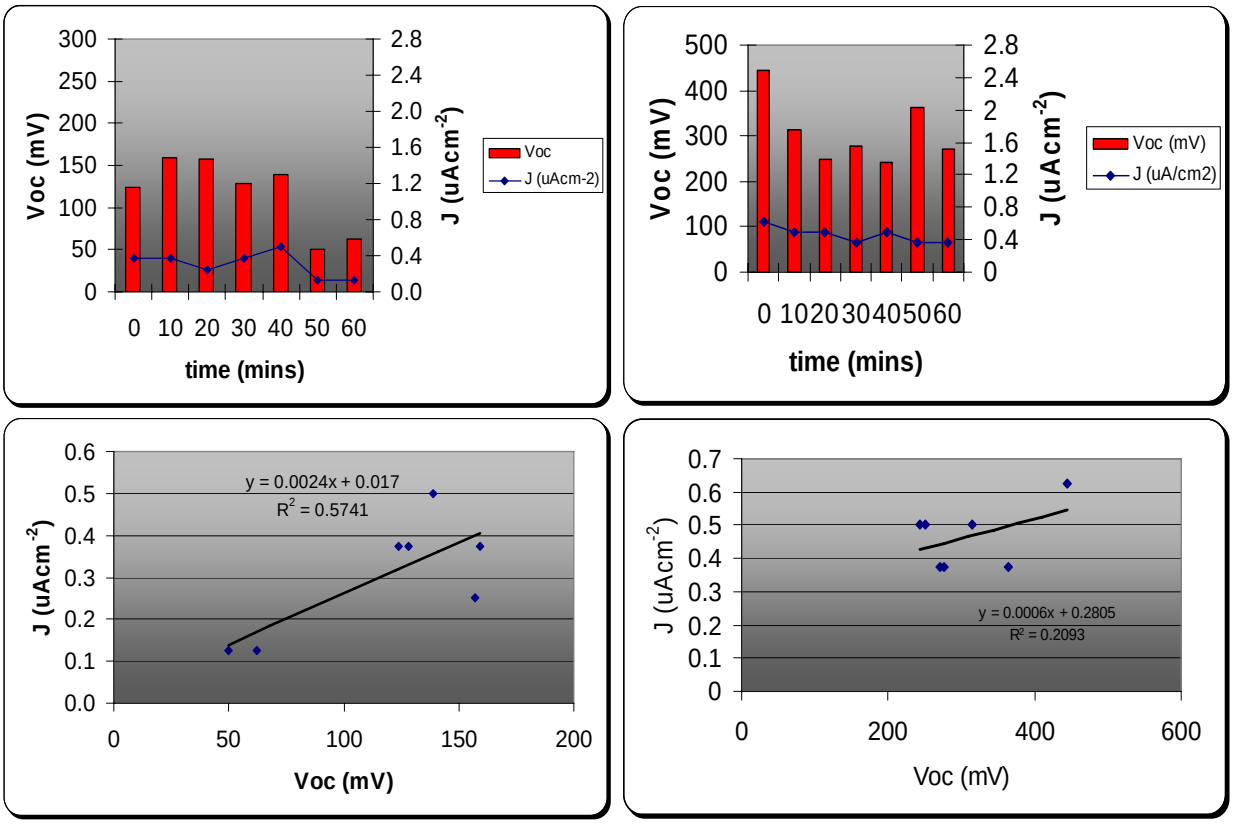


Figure 19: Stimulated (left panels) and non-stimulated PBMCs (right panels).

Table 4: Correlation coefficient values (expressed as R^2) for Voc vs. J

Anode solution	R^2
Activated white blood cells	***0.99***
Non-activated white blood cells	***0.30***
Activated PBMCs	***0.57***
Non-activated PBMCs	***0.21***

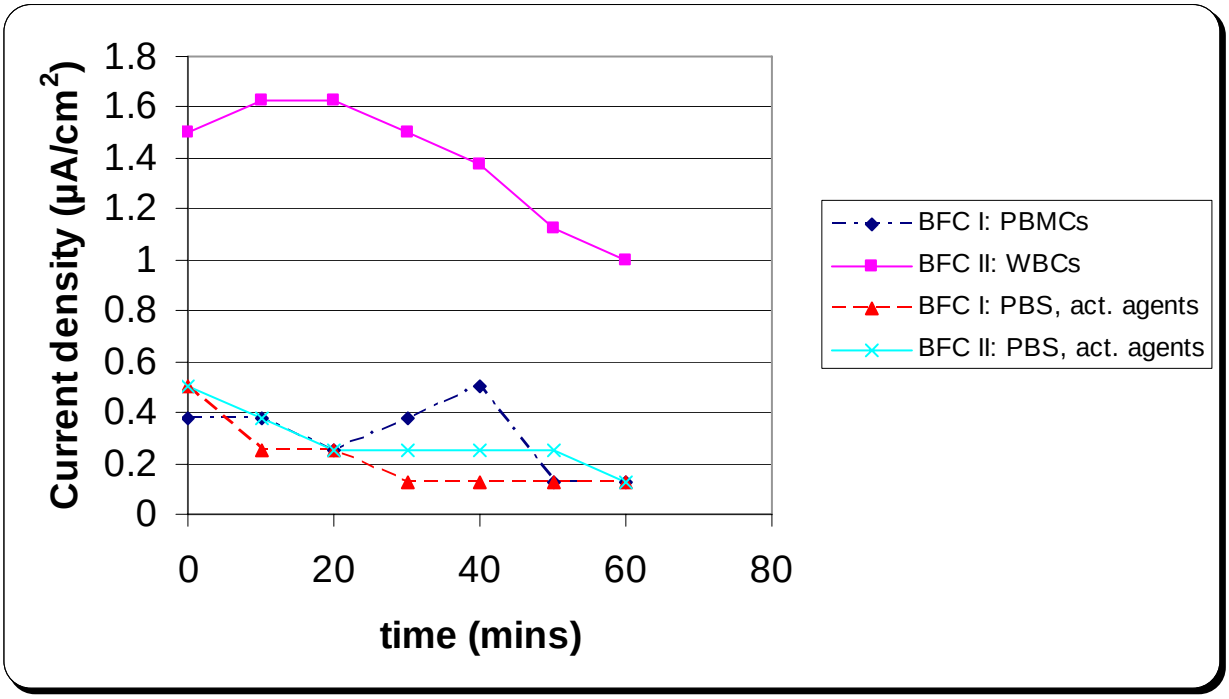


Figure 20: **Comparison of currents acquired from experiments (PMA activated cells) and controls (PBS with PMA).**

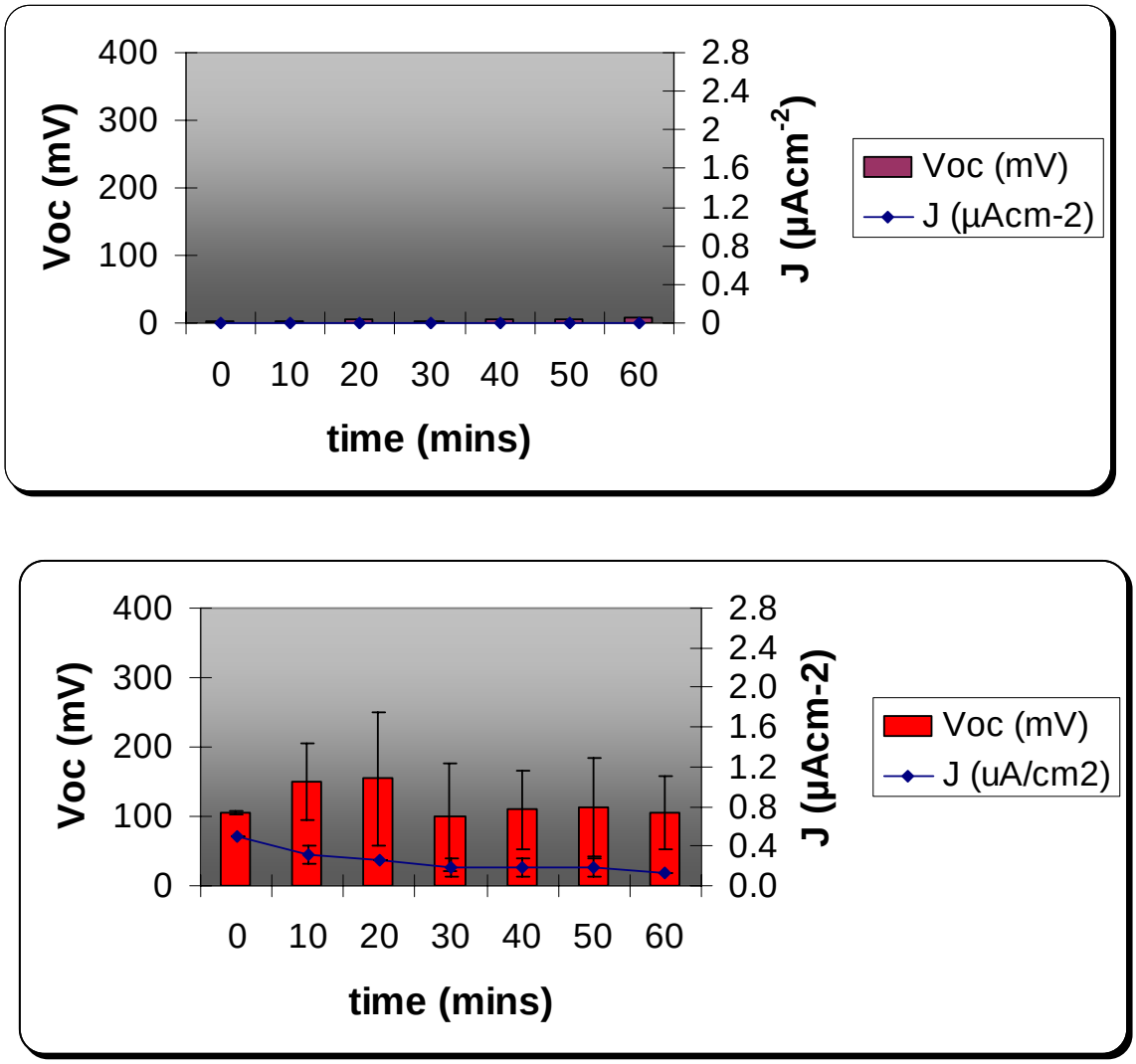


Figure 21: Plain PBS (top) and PBS with PMA and ionomycin (bottom).

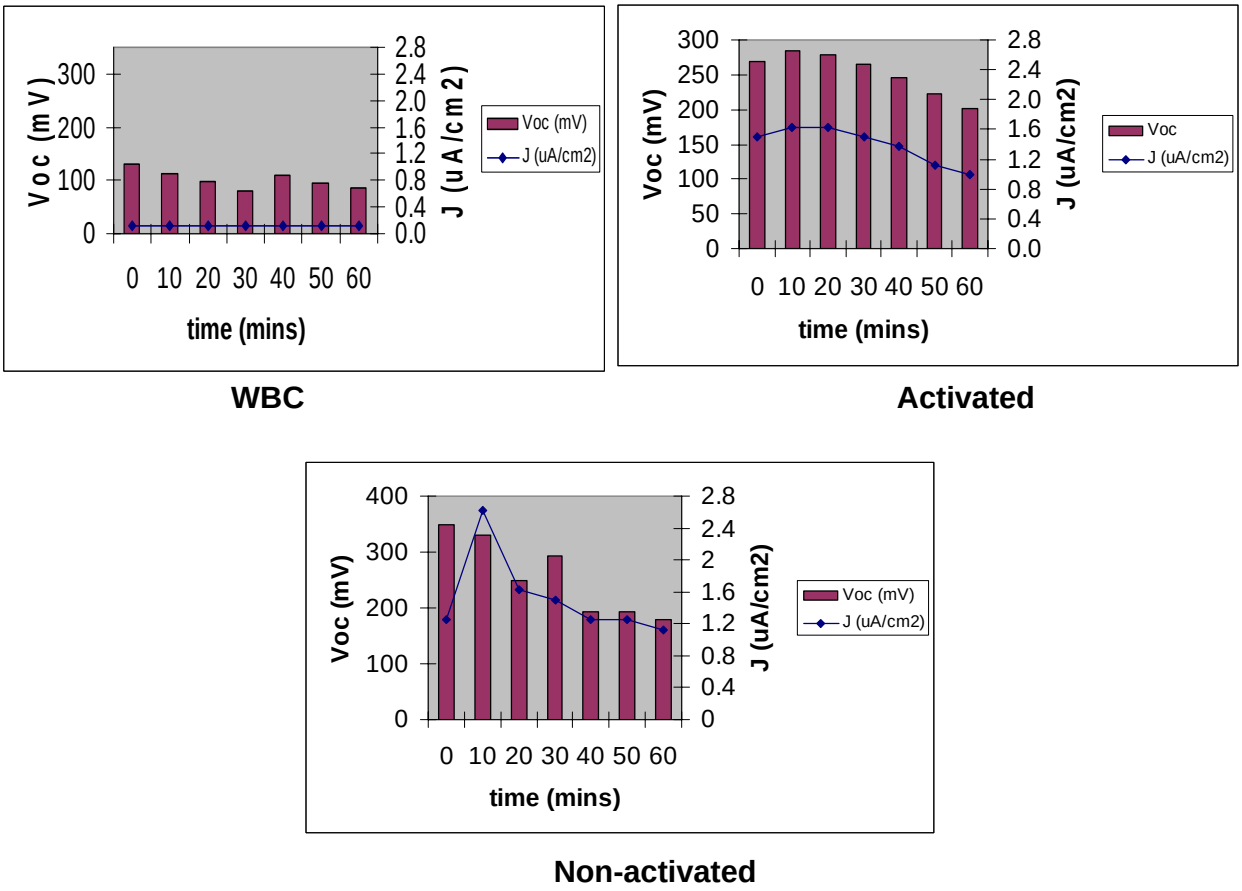


Figure 22: Comparison between BFCs containing WBC supernatant, non-activated WBCs and activated WBCs.

