Electrochemical Instrumentation for Real-Time Monitoring of Biological Model Systems

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LIST OF ABBREVIATIONS

Ag/AgCl	Silver / Silver Chloride
BBB	Blood-Brain Barrier
DI	Deionized Water
HFBx	Hollow-Fiber Bioreactor
H_2O_2	Hydrogen Peroxide
IPA	Isopropyl Alcohol
$IrCl_4$	Iridium Chloride
IrOx	Iridium Oxide
MAMP	Multianalyte Microphysiometer
SPE	Screen-Printed Electrode
TEER	Transepithelial electrical resistance
VIIBRE	Vanderbilt Institute for Integrative Biosystems Research

CHAPTER 1

Introduction

1.1 Organ Analogues

Sustained interest for alternatives to human testing for the validation of pharmaceuticals and for other directions of study have led to the development of many potential substitute models, both as a means to avoid the ethical concerns of testing potentially dangerous compounds on humans and to provide an affordable platform for determining the pharmacokinetic and pharmacodynamics properties of compounds of interest. Evaluation of therapeutics has traditionally relied on preclinical testing using *in vitro* human cell cultures and *in vivo* studies in animal models to screen drug candidates for efficacy and safety prior to the beginning of clinical trials[1]. These approaches provide a viable screening method to identify potential activity and obvious toxicity without risk to human subjects but also suffer from fundamental limitations. The metabolism and biological structures of animal models obviously differ from corresponding human biology[1] and consequently both the efficacy and potential toxicity of a given compound can vary widely between species. Cell cultures can provide an environment with greater fidelity to human *in vivo* conditions than animal models but historically have provided only an incomplete picture of a drug's metabolic pathway as they fail both to model organ to organ interactions and to provide compelling whole-organ environments[2].

These deficiencies have led to the development of new approaches in cell culturing to provide more complete testing models. While bioreactors have long provided a means to generate biological compounds of interest[3], there is an emerging use of purpose-designed

bioreactors intended to model whole organs for the study of cellular processes and metabolism[4]. Bioreactors utilized in this application tend to be small, with functional volumes of milliliters and total media reservoirs of less than 100 mL, and possess specialized structures for adherent mammalian cell culturing such as hollow-fiber[5] or dynamic compression[6] elements, in contrast to the multi-liter volumes and free-floating bacterial, yeast, or algal cultures of industrial bioreactors. These bioreactors can accurately model both the anatomy of organ environments, using their structure to allow appropriate cell-to-cell interactions between the various cell lines that compose a given organ, and the environmental conditions of the organ, providing substitutes for mechanical stresses responsible for cell differentiation and orientation in real organs[4]. A variety of approaches are used to achieve more *in vivo*-like conditions, ranging from substrates such as hydrogels[7] and porous films[8] that direct cell growth and orientation, fluidics that replicate shear stress[9], and segregation structures that allow multiple cell lines to interact[10]. Changes in the environment of the cell dramatically alter the metabolism and secretome of the culture.

On their own, biomimetic bioreactors provide compelling advantages over simple cultures in providing a platform both for the testing of compounds and for the elucidation of cellular mechanisms in animal tissues, but do not replicate the complex organ-to-organ interaction that are an advantage of animal models over cell cultures[2]. Some effects of these interactions can be effectively mimicked, such as the addition of expected hormones and proteins to induce appropriate behavior and development or signaling molecules to produce desired cellular response[11]. Other properties such as the effect of metabolic break-down products from an analyte from one organ on another organ, as seen in the conversion of methanol to formaldehyde and formic acid in the liver causing toxicity in optic nerves[12] or the conversion by

the liver of codeine to morphine which acts on the neurons of the central nervous system[13], are less easily anticipated and therefore difficult to test for in cell culture studies. While this deficiency can be mitigated to some extent through processes such as the use of conditioned media[14,15], a truly compelling replacement for animal models would need to reproduce the real-time interactions between multiple organ systems.