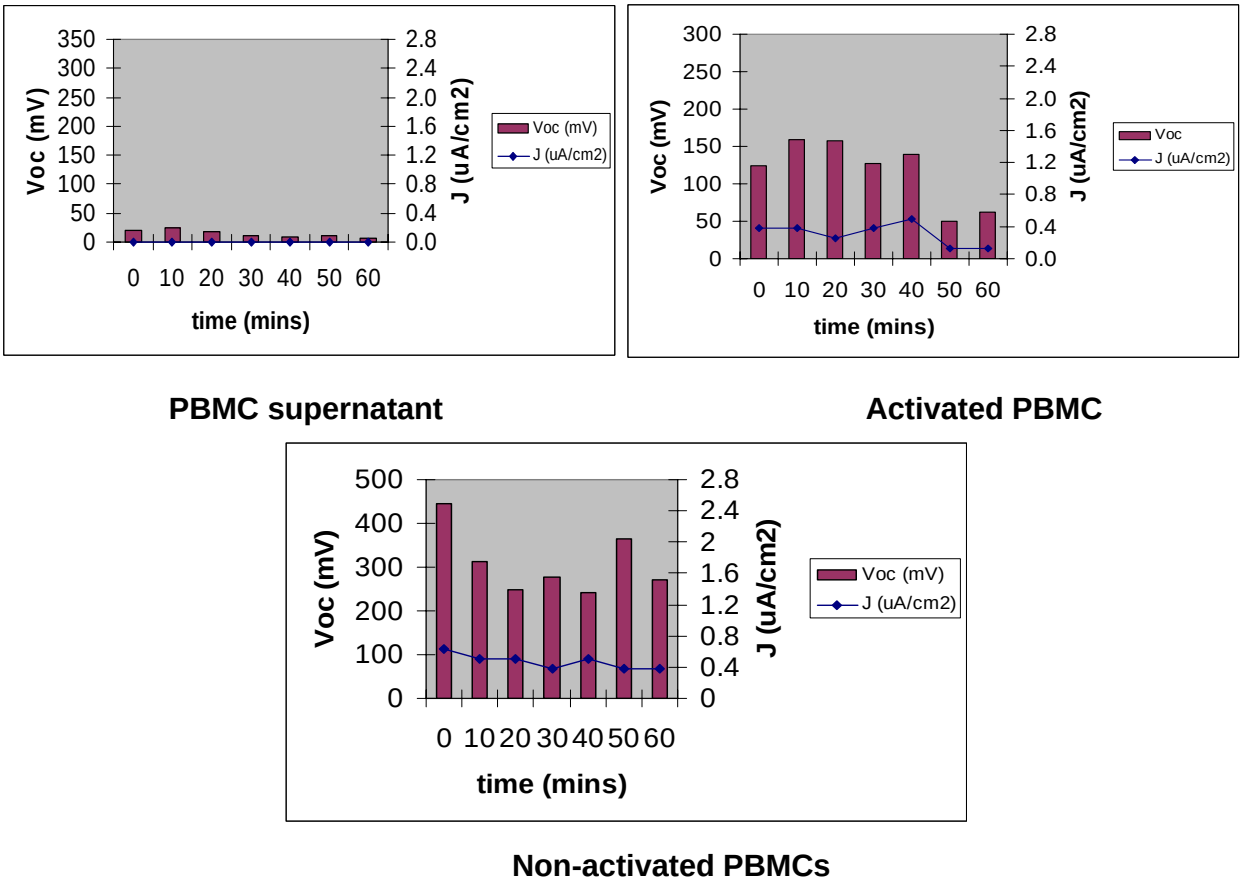


Figure 23: Comparison between BFCs containing PBMC supernatant, non-activated PBMCs and activated PBMCs.

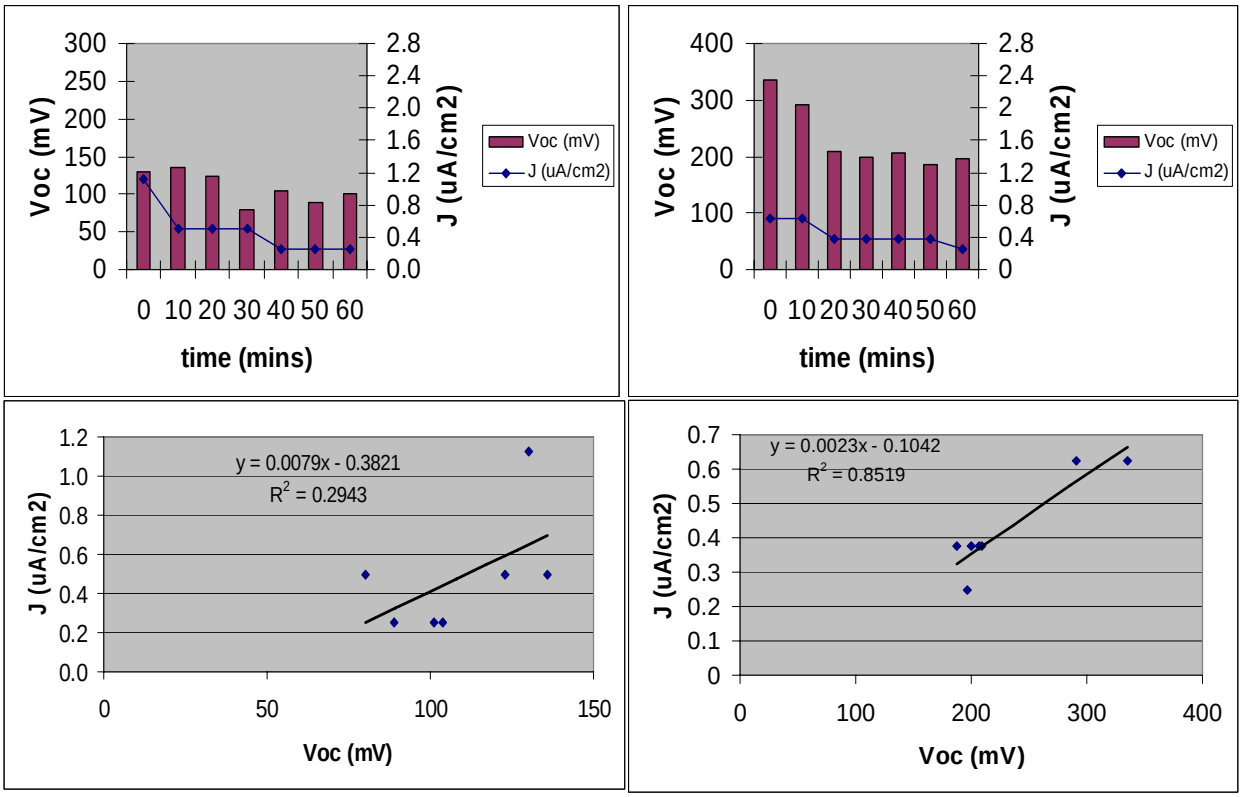
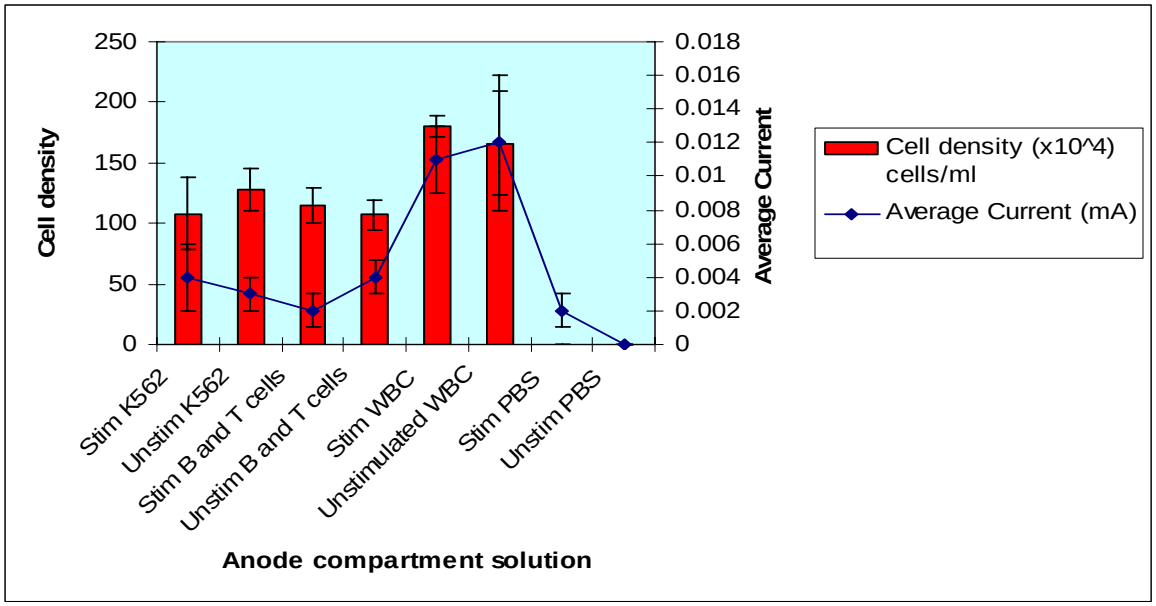


Figure 24: Activated (left panels) and non-activated (right panels) K562 cell line.



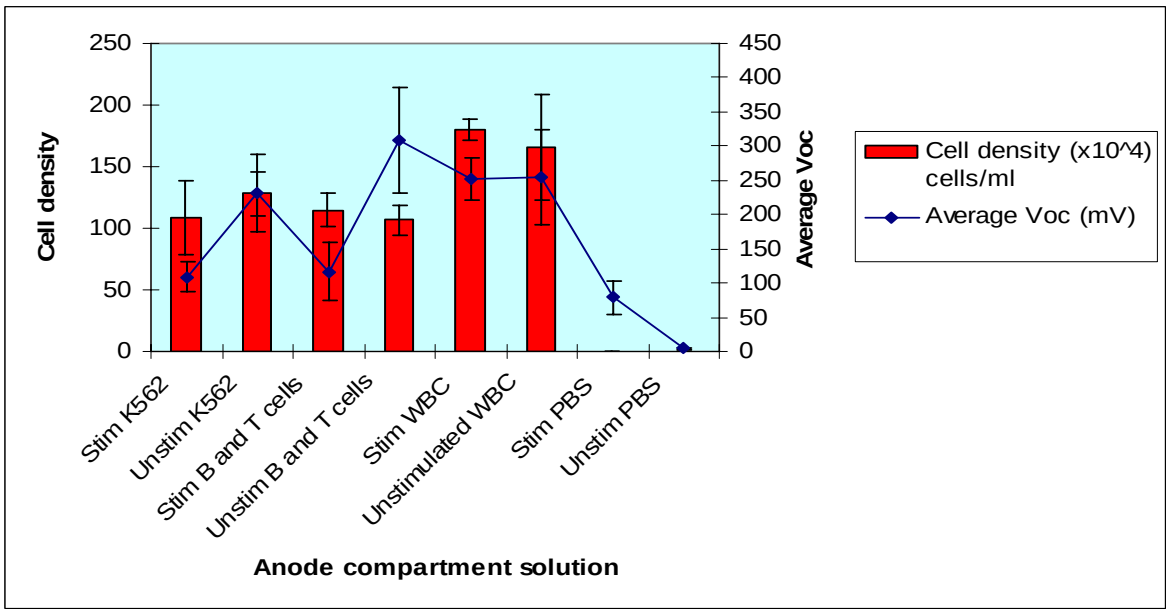


Figure 25: Relationship between cell density and electrical parameters (average current and average open circuit potential during 60 minute period of measurement).

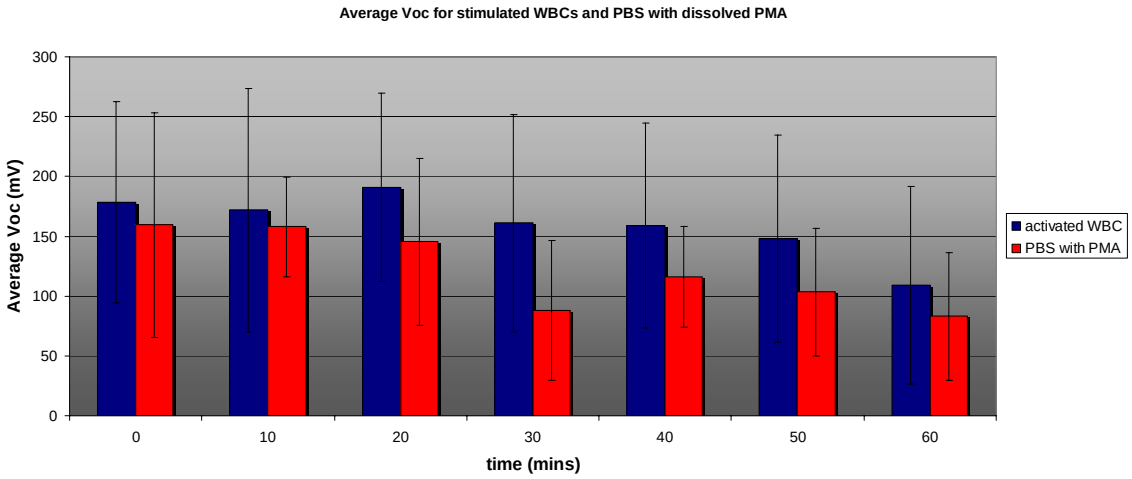


Figure 26: Comparison between average open circuit potentials (n = 3) for PEM Fuel cell with activated cells (blue) and with PBS with PMA and ionomycin (red).

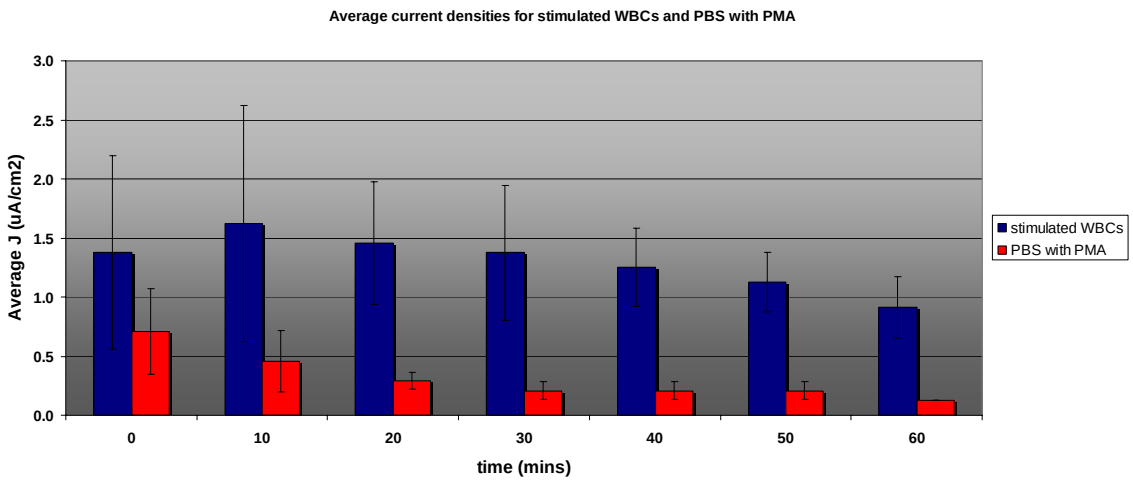


Figure 27: Comparison between average current densities (n = 3) for PEM fuel cell with activated cells (blue) and with with PBS with PMA and ionomycin (red).

5.7 CONCLUSIONS

The results of this study suggests that human white blood cells isolated from whole human blood are capable of generating small electrical currents and potentials when introduced into the anode compartment of a PEM fuel cell bioreactor. Average open circuit potentials with the white blood cells ranged between 100 and 200mV (Figure 26), while average current densities (calculated with respect to the geometric surface area of the carbon fiber electrodes) ranged between 0.8 and 1.8μAcm^{-2} (Figure 27). The magnitude of these currents and potentials are insufficient for current applications in the field of medical devices (see summary of electrical stimulation parameters for implantable devices in Table 1). However, with ever decreasing power requirements for devices due to advances in the field of micro- and nano-technologies, such a power source may feasibly be implemented. The isolated PBMCs as well as cells cultured using the K562 leukemia cell line demonstrated very little electrochemical activity when introduced into the PEM fuel cell bioreactor. The fact that there was a lack of strong evidence suggesting that PBMCs are electrochemically active imply that some other white blood cell type, such as the human granulocytes (neutrophils, basophils, eosinophils) might play a greater role in the generation of the observed currents. A major concern in investigating such an electrochemical system is the fact that background currents may at times be larger than expected (such as was observed in Experiment I), and could essentially mask smaller currents elicited by the cells themselves and/or confound the results of the experiments. The enzyme complex NADPH oxidase has been reported to generate electronic currents on the order of 10 to 20pA by various research groups. For cell densities on the order of 10^6 cells/ml in 10ml volumes of the cell suspensions (10^7 cells) maximum currents of only 200μA would be expected. With

limitations within the biofuel cell system related to low electron transfer efficiencies at the cell-electrode interface, high internal resistance associated with the PEM and electrodes, low cathode reaction rates, the current output would end up being several orders of magnitude smaller than this ideal case value. An attempt was made in this study to determine whether electron transfer can occur between human white blood cells and carbon fiber electrodes within the context of a biofuel cell system. Employing such a system, however, is inadequate in itself to prove conclusively that such electron transfer can occur and to verify the mechanism of electron transfer. As a result, electrochemical techniques such as cyclic voltammetry will become necessary to further explore the electron transfer capability of human white blood cells.

6.0 CYCLIC VOLTAMMETRY AND HPLC OF LEUKOCYTES

6.1 ABSTRACT

In previous studies by our group, the ability of human white blood cells (WBC) to generate electrical currents when introduced at the anode of a proton exchange membrane (PEM) fuel cell has been investigated. The primary goal of those studies was to promote electron transfer (ET) between intracellular metabolic processes and extracellular carbon felt electrodes. In this study, cyclic voltammetry (CV) is used to further investigate the electrochemical activity of activated human WBCs to identify possible mechanisms of ET, as well as to identify species that are released by these cells, which may undergo oxidation-reduction reactions at the electrode surface. Cyclic voltammograms were obtained for human WBCs, specifically PBMCs (lymphocyte-monocyte mixture isolated on a Ficoll gradient), a B lymphoblastoid cell line (BLCL), and two leukemia cell lines, namely K562 and Jurkat. Cyclic voltammograms of the PBMCs revealed oxidation peaks at an average potential of 368mV vs. SCE for PMA and ionomycin activated cells isolated from five human subjects. Corresponding reduction peaks were not observed in the cyclic voltammograms of the activated cells. Similar oxidation peaks to those observed for PMA activated PBMCs did not appear in the cyclic voltammograms of the BLCL, K562 or Jurkat cell lines regardless of whether they were activated or not. Previous studies by other research groups have described oxidation peaks associated with both human and

rat leukocytes that have been attributed to the release of serotonin (5-HT) from intracellular stores. The identity of serotonin was verified by high performance liquid chromatography (HPLC) with electrochemical detection.

Keywords: Serotonin, Cyclic voltammetry, HPLC, PBMC, PMN, BLCL, Jurkat, K562

6.2 INTRODUCTION

Electrochemical analyses of biological systems can be rather complicated due to the vast number of electrochemically active species that are either released by biological cells into the extracellular environment or that are present in the plasma membranes of these cells. Previous research by our group has sought to demonstrate the feasibility of generating small electrical currents when white blood cells (WBC) are introduced to the anode of a proton exchange membrane (PEM) fuel cell [13, 14]. The goal of these previous studies was to explore whether the plasma membrane bound protein complex, NADPH oxidase, is capable of self-mediating electron transfer between intracellular NADPH and an interfacing electrode. Establishing electrical communication channels between the redox centers of membrane bound proteins and an electrode is, however, one of the fundamental difficulties facing the development of bioelectronic systems. Undertaking an electrochemical analysis of membrane protein complexes can be confounded by the activity of myriad other species that may be present in the plasma membranes of the cells - such as coenzymes - or by various species that are released by cells in their activated states, such as cytokines, histamine and serotonin (normally released by leukocytes during an allergic or inflammatory response). Cyclic voltammetry (CV) studies performed by other research groups have identified oxidation peaks associated with serotonin (5-HT) release by white blood cells [72-74], specifically human neutrophils and rat basophilic cells. The peaks were normally observed with the application of various allergens to the WBC population at a potential of 330mV vs. SCE [73]. In this work, results from recent studies investigating the electrochemical activity of human WBCs are presented and discussed. Of particular interest was the lymphcoyte and monoycte cell population – or peripheral blood

mononuclear cells (PBMCs) – and the release of biochemical species, such as 5-HT, upon activation with PMA and ionomycin. Phorbol esters, such as PMA, are used to activate cells via a protein kinase C (PKC) pathway [75]. The electrochemical activity and release of 5-HT from BLCL (B lymphocyte cells), K562 (a monocyte progenitor cell line) and Jurkat (a T lymphocyte progenitor cell line) were also investigated. HPLC was used to verify the presence of 5-HT.

6.3 MATERIALS AND METHOD

6.3.1 Cyclic Voltammetry

A three-electrode electrochemical setup was employed for the cyclic voltammetry study. The system consisted of a working electrode, which is the site of charge transfer (where the reaction under investigation occurs), and a counter electrode, which serves to complete the circuit. The third electrode, the reference, functions just as its name implies. It is used as a reference for measurements of potential recorded at the working electrode. The reference electrode is designed to have a very high input impedance and as a result, remains unsusceptible to changes in its potential due to the affects of polarization, as only very small currents can flow through. The fact that the reference potential remains relatively constant means that accurate measurements of potential at the working electrode can be made. The majority of the current that flows within the three-electrode electrochemical cells flows between the working and counter electrodes and can be simultaneously measured along with potential. Cyclic voltammetry provides plots reflecting the relationship between current (i) and potential (E). The resultant i-E plot for a redox species in solution has a characteristic "duck" shape, consisting of an oxidation and reduction peak. The potential that corresponds to the middle of the two peaks is usually taken as the midpoint potential, E_m. Using the Gamry interfacial software, parameters such as the voltage scan range, scan rate, maximum current and electrode surface area can be specified.

Cyclic voltammetry was performed using the Gamry Potentiostat, FAS2/Femtostat under control of the Gamry Framework Software from Gamry Instruments. The Gamry Software is installed on a Dell Dimension 2400 Desktop Computer. A three-electrode setup was used, where

carbon felt and platinum served as the working and counter electrodes, respectively, while a saturated calomel electrode (SCE) served as the reference. Phorbol-12-myristate-13-acetate (final concentration of 5ng/ml) and calcium ionomycin (final concentration of 500ng/ml) were used to activate the white blood cells. White blood cell activation occurs as a result of non-specific Protein Kinase C (PKC) pathways. Four WBC samples were studied, namely human peripheral blood mononuclear cells (PMBC), cells derived from K562, a BLCL and Jurkat cell line.

This study was approved by the Institutional Review Board for Human Subject Research of the University of Pittsburgh. Human white blood cells were isolated from approximately 12mL of whole human blood using a Ficoll-Paque^{TM} density gradient. Briefly, whole blood was gently added, using a pipette, to an equivalent volume of the Ficoll-Paque^{TM} solution to obtain two clearly defined layers. After centrifugation at 2000 rpm for 20 minutes, four clearly defined layers can be discerned for the gradient – red blood cells at the bottom, followed by a larger volume of the Ficoll solution, then a thin layer of white blood cells (consisting primarily of PBMCs) and finally a larger volume of blood plasma. The thin PBMC layer comprising the B and T lymphocytes was carefully recovered using a pipette. The cells were washed twice in phosphate-buffered saline (PBS) solution (pH 7.4) by centrifugation at 1600 rpm for 15 minutes and were finally resuspended in 15 mL of the PBS solution. A cell count was then performed using a light microscope and hematocytometer to determine the final cell density in the PBS solution. K562, a BLCL and Jurkat cell lines were also cultured over a period of one week in RPMI with 10% fetal bovine serum (FBS). The cells were isolated from the culture medium by centrifugation, repeated washing (as described previously) and finally resuspension in 1X PBS. A light microscope and hematocytometer was used to determine the final cell densities in PBS.

In this study, the white blood cells suspended in PBS solution were scanned within a potential range of -0.5V to 1.2V at a scan rate of 100mV/s. The white blood samples were acquired from 5 human subjects and the cyclic voltammetry studies conducted over the course of several days. A new carbon fiber electrode was used for each sample. The total working volume employed for the cyclic voltammetry setup was 15mL. The white blood cells were activated by addition of 1 μL each of phorbol-12-myristate-13-acetate (PMA) and ionomycin per 1mL of the total working volume to achieve final concentrations of 5ng/ml and 500ng/ml, respectively. The effect of variable glucose concentrations was investigated. The peak potentials and currents associated with the observed oxidation peaks were determined using MATLAB® software.

Any oxidation peaks present in the cyclic voltammograms were identified and the electric potential range for the region of interest corresponding to the oxidation peak defined. Upper and lower boundaries corresponding to the region of interest were determined by visual inspection to identify the highest and lowest electric potentials corresponding to the width of the identified oxidation peak. Linear regression and interpolation was then used to fit a baseline bounded by the two points corresponding to these upper and lower potentials. The interpolated current values for the baseline were subtracted from the original current values of the cyclic voltammogram within the region of interest corresponding to the oxidation peak. As a result, adjusted values for the peak current were obtained.

6.3.2 Microdialysis and High Performance Liquid Chromatography

In vitro microdialysis [76] was used in these experiments to extract the released biochemical content from the cell suspension, while high performance liquid chromatography (HPLC) with electrochemical detection was subsequently used to validate whether or not serotonin was released by the cells, as well as to quantify the amount of serotonin released upon cell activation. The microdialysis probe is constructed from 23-gauge stainless steel tubing, with a hollow fiber that runs through the length of the tubing (Figure 28). A small length of the hollow fiber (approx 1.5-2.0mm) is allowed to protrude from the end of the stainless steel tube and remains in contact with the surrounding solution. Within the hollow fiber is vitreous silicate tubing. This silicate tubing serves as an inlet section for perfusing buffer, while the outlet section is housed in the 23-G steel tubing. Since the cells are suspended in PBS, a PBS buffer solution can also be used for perfusion. HPLC can be used to identify and quantify biochemicals in solution. It comprises a column containing the stationary phase and an injector used to introduce the mobile phase containing the dissolved species of interest into the column. If the mobile phase contains a mixture of chemicals, separation of the chemicals occurs using this chromatography technique due to their variable solubilities. Retention time is important. If the chemical is more soluble in the stationary phase, it is going to remain in the column longer (longer retention time) than another chemical that is more soluble in the mobile phase. An electrochemical detector outputs a signal that provides information related to the quantity detected as well as the retention time (used to identify the species).

Approximately 12ml of whole human blood was obtained from healthy volunteers and the white blood cells isolated on a ***Ficoll-Paque™*** gradient (as described previously). The cell

samples were placed on ice for later microdialysis and HPLC analysis. Cells derived from K562, Jurkat and BLCL cell lines were also analyzed in order to determine whether 5-HT is normally released from these cells.

1.5mL samples of each of the cell suspensions were placed in microcentrifuge tubes. The hollow fiber to be used for microdialysis was allowed to sit in fresh distilled water (dH_2O) for an extended period of time. For cell stimulation, PMA and ionomycin are introduced and 20-30 minutes allowed for complete activation. Microdialysis was used to isolate 5-HT released by the cells. 3 samples were collected from microdialysis of the cell suspensions and controls (perfusion rate of 5uL/min for 2 minutes) in autosampler tubes. The hollow fiber of the microdialysis probe was made about 1 cm long to improve the rate of recovery. Percent recovery was determined from the standard solution. HPLC was used to analyze the following samples:

a. PBS
b. PBS + PMA (5ng/ml) + ionomycin (500ng/ml)
c. PBS + PMA + ionomycin + glucose (1mg/1ml)
d. Non-activated PBMCs and cell lines
e. Activated isolated PBMCs and cell lines
f. Non-activated PBMCs and cell lines with D-(+)-glucose (1mg/1ml)
g. Activated PBMCs and cell lines with D-(+)-glucose (1mg/1ml)
h. Blood plasma

For washing, the hollow fiber was placed in dH_2O and allowed to dialyze at a rate of 20ul/min for 2 minutes. It was then placed in fresh dH_2O for 3 minutes between samples to wash out any residual 5-HT. 8.2uL sample volumes were injected into the HPLC by a FAMOS

autosampler. Detection of any 5-HT was determined electrochemically using an Antec-Leyden Intro amperometric detector (Zoeterwoude, The Netherlands).

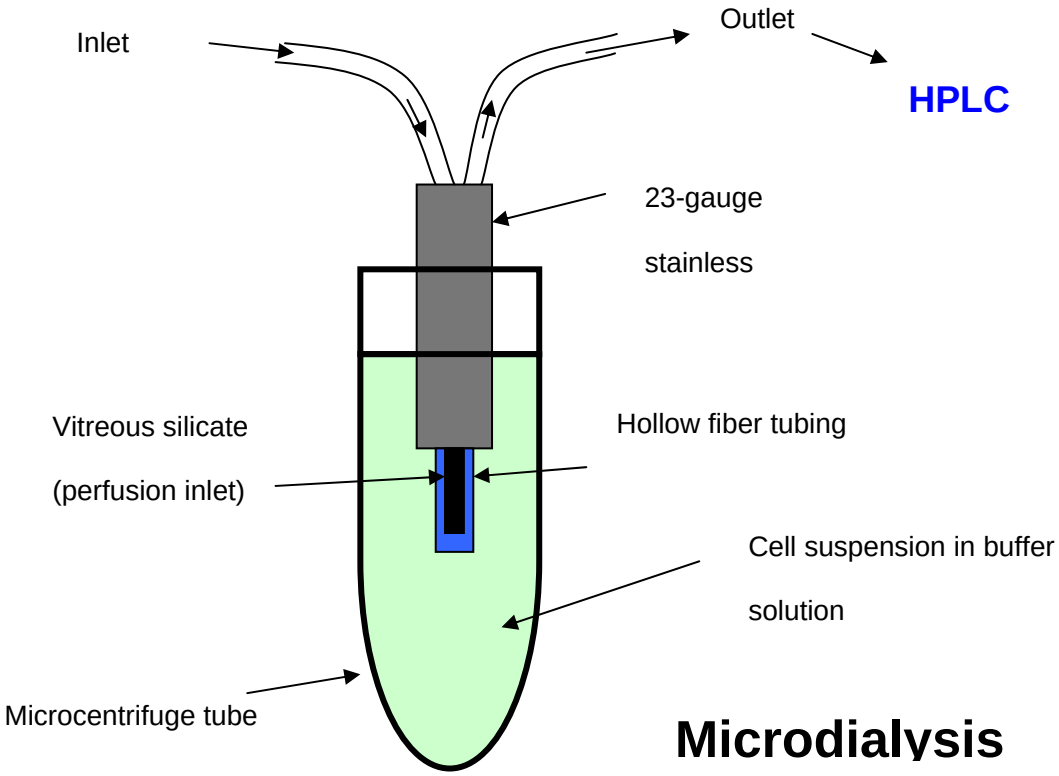


Figure 28: *In-vitro* Microdialysis

6.4 RESULTS AND DISCUSSION

6.4.1 Cyclic Voltammetry

Cyclic voltammetry of PBMCs, K562, Jurkat and BLCL

Cyclic voltammograms acquired from isolated human PBMCs suspended in 1X PBS and activated with PMA (5ng/ml) and ionomycin (500ng/ml) revealed oxidation peaks at an average potential of 368mV (+/- 18mV) vs. SCE. Peak currents up to 12 μA within the 350mV to 400mV range were recorded for cell densities on the order of 10^6 cells/ml (Figure 29 and Figure 30). Oxidation peaks were not observed for any cultured cell type, i.e. for the K562, Jurkat and BLCL cells. There was a noticeable absence of a reduction peak in the voltammograms, which normally implies that irreversible oxidation of the species has occurred. Peaks were neither observed in the absence of cells nor before activation of the cells with PMA. The results of our experiments strongly suggest that the activated lymphocyte-monocyte mixture comprising the PBMCs releases redox active species into the extracellular environment upon activation. Based on studies performed by previous research groups (see Table 5), it is likely that the redox species responsible for the 368mV oxidation peak is 5-HT [72-74]. It has been suggested that 5-HT is released from certain human leukocytes during inflammatory responses, particularly in response to allergens during allergic reaction. In the literature, the oxidation peak for 5-HT was normally reported at around 330 mV vs. SCE, which is a slightly lower value than what we have recorded in our own studies [73]. The absence of a reduction peak was often noted, and has been attributed to the irreversible oxidation of serotonin (5-HT) to 5-hydroxyindoleacetic acid (5-HIAA) [77].