Increasingly compelling organ models have drawn interest towards models which integrate multiple artificial organs to create test platforms which more fully recreate the *in vivo* condition[4]. Fluidic systems may recreate organ-to-organ interaction between multiple cell cultures by the distribution of running media from culture to culture in rates and pathways consistent with human physiology[16]. Such a system would recreate the molecular conditions that help cells maintain differentiation and function and allow for the multi-organ metabolism that is responsible for both the efficacy and the side effects and toxicity of pharmaceutical compounds. With appropriate instrumentation, the system would provide a means to monitor the behavior of these cultures and thereby identify the effects and mechanism of action for compounds of interest in an environment considerably closer to a human subject than other current models. Research efforts towards the construction of such systems utilize both bioreactors at conventional biochemical scales of mL volumes, for ease of study and size of yield, and organ-on-a-chip devices[16] at μL volumes, with microfabrication and microfluidic technologies allowing for rapid prototyping[17] and precise environmental control[18].

1.2 Blood Brain Barrier

The properties of the blood-brain barrier (BBB), the cells at the interface of capillaries and the brain responsible for transport between these regions, make it a subject of particular

interest in pharmaceutical research. Unless they are to be delivered intracranially, potential therapeutics targeting the brain must be able to cross the BBB to have effect. As many significant medical conditions, such as majority of psychiatric disorders, Alzheimer's disease, epilepsy, and others, originate in the brain, and because diseases of the brain very often result in substantial loss of quality of life the behavior of the BBB is a vital aspect to disease treatment[19]. While conventional chemical principles can guide the process of drug design through appeals to properties such as hydrophobicity and molecular analogy, the ability to test whether a novel compound will be able to cross the human BBB either through *in silico* or *in vitro* means provide substantial advantages in the early stages of drug development and testing[20].

The selective abilities of the BBB serve both to protect the brain from potential toxicity and to allow it to maintain a controlled environment where changes to local chemical concentrations are caused exclusively by cellular signaling rather than as a consequence of metabolism[21]. This selectivity arise from the interaction of multiple cell types which work in conjunction[22] to transport select molecules into or out of the brain through active and passive transport while excluding other materials by means of impermeable cell junctions and low rates of endocytosis[15]. The selective barrier is composed of specialized endothelial cells, very similar to the endothelial cells that form the lining of all blood and lymphatic vessels but differing in that endothelial cells of the BBB produce a network of interlocking proteins which resemble those of epithelial cells and create an impermeable barrier to diffusion[15]. These networks consist of a combination of adherens junctions, found in most endothelial cells and formed by the anchoring of proteins included cadherens and catenins to the actin filaments of the cytoskeletons of adjacent cells to provide stability, and tight junctions, protein complexes consisting primarily of

claudins and occludins which are embedded in the cell membranes of adjacent cells and which form a strongly hydrophobic barrier to prevent fluid diffusion[15].

Beyond the endothelial cells are pericytes and astrocytes. Pericytes are contractile cells with similarities to smooth muscle cells that provide additional structural support to the endothelium and allow the rate of blood flow through capillaries to be controlled through dilation and contraction of the capillary[21]. Pericytes line the endothelial cells of the BBB as well as the rest of the vascular system[21], though their importance to the function of the BBB is demonstrated by the barrier's failure in pathological conditions caused by their absence or impaired function[23]. Astrocytes are process-bearing cells that act as an intermediate between the endothelial cells of the BBB and the neurons of the brain, providing chemical support and signal transfer to both[22]. Both pericytes and astrocytes have a roll in the establishment of the BBB, with the former providing additional physical junctions to supplement those of the endothelium[21] while the later provides a means for the regulation of the BBB in response to changing conditions within the brain[22]. Both maintain chemical contact with the endothelium and contribute biological factors primarily responsible for the generation and regulation of the junctions in between these cells[22], though evidence suggests that astrocytes are responsible for the majority of this process[15].

Conventional approaches to cell culturing have largely failed to reproduce the robust junctions of the BBB during *in vitro* experiments[14,24]. The permeability of the BBB is typically evaluated in by the measurement of electrical resistance across the barrier, a value given the label transepithelial electrical resistance (TEER) which is observed to range from 1000-2000 Ω-cm^2 in a healthy BBB[14]. In both *in vivo* endothelium outside the BBB and in *in vitro* monolayer cultures of endothelial cells originating from the BBB, TEER measurements of 5-10 Ω-cm^2 are consistently observed[14]. This low resistance indicates that substantial diffusion is possible

through the extracellular space within these samples. Attempts to recreate the conditions of the BBB in culture have focused on cellular interactions, particularly by the addition of astrocytes, and mechanical stress, as there is substantial precedent that endothelial cells, like many other cells[9], orient under the influence of shear stress[25]. It is noted that most experiments which have achieved a substantial degree of success in emulating the BBB have managed to do so without the inclusion of pericytes, despite the demonstrated importance of these cells in *in vivo* conditions. This is likely because the major chemical role of pericytes is protection of the endothelium from the immune system activity of microglia[23], which are absent in these experiments, while the structural support provided by pericytes is to some degree supplied by the substrates used in these experiments.