The oxidation peaks obtained from the cell suspensions were barely discernable against the background currents, as the total area bounded by each CV curve was very large by comparison to the area bounded by the oxidation peaks within the 350mV to 400mV range (Figure 29). The large background currents are a direct consequence of two factors, namely the scan rate (set at 100mV/s) and the large surface area of the carbon felt electrodes. The larger the surface area of the electrode, the larger the electrode's capacitance. The relationship between the scan rate, capacitance and peak current can be expressed by the following equation:

$$i = vC_d[1 - \exp(-t / R_s C_d)] \qquad \mathbf{1}$$

where i is current, v is the scan rate, C_d is the capacitance of the electrode, t is time and R_s is solution resistance [78]. The peak current for an irreversible reaction is described by the following equation:

$$i_p = (2.99x10^5)\alpha^{1/2} n^{3/2} A D_O^{1/2} C_O^{*} v^{1/2} \qquad \mathbf{2}$$

where i_p is peak current, α is the electron transfer coefficient, n is the number of electrons transferred, A is surface area, D_o is the diffusion coefficient, C_o* is the concentration of the species in the bulk solution and v is the scan rate [78].

Differences between the results of our own studies and those of other researchers

A reason for the obvious difference between the peak potentials reported in the literature and our own is not immediately apparent. The 30mV difference may not be significant, since slight changes in the electrode surface can significantly impact peak potential. However, a possible explanation may be found in the difference in techniques used to define the location of the peak. Another possible explanation may be related to differences in the diffusional effects associated with serotonin transport to the electrode surface. It was observed that for the first three PBMC samples, there appeared to be a good correlation between the cell density and the magnitude of the peak current (Figure 31). For the fourth and fifth samples, however, the magnitude of each of the peak currents recorded was significantly lower than expected. In the case of one of the samples (Sample 4), a peak was not observed at all, despite activation with PMA and ionomycin. We have not been able to conclusively determine why an oxidation peak was not observed in this instance. However, one reasonable explanation may include the possibility that the PBMCs are not solely responsible for the observed currents, but that some other cell type is involved. During the isolation process on the Ficoll-Paque gradient, the PBMC layer extracted may have been contaminated with other cell types, including polymorphonuclear leukocytes (PML), such as neutrophils, leading to the observation of oxidation peaks associated with 5-HT release in the first three subjects. One study published by Nakamura et al. [73] reported that oxidation peaks associated with 5-HT release were only observed for isolated neutrophils and not for the isolated lymphocyte-monocyte mixture. In the fourth and fifth subjects, the neutrophil content may have been significantly less than the previous three subjects, resulting in much lower oxidation peak currents than expected. It should be noted that the experiments were performed on five separate days, with the electrochemical analysis of each cell performed on separate days. It was ensured,

however, that the techniques used to isolate the cells and to study them remained relatively unchanged. The theory that contamination by PMLs has occurred is, however, unlikely since centrifugation of blood on a Ficoll gradient would result in a well defined layer of erythrocytes containing PMLs such as neutrophils, basophils and eosinophils at the very bottom of the tube. PBMCs form a very well-defined layer at the top of the Ficoll gradient that can easily be extracted without contamination from other layers.

Establishing a relationship between the oxidation peak and glucose metabolism

An attempt was also made to verify whether or not the peaks were directly associated with glucose metabolism, as this may be an alternate source of oxidation current. It is well known that several types of cells contain protein complexes in their cell membranes that function as electron transport chains. The NADPH oxidase complex, for example, comprises structures within the plasma membrane that upon association in an activated state transfer electrons between the pentose phosphate pathway of glucose metabolism and extracellular oxygen. Many white blood cells, particularly human PMLs (neutrophils [57],[55] and eosinophils [56],[54]), express this enzyme complex. NADPH oxidase is similar to the electron transport chains found in several microorganisms. Previous research performed by other authors demonstrated that increasing the concentration of metabolizable fuels in bacterial cell cultures is associated with decreasing peak current in their cyclic voltammograms. It is known that a number of microorganisms can transfer electrons to electrodes by several mechanisms, including: i) the use of artificial electron mediators (electronophores) such as neutral red; ii) using mediators produced by the microorganisms themselves; iii) direct electron transfer across the bacterial cell membrane [33]. In one study by Prasad et al., the direct electron transfer ability of yeast cells, Hansenula

anomala, was demonstrated by cyclic voltammetry [36]. In the study, the authors reported oxidation peaks at 0.09V and 0.38V. Several additions of lactate stock resulted in a decrease in the magnitude of the peak currents. The explanation provided was that when nutrients such as lactate or glucose are introduced to the cells, the peak currents decrease indicating that direct electrical communication exists with the electrode. Electroactive enzymes in the extracellular membrane of the cells accept electrons generated through the oxidation of the fuel and are subsequently reduced. The reduced enzymes then transfer electrons to the electrode becoming oxidized themselves. The smaller peak currents reflect the fact that the majority of the enzyme population is unavailable due to their reduction resulting from increased rates of lactate metabolism.

In our study, addition of glucose to the WBC samples, however, was not normally associated with a clear decrease in the peak current. In most cases, addition of glucose did not appear to be correlated with the peak current at all (Figure 32). In a further investigation of Sample 5, however, a clear decrease was observed over the course of several successive CV cycles (sequence of 15 CV cycles) in spite of the addition of glucose to the cell suspension (Figure 33). The variability in the peak currents observed in Figure 32 reflects a possible role of mass transport to the electrode surface. In the event that inadequate mass transport occurs (such as when there is insufficient stirring of the sample solution), peak currents may be smaller than expected for certain cyclic voltammograms due to a lack of the reduced species at the electrode surface. These results suggest that the oxidation peak is due to a species that has been released by the cells into the extracellular environment. Irreversible oxidation of 5-HT to 5-HIAA is the most likely reaction.

Extracting the oxidation peak current

One problem that was encountered while conducting the CV studies was a rather unstable i-V curve for the cell suspensions. The overall shape of the CV curves and the absolute values of the current magnitude tended to differ appreciably from cycle to cycle for the same sample. Therefore, a control curve (obtained from performing CV on a non-activated cell population) often did not provide an appropriate overlap (background current-voltage curves) that could have been used to subtract from the experimental case to obtain the true peak current value. Instead of relying on this background current, MatLab was used to create a linear baseline fit within the potential range corresponding to the width of the peak (Figure 30). The boundary points of the peak, however, were determined solely by visual inspection and consequently there is an associated error that should be considered. This error, however, does not exceed a potential range of approximately +/- 10mV at each boundary point, and thus +/- 20mV for the entire width of the peak. The magnitude of the peak current was obtained by subtracting the original experimental values of the peak, from the current values that lay along the baseline fit that was previously established. Due to the fact that visual inspection was required in order to identify the boundary points of the peak current and to create the baseline current, errors may be expected in terms of the peak potential. Notable, however, was the fact that the average peak potential appeared to increase with increasing average cell density (Table 6). One thing that should be taken into consideration is the fact that the greater the cell density, the more viscous the cell suspension becomes. Therefore, it is more than likely that the peak potential becomes higher with increasing cell density due to the greater diffusion effects that accompany the changing fluid viscosity.

Cyclic voltammetry of 5-HT samples

Cyclic voltammetry of samples of 5-HT dissolved in PBS revealed oxidation peaks at about 400mV. The peak potentials were evidently slightly higher than that observed for the WBC samples. However, there were very similar characteristics, in that an oxidation peak was observed with the absence of a corresponding reduction peak. On successive cycles (2^{nd} and 3^{rd} CV cycles), the height of the peak decreased by more than 50%, indicating that i) there is less 5-HT available in the solution for oxidation and ii) that electrode filming might have occurred, a very common problem associated with carbon-based electrodes. In Figure 29, peaks were not observed in the 2^{nd}, 3^{rd} and subsequent cycles. Since only a very small quantity of 5-HT would be expected to be released by the cells, it is more than likely that the sensitivity of the Gamry was not sufficient to detect the smaller concentrations subsequent to the initial cycle. By varying 5-HT concentrations and measuring the peak currents from the cyclic voltammograms, a direct proportionality relationship was established between 5-HT concentration and peak current (Figure 34). Addition of glucose to the 5-HT solution did not affect the decreasing trend of the peak current significantly. There was some variation in the measurements made of peak current with addition of glucose, but over time (or over successive CV cycles) there was a clear decrease in the peak current.

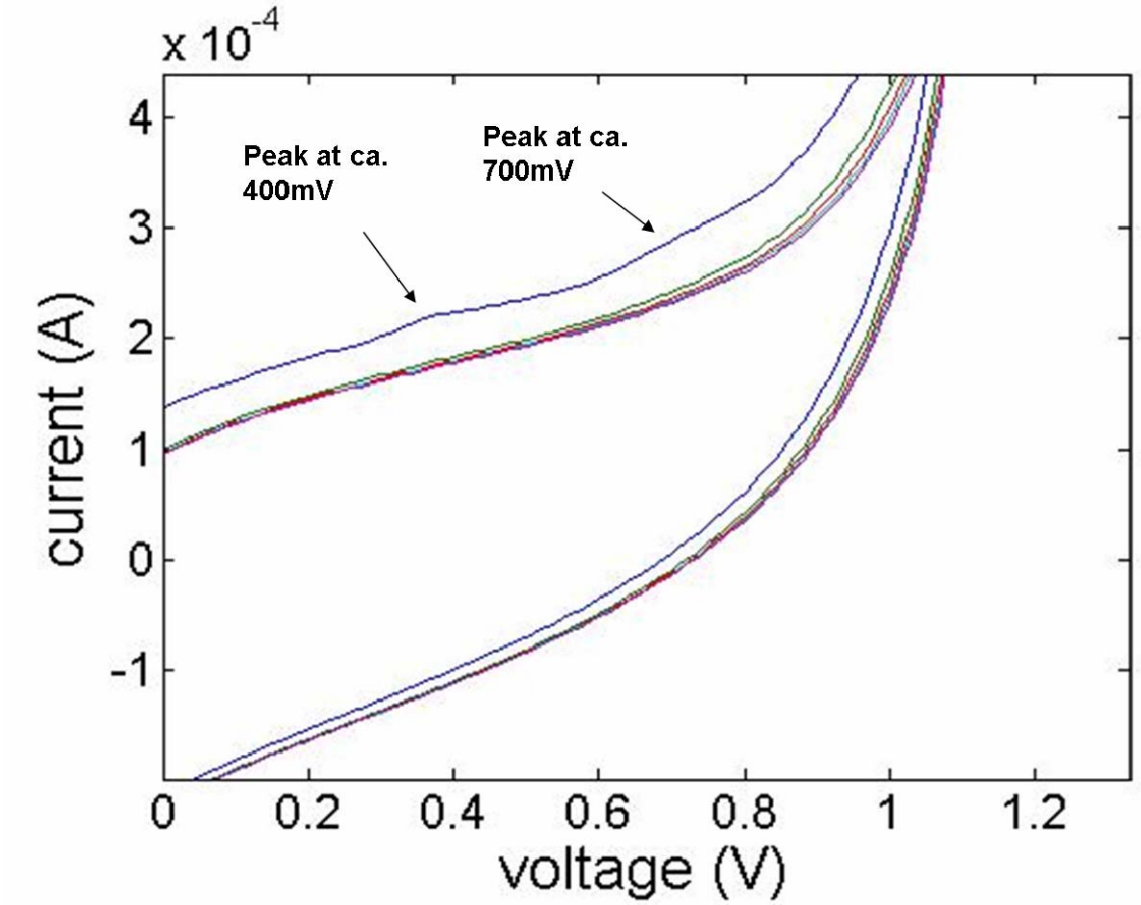


Figure 29: **i-E (or I-V) curves (5 cycles are shown) from the cyclic voltammetry of activated human lymphocytes. Two peaks can be perceived at about 400mV and 750mV. The first peak is due to serotonin released by the cells in their activated state. The second has been reported in the literature to be associated with CoA in the extracellular membrane.**

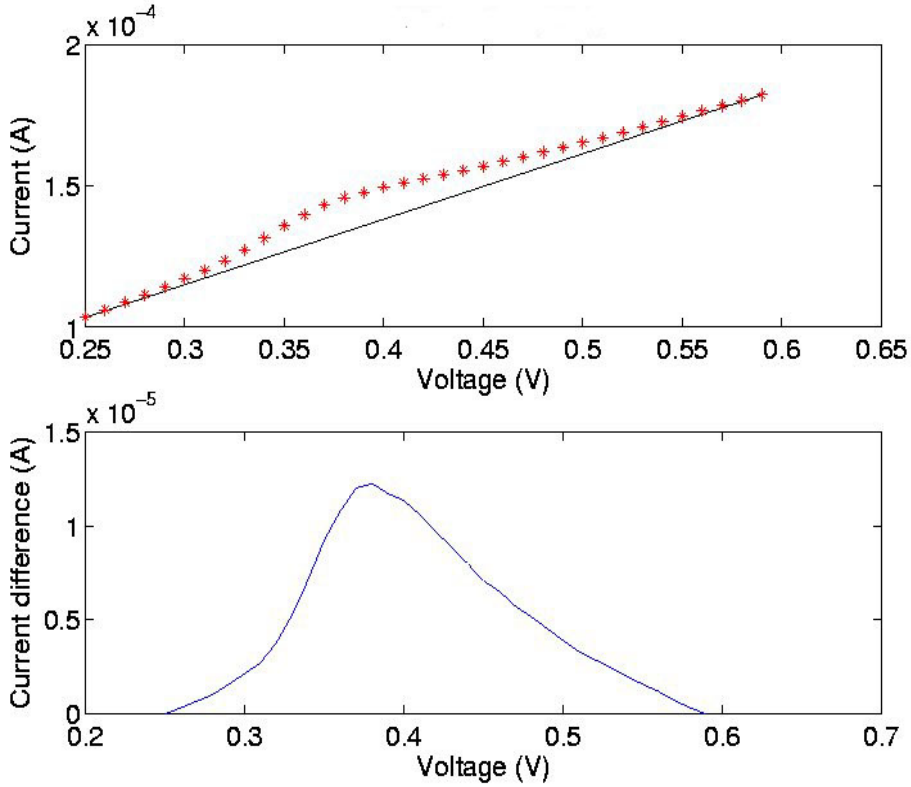


Figure 30: Oxidation peak at around 400mV vs. SCE was identified. An approximation to the current amplitude of the observed peak was determined by creating a linear baseline fit spanning the width of the oxidation peak. The boundary points corresponding to the width of the peak were identified by visual inspection and the linear regression and interpolation performed using MatLab software. Subtraction of the baseline from the actual cyclic voltammogram current values gave an approximation of the current amplitude of the oxidation peak.

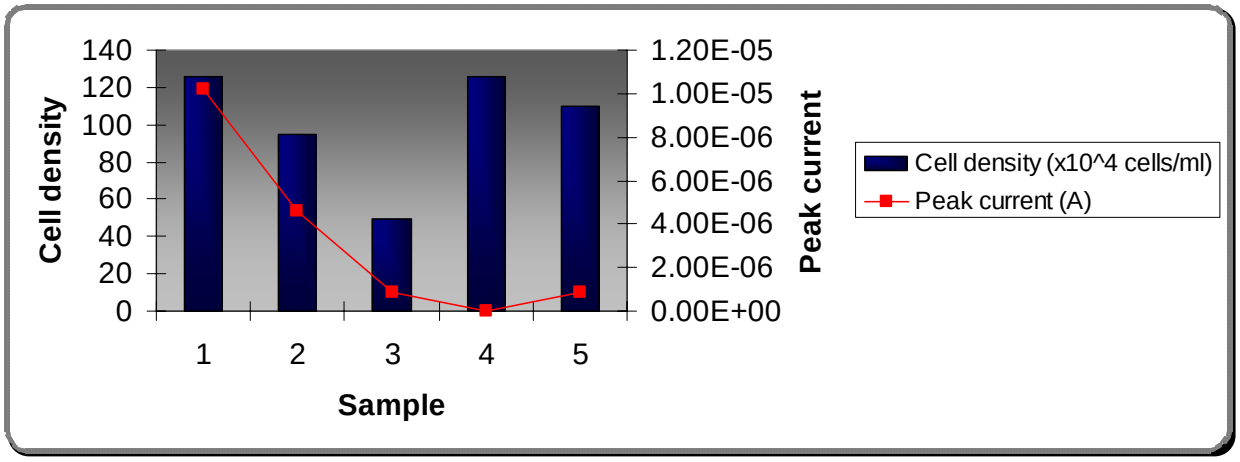


Figure 31: Variation in the amplitude of the oxidation peak current is plotted with PBMC density for blood samples acquired from five different individuals. Each sample was studied using a fresh carbon fiber electrode and conducted over the course of several days. Inspite of the large number of cells present in Sample 4, oxidation peaks were not detected for the activated lymphocytes. This may indicate an absence of cells that serve as the major contributor to the peak current. If the currents are produced by the lymphocyte-monocyte mixture, the lower than expected currents for Samples 4 and 5 may indicate failure of the cells to activate.

Table 5: **Summary of previous cyclic voltammetry studies of white blood cells acquired from human beings, as well as from various other animals. All measurements were made in PBS with a saturated calomel electrode (SCE) as the reference electrode.**

Article	Cells	Working electrode	Ep	Ip	Electroactive species
Ci et al. (1998)	White rabbit leukocytes and erythrocytes	Graphite	0.32V (leukocytes) 0.73V (erythrocytes)	1µA per 10^5 cells for Ep = 0.32V	Membrane-bound proteins?
Matsunaga et al. (1989)	Rat basophilic leukemia cells (RBL-1) and mouse lymphocytes	Basal plane pyrolytic graphite	0.34V and 0.68V (RBL-1) 0.65V (mouse lymphocytes)	0.76µA per 10^5 cells for Ep = 0.34V	Serotonin (0.29-0.34V) CoA (0.65-0.74V for microorganisms)
Nakamura et al. (1991)	Human leucocytes	Basal plane pyrolytic graphite	0.33V and 0.68V (leucocytes) and 0.68V (erythrocytes)	0.36-0.58 µA per 10^6 cells for Ep = 0.33V	Serotonin (0.29-0.34V) CoA (0.65-0.74V for microorganisms)

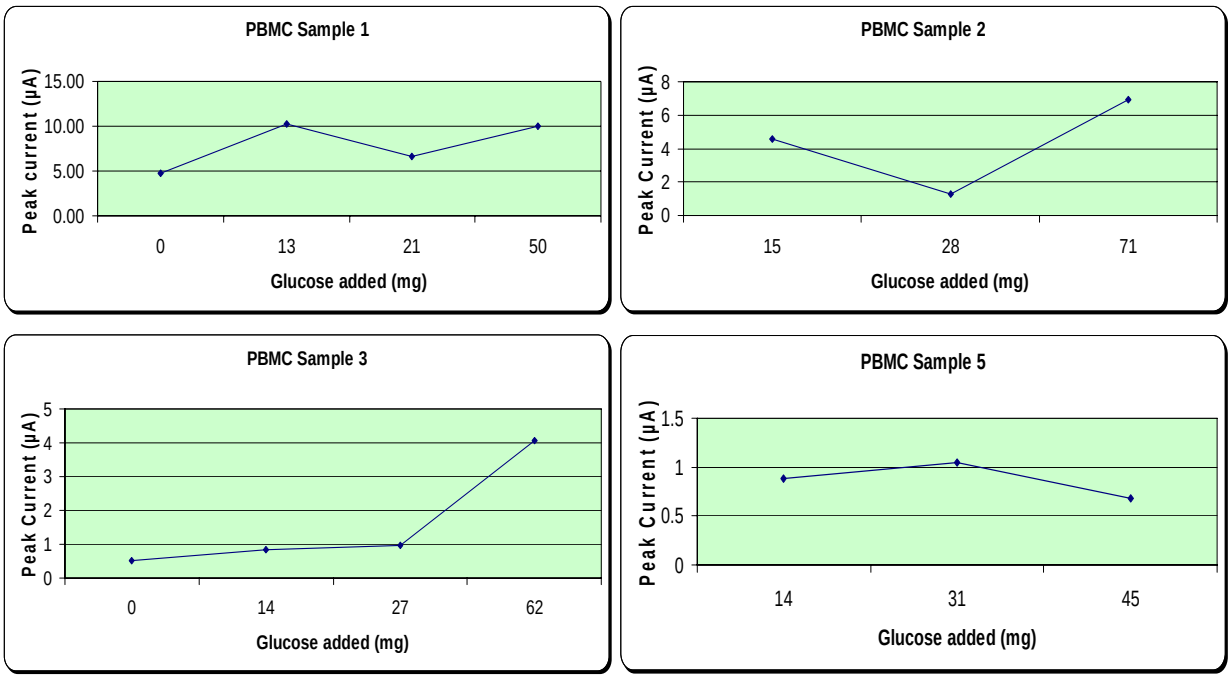


Figure 32: **The relationship between glucose concentration and the peak current magnitude is explored for four white blood cell samples suspended in PBS (PBMC Samples 1, 2, 3 and 5). A consistent increase in peak current with glucose concentration was observed only for Sample 3. A correlation could not be established for the other 3 samples. These results imply that there is little to no correlation between glucose metabolism and the oxidation peak observed from the cyclic voltammograms. As a result, a possible role of NADPH oxidase or any other membrane-bound proteins as a direct source of electrons is eliminated.**

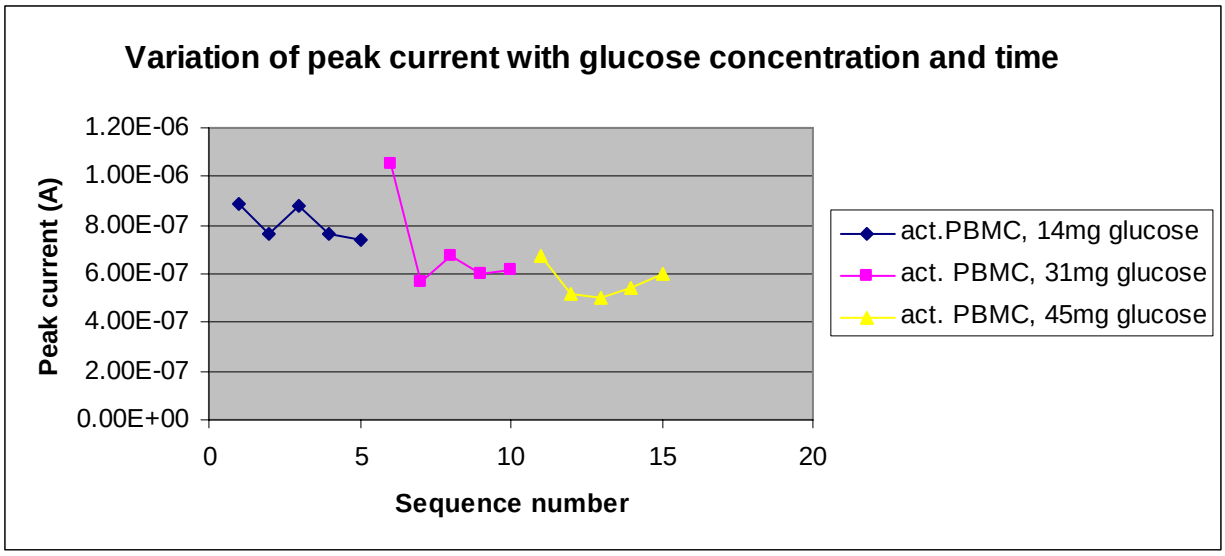


Figure 33: In spite of the addition of glucose to the PBMC sample (Sample 5), there was a continual decrease in the peak current with each successive CV cycle. The sequence number on the x-axis indicates the total number of CV cycles performed (16 cycles) in succession. After running 5 cycles, the conditions were changed by addition of glucose, in an attempt to determine whether or not a correlation can be made between metabolism and the observed oxidation peaks. The decreasing peak current is most likely a reflection of decreasing availability of 5-HT in the solution (due to irreversibility of the oxidation reaction).

Table 6: Peak potentials for PBMC samples. Sample 4 excluded since no peak was detected.

Sample	Cell density ($x10^4$), cell/mL	Peak Potential, mV
1	126 +/- 11	379 +/- 7
2	94 +/- 8	367 +/- 11
3	49 +/- 1	360 +/- 9
5	110	365 +/- 8

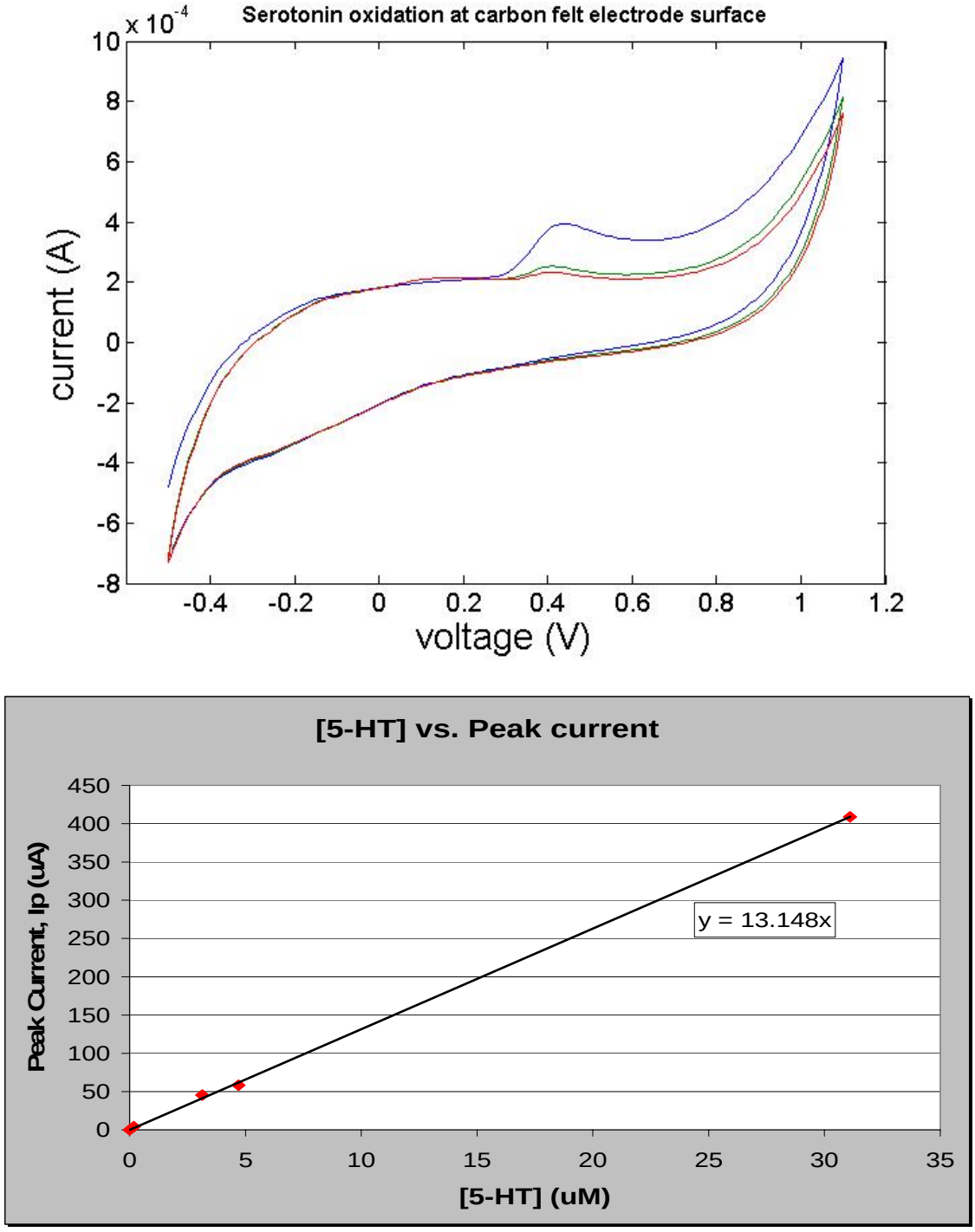


Figure 34: **(Top) Cylic voltammogram of 5-HT in 1X PBS. (Bottom) Relationship between peak oxidation current and serotonin concentration.**

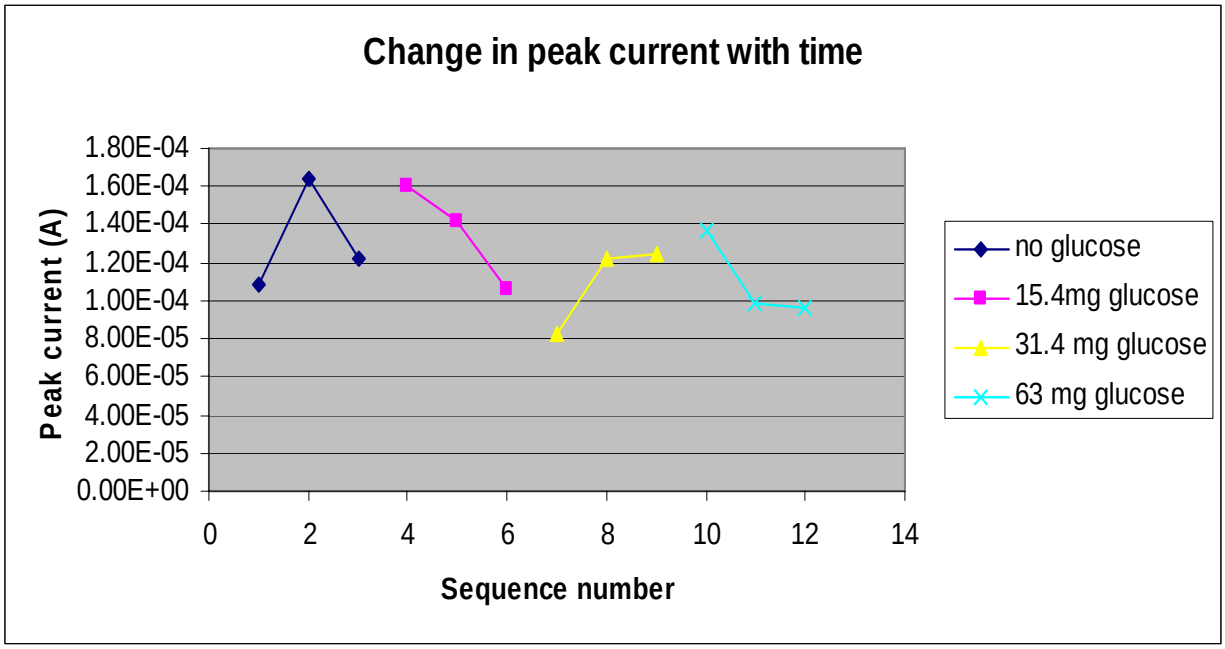
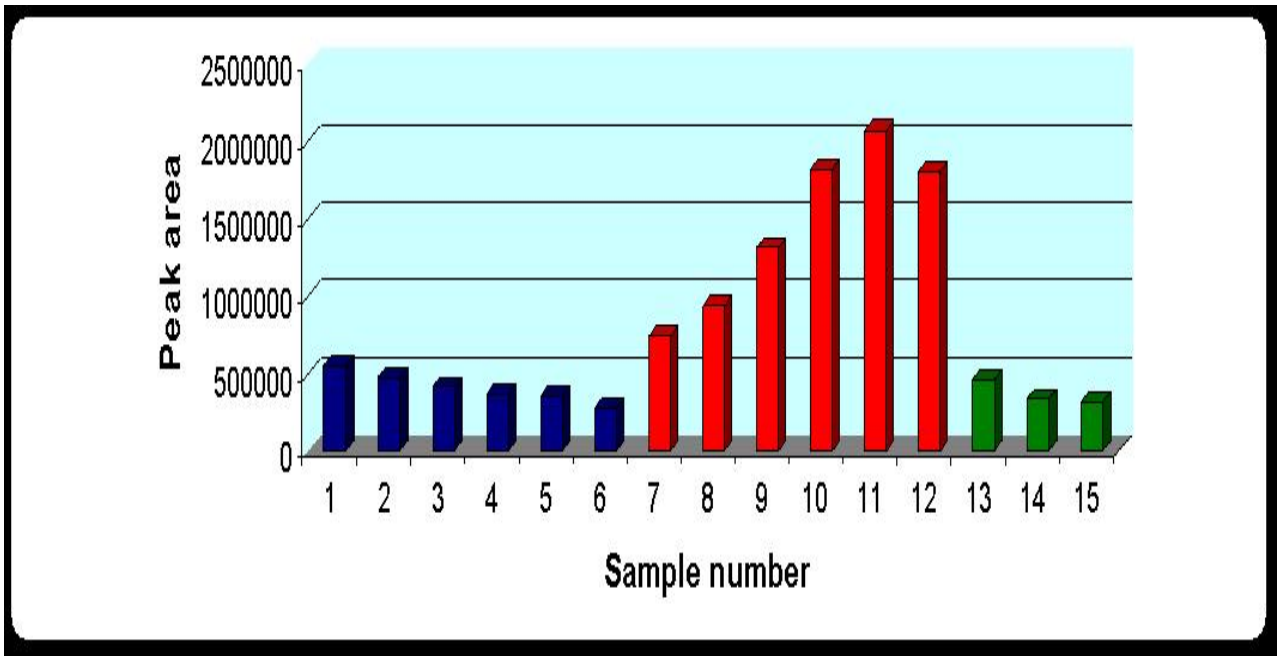


Figure 35: **Decreasing oxidation peak current of 5-HT over time with addition of varying glucose concentrations.**

6.4.2 Microdialysis and HPLC

HPLC was used to confirm the release of serotonin from the PBMC population. Any 5-HT present was first extracted from the WBC suspension by microdialysis (Figure 28). The HPLC results confirmed that 5-HT was in fact released only from PMA activated white blood cells, specifically PBMCs (Figure 36). The cells also did not require physiological glucose concentration in order to initiate release. These results go hand in hand with the previously performed cyclic voltammetry studies, as they indicate increased extracellular 5-HT concomitant with the observed oxidation peaks in the cyclic voltammograms. The HPLC results also indicated that 5-HT was not released from the BLCL, K562 or Jurkat cells.



Sample	*Sample Number*
PBS, PMA, Ionomycin	1-3
PBS, glucose, PMA, Ionomycin	4-6
PMA Activated PBMCs	7-9
PMA Activated PBMCs + glucose	10-12
Blood plasma	13-15

Figure 36: **Peak areas corresponding to the electrochemical detection of 5-HT released by human PBMCs. 5-HT release was associated with activation of the PBMCs with PMA and ionomycin. HPLC of PBS and blood plasma reveals baseline peak area values.**

6.4.3 Comparison with literature results

The observation of oxidation peaks and positive HPLC results corresponding to 5-HT in our studies with human lymphocytes and monocytes disagrees with the results from previous work performed by Nakamura et al. (1991) [73] on human neutrophils and human lymphocyte-monocyte mixtures. In their study, the authors reported that oxidation peak currents were not obtained from the lymphocyte-monocyte mixture. However, peaks were observed for the isolated neutrophil population. Our own studies indicate otherwise. Upon activation of the lymphocyte-monocyte suspension with PMA and ionomycin, there were discernable oxidation peaks at around 368mV vs. SCE. There were, however, differences in the implementation of the two studies. In our study, a phorbol ester was used to activate the cells, which had been suspended in PBS. Carbon felt was used as the working electrode. In the study by Nakamura et al., however, a basal pyrolytic graphite electrode was employed with the leucocytes attached to the electrode surface through immobilization on a porous nitrocellulose membrane filter. The authors did not use a phorbol ester, but rather applied known allergens to the blood samples before performing CV. The allergen was expected to induce an allergic response by the cells, resulting in the release of various compounds normally associated with cell activation. The difference between our results and theirs cannot be explained at the present time. The variable conditions between the two experiments may play a significant, but as yet, unclear role and one can only surmise at the moment. Another possibility for the observation of peak currents in our experiments is that the PBMCs contained some neutrophil content that would have accounted for the majority of the serotonin released. As seen in Figure 31, the cyclic voltammogram of PBMC Sample 4 did not demonstrate an oxidation peak, despite the high cell density at which the lymphocyte-monocyte

mixture was suspended. Smaller peaks than expected were also evident for the PBMC Sample 5 acquired from a different subject. Two possible explanations are that the cells from Samples 4 and 5 were not sufficiently activated or that there was a significant lack of neutrophils that might have contaminated the first three PBMC samples but was not present in sufficiently large numbers in the last two samples obtained.

6.5 CONCLUSIONS

This work builds upon work performed by previous authors demonstrating that serotonin release from human white blood cells can be detected using electrochemical techniques such as cyclic voltammetry. Previous work by other researchers has similarly been performed for white blood cells isolated from rodents and white rabbits. In these studies, peaks were also reported within the 300 to 400mV range; however, in one study the oxidation peak was attributed to serotonin, while in the other study, the peak was attributed to electroactive membrane-bound proteins. In this study, it was shown that human lymphocyte-monocyte mixtures (PBMCs) isolated from whole human blood on a Ficoll gradient release serotonin upon activation with a phorbol ester, namely PMA, and calcium ionomycin. Identification of serotonin was verified by HPLC with electrochemical detection. Well-defined peaks were observed on HPLC chromatograms, which was a clear indication of the presence of serotonin in the injected samples. In previous studies, activation of the white blood cells was normally achieved by application of allergens. In order to refute the theory that the peak was due to direct electrical communication between the cells and the electrode surface through membrane-bound proteins, various quantities of glucose were added to the WBC suspension and cyclic voltammograms subsequently obtained. According to work performed by other research groups such as Prasad et al., addition of a metabolizable biofuel, such as lactate or glucose, should decrease the height of the peak current if the peak is in fact due to direct electrical communication between cellular metabolism and the electrode. Tremendous variation was observed for the height of the peak current with the increasing glucose concentration. A clear decrease was not apparent from the four PBMC samples that demonstrated oxidation peaks. In fact, a decrease in the oxidation peak

height would normally be expected, since 5-HT is irreversibly oxidized to 5-HIAA. The variation observed in most of the samples; however, appear to be indicative of diffusion limiting effects, associated with the mass transport of the electrochemically active species to the electrode surface. Insufficient stirring of the solution and/or time dependent release of serotonin quantities from intracellular stores would be likely contributors to the variation in the peak height, which offers a direct indication of the concentration of the species in the bulk solution. Noteworthy was the fact that oxidation peaks were not observed for BLCL, Jurkat or K562, despite activation with the phorbol ester. The absence of oxidation peaks from the cyclic voltammograms of these cells suggests that 5-HT (derived from the amino acid tryptophan) is not a normal metabolic product. The majority of the 5-HT released by the white blood cells might have actually been absorbed and subsequently compartmentalized by the cells for later release. These results are also suggestive of 5-HT as a possible source of electrons in the previous leukocyte-based biofuel cell experiments.

7.0 SUMMARY AND FUTURE WORK

Research project goals

The primary goal of this research was to explore a possibly novel method for generating electricity within the physiological environment for low power implantable medical devices, such as miniature biosensors. Several technologies have previously been developed to power larger implantable devices such as cardiac pacemakers and deep brain stimulators [2, 15]. The power densities required for these devices are rather significant, and as a result high capacity energy sources such as lithium ion batteries have been commonly employed [5, 15, 17]. Throughout the literature, many research groups have explored other potential power sources to replace internal batteries, due to the problem of size (batteries are rather bulky) and limited lifespan (lithium ion batteries can last for up to 10 years, however, a longer lasting power source may eliminate the need for surgical explantation). Apart from the various modalities explored for transcutaneous energy delivery, other devices such as nuclear devices, piezoelectric devices [8, 9, 18] and implantable biochemical/biofuel cells [3] have been studied. Nuclear batteries have achieved incomparable success from a technical standpoint [6, 7], however, regulatory issues quickly stunted their wider employment in the medical device field. Piezoelectric devices have been plagued by their very low current and power densities, until very recently [18]. Biofuel cells have also been achieving a reasonable amount of recognition as potential power sources,

particularly since the development of an enzyme-based biofuel cell for continuous glucose monitoring and of another enzyme-based BFC that runs on alcohol for powering small, portable devices. As our group became more knowledgeable about the theories (thermodynamic and kinetic) that govern electrochemical devices such as biofuel cells, it became more evident that the challenge of powering implantable stimulating devices, including pacemakers, would be difficult to overcome. We have, therefore, attempted to focus our attention increasingly on the novel fields of micro and nano-technology, which are creating unique opportunities for the development of small, minimally invasive biosensors for the detection of pathologies such as cancer. For such biosensors, small amounts of power may be needed for detection, as well as for communication with extracorporeal devices. Biofuel cells appear to be promising, due to the fact that they are essentially biomimetic devices that generate electricity in a similar manner to the way that cells generate energy through respiration. A biofuel cell can transduce the energy stored in various organic substrates such as glucose into electricity, by coupling the oxidation of the fuel with the reduction of the essential universal biological electron acceptor, oxygen.

Electron transport between biological species and electrodes: Challenges

Electron transfer between biological species and electrodes remains a challenging problem, but not an insurmountable one. In this work, we have actively sought to determine the feasibility of developing a novel type of biofuel cell, one that is based on electron transfer between human white blood cells and an electrode surface. A significant amount of research performed by other groups has previously focused on microbial [40] and enzyme [49] based biofuel cells that generate electricity through the catabolism of organic substrates such as glucose. Our studies described here are a unique contribution to the field in that these were the first to suggest and

attempt to employ whole eukaryotic, and more specifically, human white blood cells to generate electricity through electrochemical interactions with the anode of a biofuel cell. The unique concept that we presented was that NADPH oxidase, a plasma membrane based enzyme complex expressed extensively among phagocytic white blood cells, can self-mediate electron transfer between intracellular NADPH and an extracellular electrode. The hypothesis stemmed from the many studies that indicated certain microbes, such as those belonging to the metal reducing Geobacter family, are capable of self-mediating electron transfer to metals and metal electrodes due to the presence of an electron transport chain proteins in their outermost membrane [30]. Electron transport is typically not characteristic of the plasma (extracellular) membranes of eukaryotic cells in general. It is an important aspect, however, of glucose metabolism in the mitochondria. Prokaryotic organisms such as bacteria, unlike eukaryotic cells, do not possess mitochondria; neither do they possess any other organelles typically found in the cells of eukaryotes [69]. Glucose metabolism in its entirety occurs within the intracellular environment of prokaryotes and the final electron transport process is characteristic of the extracellular membranes of microbial organisms.

A number of microbes have been shown to be capable of self-mediating electron transfer to electrodes. Various mechanisms for electron transfer have been proposed, including indirect electron transfer through mediators excreted by the cell and direct electron transfer through membrane bound electron transport proteins associated with metabolism. The latter has commonly been considered as a very likely mechanism in the literature. Normally, electron transfer through the direct method described previously occurs at very low efficiencies, and as a result artificial electron mediators (or electronophores) are commonly employed to improve the electron transfer efficiency. This low electron transfer efficiency is an extremely important

problem to consider, as this explains the very low currents observed from microbial fuel cells that did not employ artificial electron mediators (such as methylene blue and neutral red) and also is a more than probable explanation for the very low currents observed from our own biofuel cell studies with white blood cells.

The issue of low electron transfer efficiencies is a fundamental difficulty that faces bioelectronic systems [53]. Low currents obtained from bioelectronic systems are largely associated with i) the distance between the electron donor and acceptor (rate of electron transfer decreases exponentially with distance), ii) the aqueous environment characteristic of many electrochemical systems and iii) inaccessibility of redox active centers – embedded within the three dimensional protein structures - to the electrode surfaces [53]. The low currents that we have reported for our biofuel cells is likely a consequence of the inability of the carbon fiber electrodes to achieve good electrical communication with the redox centers of the NADPH oxidase enzyme complex. Cytochrome b558 contains the redox active center of NADPH oxidase that is normally responsible for transferring electrons to the final electron acceptor, molecular oxygen [58]. Now, consider the structure and characteristics of molecular oxygen. It is small, mobile and interacts in a very specific orientation with cytochrome b558. These are characteristics that a carbon fiber electrode can rarely, if ever, compete with without modification. In the context of a biofuel cell, where the white blood cells are in suspension around the carbon fiber electrode and may even attach to the electrode, many electrons derived from intracellular NADPH are likely to be preferentially transferred to oxygen in the surrounding solution, rather than to the electrode. This is a very important problem that must carefully be considered in our current study. Bacteria such as Escherichia Coli are capable of producing comparatively larger currents than our own fuel cell because they 1) can survive in an anaerobic

environment (nitrogen gas is often passed through the anode compartment to remove any dissolved oxygen that may interfere with the electron transfer process to the electrode) and 2) artificial electron mediators may be employed as a means of bridging the gap between the metabolic processes of the cells and the electrode. Neither of these techniques can be applied to white blood cells primarily because 1) cells can only function anaerobically for a short period of time and therefore will always require oxygen for mitochondrial glucose metabolism and 2) many electron mediators can be toxic, which is undesirable for any *in-vivo* human applications. Depriving cells of oxygen would quickly lead to cell death as oxidative phosphorylation in the mitochondria would cease. Maintaining sufficient quantities of oxygen in the extracellular environment would mean that electrons from the pentose phosphate pathway – the primary source of NADPH – would ultimately be preferentially transferred to the smaller, more mobile oxygen molecules. The situation can essentially be regarded as a double-edged sword. In order to attain higher electron transfer efficiencies, an artificial electron mediator would become absolutely necessary. Employing an artificial electron mediator on the other hand may lead to detrimental effects to the biological system.

Improving the efficiency of electron transfer

Earlier in this work, it was reported that several techniques had previously been employed by other groups to improve the electron transfer efficiency between bacteria and electrodes. Apart from simply introducing free roaming mediators in the anode compartment of the BFC, the mediators were immobilized to the electrode. In studies by Park et al. [79], neutral red was the electron mediator of choice and was immobilized onto carbon-based electrodes. Immobilization of the electron mediators was not the only method used. In many studies, attempts were made to

immobilize the bacteria themselves to the electrode surface. Biofilms were applied to the electrode surface, allowing the growth of bacterial colonies that could survive and thrive by transferring the electrons derived from the metabolism of various substrates. Special permeable membranes could be used to keep the biofilm against the electrode, but still allowing nutrients to traverse their structure. Although this technique could achieve a reasonable amount of success with bacteria, the same would be impossible for white blood cells. The idea that we are pursuing for an implantable biofuel cell would be one that could be placed within the physiological environment, without the need for any specific attachment of cells to the surface, a process that would be a very difficult problem to overcome. The goal would be to do very little to disrupt the physiological environment, but to still encourage good electrical communication with the cells in close proximity to the BFC electrode. Attachment of cells to the electrode surface would not be beneficial since the cells multiply and are eventually replaced by daughter cells. The BFC should, therefore, be capable of interacting with any cells in its immediate environment and of producing electricity, irrespective of the dynamic conditions within which it is placed.

Schroeder et al. also recognized the low electron transfer efficiency problem facing microbial fuel cells and formulated their own perspectives and implementation in tackling the issue [40]. Carbon-based electrodes modified with a platinum black overlay and electrically conducting polymers were employed in a microbial fuel cell with Escherichia Coli. Glucose was used as the fuel. At the time of their publication, Schroeder and co-authors reported the highest current and power densities ever attained by a microbial fuel cell. The specific conducting polymer used was polyaniline. The use of conducting polymers is an interesting and potentially promising concept that could be applied to solving a number of problems with respect to biofuel cell technologies for implantation within the body. Electrodes could be modified with

electrochemically deposited conductive polymers (PEDOT, polyaniline and polypyrrole) to promote good cell adhesion and improved cell to electrode electron transfer. The presence of the conducting polymers also makes it possible to incorporate bioactive molecules to promote good biocompatibility and reduce scar tissue formation around the implant. This has been shown in studies by Wadhwa et al., where dexamethasone (a drug shown to effectively reduce glial scar formation in the brain) was incorporated into an electrode containing polypyrrole – a well-known and studied conducting polymer [80]. Upon application of an electric potential to the electrode, small quantities of the drug could be released. A similar concept may be applied in the case of biofuel cell electrodes, where scar tissue formation could effectively be halted with the release of bioactive drugs from the electrode surface during the biofuel cell's operation. In the original study by Schroeder et al., it was noted that the higher current densities may have been due to ability of the conducting polymer chains to access the redox active centers embedded within the proteins of the cell membrane. In spite of the possible improved electrical interactions, however, between the electrodes and the cells, other factors may have played just as significant a role in the authors attaining such high current and power densities. One of those factors is that an anaerobic atmosphere was maintained at the anode during the biofuel cell operations. The presence of oxygen at the anode may have still been a very prominent problem. The other factor was that electric potential pulses on the order of 1V were periodically applied to the electrode surface. The potential pulses were applied in order to remove adsorbed species from the electrode surface that could contribute to electrode fouling, hence reducing the electron transfer efficiency, rates and overall current and power. Electrode fouling can be a significant problem for biofuel cells, due to the presence of several proteins and other species that could adhere to the

electrode surface, particularly to surfaces that are carbon based, which tend to be rather hydrophobic.

Activation of the NADPH oxidase complex

Another significant hurdle associated with using the phagocytic white blood cell NADPH oxidase as a source of high energy electrons is the fact that the individual components of the enzyme associate into their final functional form only during the enzyme's activated state. The purpose of the NADPH oxidase enzyme complex should be kept clearly in mind. As described earlier in this work, NADPH oxidase is an important aspect of the host immune or inflammatory response. It is responsible for the generation of free oxygen radicals (reactive oxygen species) such as superoxide (and consequently hydrogen peroxide). In order to produce the reactive oxygen species (ROS) that are used to attack invading organisms, NADPH oxidase must become activated after which it can bind molecular oxygen. During this binding, electrons derived from the pentose phosphate pathway of glucose metabolism are transferred to NADPH oxidase via the coenzyme NADPH and to the final extracellular electron acceptor. Protons of equivalent but opposite (positive) charge are simultaneously pumped out of the cell through ion channels in the membrane to complete the circuit. The electrons are accepted by the final electron acceptor, oxygen, to form the free radical superoxide. In vitro, activation of the NADPH oxidase complex is achieved through the application of specific agents, namely the phorbol ester, PMA, and calcium ionomycin. Such activating agents cannot be applied in vivo as they can cause non-specific activation. PMA has been shown to cause malignant tumors in the skin of rodents [81]. For our own in vitro studies, the application of PMA and ionomycin complicated our results, since either one or both are electrochemically active species. This fact leads to a further difficulty

that may be encountered during the electrochemical studies of biological systems. Electrochemical cells in general can be regarded as black boxes, since it can prove extremely challenging to determine what exactly is occurring in the system. Some insight can be gathered from electrochemical techniques such as cyclic voltammetry and electrochemical impedance spectroscopy, which are used to investigate the thermodynamics, kinetics and impedance characteristics of the system. However, for biological systems, there are so many unknowns, that it can prove difficult to even make an educated guess as to the identity of the electrochemical processes that might be occurring, which are contributing to any observed currents or electric potentials. It is clear that detailed knowledge of the biological system itself is first required in order to come to a more complete understanding as to the nature of the electrochemical interactions and insight into what might be done to optimize the kinetics and reduce energy losses within an associated electrochemical cell.

Technical limitations

It is clear that alternative techniques must be employed in order to effectively conduct *in-vivo* studies of the electrochemical activities of white blood cells and other human or animal cells. In these leukocyte-based biofuel cell studies in particular, techniques, methods and apparatuses that were previously used to study microbial fuel cells were employed. Bacteria, however, are very different from white blood cells and animal cells in general. An inability to attain cell densities much higher than a couple of million cells per cubic centimeter was not feasible, something that could easily be achieved for the microbes. Therefore, insufficient cell densities and low measuring sensitivities due to instrument limitations may be factors contributing to the very little or no currents observed for the leukocyte-based biofuel cells. Another issue with respect to the

apparatus was that in order to attain high currents, the fuel and bacteria would have to be passed at sufficiently high flow rates across the anode [82]. This would be difficult to achieve for the white blood cells, as they are not likely to adhere readily to the electrode surface during the anodic fluid flow. Maintenance of physiological levels of glucose was, therefore, difficult to achieve in our experiments.

In order to overcome the previously described challenges, a solution may be to employ microfluidics to the study of white blood cell electrochemical activity. Rather than requiring larger volumes of the cell suspensions (typically on the order of 10mL in our studies), volumes on the order of microliters could be used. An anode with microfluidic channels, such as that described by Chiao et al. [43] could be very beneficial to a biofuel cell study with white blood cells. With such small volumes, it would become important to utilize instrumentation with very high sensitivities for recording current magnitudes.

Biofuel cell design for in vivo applications

The larger problem associated with the development of biological or biofuel cells for implantable medical devices can actually be divided into smaller, yet very important problems. The first problem has been highlighted in the several paragraphs that have preceded this one and is perhaps the most important. Establishing electrical communication links between biological species and electrode surfaces will continue to be a very fundamental and significant problem. The other problems actually fall within the realm of design, specifically the design of the individual components that make up the physical structure of the biofuel cell. Apart from improving the efficiency of electron transfer between biological species (enzymes or whole cell) and electrodes, some of the major challenges facing an actual implementation of an implantable

biofuel cell also include 1) eliminating the proton exchange membrane (PEM); 2) eliminating the ferricyanide solution, while improving the rate of oxygen reduction at the cathode; and 3) miniaturization of the device while still maintaining sufficiently high current and power densities.

An in vitro implementation of our biofuel cell currently requires a PEM to separate the anode and cathode compartments. The purpose of the PEM is to i) prevent shorting of the current through physical contact between the anode and cathode electrodes; ii) separate the anode and cathode solutions (the cathode solution, ferricyanide, would be toxic to the cells); iii) selectively permit the movement of protons to the cathode, while preventing larger negatively charged ions from passing. A Nafion-117 proton exchange membrane was used in our in vitro study. One of the issues surrounding the membrane is that it was not designed to be implanted. The other problem is the fact that within the context of the physiological environment, it would be impossible to use the PEM to separate the anode and cathode reactants, since tissues are largely homogenous in their content. To tackle the problem of homogeneity with respect to the reactants presented to the electrodes of a biofuel cell, a number of researchers (including the Heller group at the University of Texas at Austin) designed electrodes that were modified with enzymes [19-21, 49, 83]. The enzymes permitted specific reactions to occur at each electrode, namely the oxidation of glucose to gluconolactone at the anode and the reduction of oxygen to water at the cathode. The PEM as well as the ferricyanide solution (normally employed at the cathode for its high redox reversibility and high electron affinity) were made obsolete with this enzyme based biofuel cell. Bilirubin oxidase or the laccase enzyme served well to replace the ferricyanide solution at the cathode and also prevented the occurrence of fuel crossover, which is problematic as it can lead to electric potential losses and kinetic inefficiencies.

Possible applications of an in vivo biofuel cell technology

This novel study of eukaryotic-based biofuel cells has potentially important implications for possible future applications of biological fuel cells to powering implantable devices. By adapting the fuel cell to operate within a physiologically relevant system, the electrical energy produced by the biofuel cell could potentially be used to power an implantable miniature biosensor or a microfabricated controlled-release device. In a paper published by Lahann et al. (2003), an electrically switchable surface was described [84]. Upon application of an electric potential to the surface, dynamic changes in the interfacial surface properties, such as wettability, could be achieved. The surface was designed by modification of a Au(111) surface with a self-assembled monolayer (SAM). The molecule of choice in this case was (16-Mercapto)hexadecanoic acid (MHA). It was explained by the authors that information regarding the potential range required for switching of the surface properties could be determined from basic energy considerations. Citing previous research, Lahann et al. presented an electrical potential range between -1.046V and +654mV vs. SCE (-804mV and 896mV vs NHE), when considering the electrochemical stability of alkanethiolates (such as MHA) adsorbed on gold and exposed to solution. It was also estimated that a potential above -118mV vs. SCE (+124mV vs. NHE) would be required to satisfy the conditions of molecular reorientation in low density SAMs. Based on these estimates, our metabolic biofuel cell would be able to provide a sufficiently large potential to satisfy these requirements.

Complexity of bioelectrochemical systems

Throughout the course of this work, it was difficult to establish an exact mechanism by which electron transfer could take place between the isolated white blood cells and the carbon fiber

electrodes. Although the release of serotonin from certain white blood cells, specifically PBMCs, was verified using cyclic voltammetry and HPLC, the results of the leukocyte-based BFCs investigated in this work suggested that some other mechanism might be employed. The PBMCs did not appear to generate currents comparable to those observed from the general white blood cell population isolated by RBC lysis. This suggests that another type of cell may be involved. Oxidation peaks associated with coenzyme A has been previously observed for human white blood cells [73]. Since coenzyme A is highly linked to glucose metabolism among eukaryotic cells, it is reasonable to consider this species as a likely electron mediator. The evident complexity of biological systems as well as electrochemical systems revealed by this research is an indication that a lot more work is left to be done in attempting to understand the process of electron transfer between biological species and electrodes.

APPENDIX A

LIST OF ABBREVIATIONS

PML	Polymorphonuclear Lymphocyte
BLCL	B Lymphoblastoid Cell Line
PBMC	Peripheral Blood Mononuclear Cell
CFE	Carbon Fiber Electrode
HPLC	High Performance Liquid Chromatography
CV	Cyclic Voltammetry
WBC	White Blood Cell
BFC	Biofuel Cell
PEM	Proton Exchange Membrane
PEMFC	Proton Exchange Membrane Fuel Cell
MFC	Microbial Fuel Cell
FC	Fuel Cell
MB	Methylene Blue
GOx	Glucose oxidase

BOD	Bilirubin Oxidase
NR	Neutral Red
PMA	Phorbol-12-myristate-13-acetate (activating agent)
ROS	Reactive Oxygen Species
CGD	Chronic Granulomatous Disease
PPP	Pentose Phosphate Pathway
RPMI	Common cell culture media (abbreviation for Roswell Park Memorial Institute)
PBS	Phosphate buffered saline
CoA	Coenzyme A
SCE	Saturated Calomel Electrode (common reference electrode for electrochemical studies)

BIBLIOGRAPHY

[1] D. Prutchi and M. Norris, *Design and Development of Medical Electronic Instrumentation*. Hoboken, NJ: John Wiley and Sons, Inc., 2002.

[2] W. Greatbatch and C. Holmes, "History of Implantable Devices," *IEEE Engineering in Medicine and Biology*, pp. 38-49, 1991.

[3] R. Drake, B. Kusserow, S. Messinger, and S. Matsuda, "A Tissue Implantable Fuel Cell Power Supply," *Trans. Amer. Soc. Artif. Int. Organs*, vol. XVI, pp. 199-205, 1970.

[4] H. Lanmuller, M. Bijak, W. Mayr, D. Rafolt, S. Sauerann, and H. Thoma, "Useful applications and limits of battery powered implants in functional electrical stimulations," *Artificial Organs*, vol. 21, pp. 210-12, 1997.

[5] H. Goto, T. Nakagawa, O. Nakamura, and S. Kawata, "An implantable power supply with an optically rechargeable lithium battery," *IEEE Transactions on Biomedical Engineering*, vol. 48, pp. 830-833, 2001.

[6] V. Parsonnet, G. Myers, L. Gilbert, and I. Zucker, "Clinical experience with nuclear pacemakers," *Surgery*, vol. 78, pp. 776-86, 1975.

[7] G. Sandberg Jr., F. Huffman, and J. Norman, "Implantable nuclear power sources for artificial organs: I. Physiological monitoring of pathologic effects," *Trans. Amer. Soc. Artif. Int. Organs*, vol. XVI, pp. 172-9, 1970.

[8] H. Goto, T. Sugiura, Y. Harada, and T. Kazui, "Feasibility of using the automatic generating system for quartz watches as a leadless pacemaker power source," *Med. Biol. Eng. Comput.*, vol. 37, pp. 377-80, 1999.

[9] P. Starek, D. White, and C. Lillehei, "Intra-cardiac pressure changes utilized to energize a piezoelectric powered cardiac pacemaker," *Trans. Amer. Soc. Artif. Int. Organs*, vol. XVI, pp. 180-3, 1970.

[10] M. Schaldach and U. Kirsch, "In vivo electrochemical power generation," *Trans. Amer. Soc. Artif. Int. Organs*, vol. XVI, pp. 184-190, 1970.

[11] J. Schuder, "Powering an artificial heart: birth of the inductively coupled radio frequency system in 1960 " *Artificial Organs*, vol. 26, pp. 909-915, 2002.

[12] S. Suzuki, T. Katane, and O. Sato, "Fundamental study of an electric power transmission system for implanted medical devices using magnetic and ultrasonic energy," *Journal of Artificial Organs*, vol. 6, pp. 145-8, 2003.

[13] M. Sun, G. Justin, P. Roche, J. Zhao, B. Wessel, Y. Zhang, and R. Sclabassi, "Harnessing the Body's Own Energy and Communication Resources," in *IEEE Engineering in Medicine and Biology Magazine: Clinical Engineering*, 2006, pp. 39-46.

[14] R. Sclabassi, Q. Liu, S. Hackworth, G. Justin, and M. Sun, "Platform technologies to support brain-computer, interfaces," *Neurosurgery Focus*, vol. 20, pp. E5, 2006.

[15] W. Greatbatch, "Implantable power-sources: A review," *J Med Eng and Tech*, vol. 8, pp. 56-63, 1984.

[16] R. Khandpur, *Biomedical Instrumentation: Technology and Applications*. New York: McGraw-Hill, 2005.

[17] H. Shill and A. Shetter, "Reliability in Deep Brain Stimulation," *IEEE Transactions on Device and Materials Reliability*, vol. 5, pp. 445-448, 2005.

[18] Z. Wang and J. Song, "Piezoelectric Nanogenerators Based on Zinc Oxide Nanowire Arrays," *Science*, vol. 312, pp. 242-246, 2006.

[19] N. Mano, F. Mao, and A. Heller, "Characteristics of a Miniature Compartment-less Glucose-O2 Biofuel Cell and Its Operation in a Living Plant," *Journal of the American Chemical Society*, vol. 125, pp. 6588-6594, 2003.

[20] F. Mao, N. Mano, and A. Heller, "Long Tether Binding Redox Centers to Polymer Backbones Enhance Electron Transport in Enzyme "Wiring" Hydrogels," *Journal of the American Chemical Society*, vol. 125, pp. 4951-57, 2003.

[21] V. Soukharev, N. Mano, and A. Heller, "A Four-Electron O2-Electroreduction Biocatalyst Superior to Platinum and a Biofuel Cell Operating at 0.88V," *Journal of the American Chemical Society*, vol. 126, pp. 8368-8369, 2004.

[22] O. DeVel, "Controlled transcutaneous powering of chronically implanted telemetry device," *Biotelem Patient Monit*, vol. 6, pp. 176-185, 1979.

[23] B. Smith, Z. Tang, M. Johnson, S. Pourmehdi, M. Gazdik, J. Buckett, and P. Peckman, "An externally powered, multichannel, implantable stimulator-telemeter for control of

paralyzed muscle," *IEEE Transactions on Biomedical Engineering*, vol. 45, pp. 463-75, 1998.

[24] K. Foster and E. Adair, "Modeling thermal responses in human subjects following extended exposure to radiofrequency energy," *Biomedical Engineering Online*, vol. 3, 2004.

[25] Q. Tang, N. Tummala, S. K. S. Gupta, and L. Schwiebert, "Communication scheduling to minimize thermal effects of implanted biosensor networks in homogeneous tissue," *IEEE Transactions on Biomedical Engineering*, vol. 52, pp. 1285-1294, 2005.

[26] R. Fischell, "Fixed Rate Rechargeable Cardiac Pacemaker," vol. US3867950. United States of America: The Johns Hopkins University, Baltimore, MD, 1975.

[27] D. Park and J. Zeikus, "Electricity Generation in Microbial Fuel Cells Using Neutral Red as an Electronophore," *Applied and Environmental Microbiology*, vol. 66, pp. 1292-97, 2000.

[28] R. Allen and H. Bennetto, "Microbial Fuel Cells," *Applied Biochemistry and Biotechnology*, vol. 39/40, pp. 27-40, 1993.

[29] D. Bond, D. Holmes, L. Tender, and D. Lovley, "Electrode-Reducing Microorganisms that Harvest Energy from Marine Sediments," *Science*, vol. 295, pp. 483-485, 2002.

[30] D. Bond and D. Lovley, "Electricity Production by Geobacter Sulfurreducens Attached to Electrodes," *Applied and Environmental Microbiology*, vol. 69, pp. 1548-1555, 2003.

[31] S. Chauduri and D. Lovley, "Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells," *Nature Biotechnology*, vol. 21, pp. 1229-32, 2003.

[32] G. M. Delaney, H. P. Bennetto, J. R. Mason, S. D. Roller, J. L. Stirling, and C. F. Thurston, "Electron-transfer Coupling in Microbial Fuel Cells. 2. Performance of Fuel Cells Containing Selected Micro-organism-Mediator-Substrate Combinations," *Journal of Chemical Technology and Biotechnology*, vol. 34B, pp. 13-27, 1984.

[33] I. Ieropoulos, J. Greenman, C. Melhuish, and J. Hart, "Comparative study of three types of microbial fuel cell," *Enzyme Micorbial Technol.*, vol. 37, pp. 238-245, 2005.

[34] D. Park, S. Kim, I. Shin, and Y. Jeong, "Electricity Production in Biofuel Cell using modified graphite electrode with Neutral Red," *Biotechnology Letters*, vol. 22, pp. 1301-4, 2000.

[35] D. Park and J. Zeikus, "Improved Fuel Cell and Electrode Designs for Producing Electricity from Microbial Degradation," *Biotechnology and Bioengineering*, vol. 81, pp. 348-55, 2003.

[36] D. Prasad, S. Arun, M. Murugesan, S. Padmanaban, R. Satyanarayanan, S. Berchmans, and V. Yegnaraman, "Direct electron transfer with yeast cells and construction of a mediatorless microbial fuel cell," *Biosensors and Bioelectronics*, vol. doi:10.1016/j.bios.2006.10.028, 2006.

[37] K. Rabaey, N. Boon, S. Siciliano, M. Verhaege, and W. Verstraete, "Biofuel Cells Select for Microbial Consortia That Self-Mediate Electron Transfer," *Applied and Environmental Microbiology*, vol. 70, pp. 5373-82, 2004.

[38] K. Rabaey, G. Lissens, S. Siciliano, and W. Verstraete, "A microbial fuel cell capable of converting glucose to electricity at high rate and efficiency," *Biotechnology Letters*, vol. 25, pp. 1531-35, 2003.

[39] S. Roller, H. Bennetto, G. Delaney, J. Madison, J. Stirling, and C. Thurston, "Electron-transfer coupling in microbial fuel cells: 1. Comparison of redox-mediator reduction rates and respiratory rates of bacteria," *Journal of Chemical Technology and Biotechnology*, vol. 34B, pp. 3-12, 1984.

[40] U. Schroeder, J. Niessen, and F. Scholz, "A Generation of Microbial Fuel Cells with Current Outputs Boosted by More than One Order of Magnitude," *Angewandte Chemie International Edition*, vol. 42, pp. 2880-83, 2003.

[41] J. Larminie and A. Dicks, *Fuel Cell Systems Explained*. Chichester: John Wiley and Sons, Ltd, 2000.

[42] K. Williams, *An Introduction to Fuel Cells*. New York: Elsevier Publishing Company, 1966.

[43] M. Chiao, K. B. Lam, and L. Lin, "Micromachined microbial and photosynthetic fuel cells," *Journal of Micromechanics and Microengineering*, vol. 16, pp. 2547-2553, 2006.

[44] D. Park, M. Laivenieks, M. Guettler, M. Jain, and J. Zeikus, "Microbial Utilization of Electrically Reduced Neutral Red as the Sole Electron Donor for Growth and Metabolite Production," *Applied and Environmental Microbiology*, vol. 65, pp. 2912-2917, 1999.

[45] H. Liu and B. E. Logan, "Electricity Generation Using an Air-Cathode Single Chamber Microbial Fuel Cell in the Presence and Absence of a Proton Exchange Membrane," *Environmental Science and Technology*, vol. 38, pp. 4040-4046, 2004.

[46] M. Allen, "Cellular Electrophysiology," in *Methods in Microbiology*, J. Norris and D. Ribbons, Eds. New York: Academic Press, 1972, pp. 247-283.

[47] S. Chaudhuri and D. Lovley, "Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells," *Nature Biotechnology*, vol. 21, pp. 1229-32, 2003.

[48] K. P. Nevin and D. R. Lovley, "Lack of Production of Electron-Shuttling Compounds or Solubilization of Fe(III) during Reduction of Insoluble Fe(III) Oxide by Geobacter metallireducences," *Applied and Environmental Microbiology*, vol. 66, pp. 2248-2251, 2000.

[49] N. Mano, F. Mao, and A. Heller, "A Miniature Biofuel Cell Operating in Physiological Buffer," *Journal of the American Chemical Society*, vol. 124, pp. 12962-63, 2002.

[50] C. Kang, Y. Xie, and F. Anson, "Catalytic pathways for the electroreduction of O2 at graphite electrodes on which a macrocyclic cobalt complex is adsorbed," *Journal of Electroanalytical Chemistry*, vol. 413, pp. 165-174, 1996.

[51] G. Palmore, H. Bertschy, S. Bergens, and G. Whitesides, "A methanol/dioxygen biofuel cell that uses NAD+-dependent dehydrogenases as catalysts: application of an electro-enzymatic method to regenerate nicotinamide adenine dinucleotide at low overpotentials," *Journal of Electroanalytical Chemistry*, vol. 443, pp. 155-161, 1998.

[52] G. Palmore and H. Kim, "Electro-enzymatic reduction of dioxygen to water in the cathode compartment of a biofuel cell," *Journal of Electroanalytical Chemistry*, vol. 464, pp. 110-117, 1999.

[53] I. Willner and E. Katz, *Bioelectronics: From Theory to Applications*. Weinheim: Wiley-VCH, 2005.

[54] T. DeCoursey, V. Cherny, A. DeCoursey, W. Xu, and L. Thomas, "Interactions between NADPH oxidase-related proton and electron currents in human eosinophils," *Journal of Physiology*, vol. 535, pp. 767-781, 2001.

[55] T. E. DeCoursey, V. V. Cherny, W. Zhou, and L. L. Thomas, "Simultaneous activation of NADPH oxidase-related proton and electron currents in human neutrophils," *PNAS*, vol. 97, pp. 6885-89, 2000.

[56] T. E. DeCoursey, D. Morgan, and V. V. Cherny, "The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels," *Nature*, vol. 422, pp. 531-34, 2003.

[57] J. Schrenzel, L. Serrander, B. Banfi, O. Nusse, R. Fouyouzi, D. Lew, N. Demaurex, and K. Krause, "Electron currents generated by the human phagocyte NADPH oxidase," *Nature*, vol. 392, pp. 734-737, 1998.

[58] Y. Groemping and K. Rittinger, "Activation and assembly of the NADPH oxidase: a structural perspective," *Biochemistry Journal*, vol. 386, pp. 401-416, 2005.

[59] G. M. Bokoch and U. G. Knaus, "NADPH oxidases: not just for leukocytes anymore," *Trends in Biochemical Sciences*, vol. 28, pp. 502-8, 2003.

[60] H. Gao, J. Hong, W. Zhang, and B. Liu, "Distinct Role for Microglia in Rotenone-Induced Degeneration of Dopaminergic Neurons," *Journal of Neuroscience*, vol. 22, pp. 782-790, 2002.

[61] K. K. Griendling, C. A. Minieri, J. D. Ollerenshaw, and R. W. Alexander, "Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells.," *Circulation Research*, vol. 74, pp. 1141-48, 1994.

[62] S. Jones, V. O'Donnell, J. Wood, J. Broughton, E. Hughes, and O. Jones, "Expression of phagocyte NADPH oxidase components in human endothelial cells," *American Journal of Physiology*, vol. 271, pp. H1626-34, 1996.

[63] C. Piccoli, R. Ria, R. Scrima, O. Cela, A. D'Aprile, D. Boffoli, F. Falzetti, A. Tabilio, and N. Capitanio, "Characterization of Mitochondrial and Extra-mitochondrial Oxygen Consuming Reactions in Human Hematopoietic Stem Cells," *Journal of Biological Chemistry*, vol. 280, pp. 26467-26476, 2005.

[64] B. Babior, "NADPH Oxidase: An Update," *Blood*, vol. 93, pp. 1464-1476, 1999.

[65] A. Abramov, L. Canevari, and M. Duchen, "Beta-Amyloid Peptides Induce Mitochondrial Dysfunction and Oxidative Stress in Astrocytes and Death of Neurons through Activation of NADPH Oxidase," *Journal of Neuroscience*, vol. 24, pp. 565-575, 2004.

[66] M. Patel, Q. Li, L. Chang, J. Crapo, and L. Liang, "Activation of NADPH oxidase and extracellular superoxide production in seizure-induced hippocampal damage," *J Neurochem*, vol. 92, pp. 123-31, 2005.

[67] C. Marshall, A. Mamary, A. Verhoeven, and B. Marshall, "Pulmonary artery NADPH oxidase is activated in hypoxic pulmonary vasoconstriction," *American Journal of Respiratory Cell and Molecular Biology*, vol. 15, pp. 633-644, 1996.

[68] D. Park and J. Zeikus, "Electricity generation in microbial fuel cell with neutral red as an electronophore," *Applied and Environmental Microbiology*, vol. 66, pp. 1292-1297, 2000.

[69] H. Lodish, A. Berk, P. Matsudaira, C. Kaiser, M. Krieger, M. P. Scott, S. L. Zipursky, and J. Darnell, *Molecular Cell Biology* Fifth ed. New York: W.H. Freeman and Company, 2004.

[70] T. Magnuson, A. Hodges-Myerson, and D. Lovley, "Characterization of a membrane bound NADPH-dependent Fe^{3+} reductase from the dissimilatory Fe^{3+} reducing bacterium Geobacter sulfurreducens," *FEMS Microbiology Letters*, vol. 185, pp. 205-211, 2000.

[71] T. Magnuson, N. Isoyama, A. Hodges-Myerson, G. Davidson, M. Maroney, G. Geesey, and D. Lovley, "Isolation, characterization, and gene sequence analysis of a membrane associated 89kDa Fe(III) reducing cytochrome from Geobacter sulfurreducens," *Biochemical Journal*, vol. 359, pp. 147-152, 2001.

[72] T. Matsunaga, A. Shigematsu, and N. Nakamura, "Detection of Rat Basophilic Leukemia by Cyclic Voltammetry for Monitoring Allergic Reaction," *Analytical Chemistry*, vol. 61, pp. 2471-2474, 1989.

[73] N. Nakamura, I. Inoue, Y. Kitajima, T. Matsanuga, T. Chiba, and T. Honda, "Detection of human leucocytes by cyclic voltammetry and its application to monitoring of allergic reaction," *Biosensors and Bioelectronics* vol. 6, pp. 431-437, 1991.

[74] M. Okochi, H. Yokouchi, N. Nakamura, and T. Matsunaga, "Electrochemical Detection of Allergen in Small-Volume Whole Blood Using an Array Microelectrode: A Simple Method for Detection of Allergic Reaction," *Biotechnology and Bioengineering*, vol. 65, pp. 480-484, 1999.

[75] M. Faellman, O. Stendahl, and T. Andersson, "Phorbol ester-induced activation of protein kinase C leads to increased formation of diacylglycerol in human neutrophils," *Experimental Cell Research*, vol. 181, pp. 217-225, 1989.

[76] C. Bradberry, J. Sprouse, P. Sheldon, G. Aghajanian, and R. Roth, "In vitro micodialysis: a novel technique for stimulated neurotransmitter release measurements," *Journal of Neuroscience Methods*, vol. 36, pp. 85-90, 1991.

[77] B. Sarada, T. Rao, D. Tryk, and A. Fujishima, "Electrochemical Oxidation of Histamine and Serotonin at Highly Boron-doped Diamond Electrodes. ," *Analytical Chemistry*, vol. 72, pp. 1632-1638, 2000.

[78] A. J. Bard and L. R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, Second ed. Hoboken: John Wiley and Sons, Inc., 2001.

[79] D. Park, S. Kim, I. Shin, and Y. Jeong, "Electricity Production in Biofuel Cell using modified graphite electrode with Neutral Red," *Biotechnology Letters*, vol. 22, pp. 1301-1304, 2000.

[80] R. Wadhwa, C. Lagenaur, and X. Cui, "Electrochemically controlled release of dexamethasone from conducting polymer polypyrrole coated electrodes," *J Control Release*, vol. 110, pp. 531-41, 2006.

[81] S. Allen, S. Florell, A. Hanks, A. Alexander, M. Diedrich, D. Altieri, and D. Grossman, "Survivin expression in mouse skin prevents papilloma regression and promotes chemical induced tumor progression," *Cancer Research*, vol. 63, pp. 567-72, 2003.

[82] K. Rabaey, G. Lissens, S. Siciliano, and W. Verstraete, "A microbial fuel cell capable of converting glucose to electricity at high rate and efficiency," *Biotechnology Letters*, vol. 25, pp. 1531-1535, 2003.

[83] T. Chen, S. Barton, G. Binyamin, Z. Gao, Y. Zhang, H. Kim, and A. Heller, "A Miniature Biofuel Cell," *Journal of the American Chemical Society*, vol. 123, pp. 8630-31, 2001.

[84] J. Lahann, S. Mitragotri, T.-N. Tran, H. Kaido, J. Sundaram, I. S. Choi, S. Hoffer, G. A. Somorjai, and R. Langer, "A Reversibly Switching Surface," *Science*, vol. 299, pp. 371-74, 2003.