Past experiments have shown that culturing endothelial under conditions of fluid flow can raise TEER to values of 10-50 Ω-cm^2, while the addition of media that has been conditioned by growth of astrocytes can induce endothelial cells to form junctions, increasing TEER values to the range of 50-200 Ω-cm^2 [14]. Models co-culturing endothelial cells and astrocytes in environments that recreate fluidic stress on the endothelial cells have produced cultures with TEER in the range of 500-1000 Ω-cm^2, indicative of substantial recreation of the BBB in *in vitro* conditions[14]. It is noted that immunocytochemistry techniques have also shown an increase in the expression and organization of tight junction proteins as endothelial cells are subjected to conditions that more closely resemble the BBB[14].

1.3 PC-12 and HTB-10 (SK-N-MC) Cell Lines

A variety of immortalized cell lines are available as model substitutes for any particular type of primary cell. In selecting a linage for a particular experiment, consideration should be

given to what particular properties are needed from the cells for the purpose of the model and whether the conditions of the model are conducive to the survival of the cell line. Some lineages better reproduce the secretion profile of primary cells, while others are more morphologically similar or respond to stimuli more accurately. Lines also have different requirements for use in experimentation. Some lines require a stable substrate to which they may adhere and may or may not require adhesives such as a collagen film in order to thrive. Cell lines may require particular nutrients or signaling factors either to survive or to maintain differentiation. Variations between organisms require consideration as to whether animal-derived cell-lines may substitute for human in a particular experiment or whether an immortalized cell line can even serve as a replacement for primary cells in a given model.

PC-12 cells and the derivative PC-12 adh line are an immortal cell line derived from a rat adrenal pheochromocytoma[26]. PC-12 cells are non-adherent but PC-12 adh cells, which were developed by selection across multiple PC-12 cells, are weakly adherent. Both lines can adhere to collagen-treated surfaces while the adh line can also adhere to Corning CellBIND® surfaces. The line can be maintained in RPMI-1640 medium supplemented with both horse serum and fetal bovine serum. PC-12 cells exhibit many neuron-like properties[26]. The line responds reversibly to nerve growth factor by developing processes and becoming susceptible to electrical excitability[27,28]. PC-12 cells synthesize acetylcholine and the catecholamines dopamine and norepinephrine and will secrete these compounds in response to chemical or electrical stimulation in a manner consistent with neurotransmission[28,29]. These properties make PC-12 cells useful as a general surrogate for sympathetic neuronal cells, though it is noted that they are deficient in certain cell receptors found in neurons and in the production of the neurotransmitter epinephrine[26]. The PC-12 cell line has been characterized by Multianalyte Microphysiometer

(MAMP) analysis[30] and as a consequence its baseline metabolic activity and its response to certain stimulants are documented, providing a point of comparison for metabolic studies.

HTB-10 cells (also referred to as SK-N-MC cells) are an immortalized cell line derived from a metastasized human neuroblastoma[31]. The lineage is robust, with a population doubling-time of 32 hours, high achievable densities, and marked stability through culture passaging[31]. The cells can be maintained in Eagle's minimum essential medium supplemented with fetal bovine serum. Being of neurogenic origin HTB-10 cells possess neuronal properties, particularly the synthesis and storage of catecholamines[31]. The cells are naturally adherent as a consequence of fibroblast-like character[31] and do not require a collagen-coated surface to adhere or thrive in culture. A related line, HTB-11 (SK-N-SH), is derived from a metastatic site of the same original tumor and displays similar morphology and behavior to the HTB-10[31]. The HTB-11 line has been characterized by MAMP analysis[32] and as a consequence its baseline metabolic activity and its response to certain stimulants are documented and provide a standard of comparison for HTB-10 behavior.

CHAPTER II

Development and Validation of Multianalyte Screen-Printed Electrode

2.1 Introduction

The means to monitor the behavior and condition of an *in vitro* biological system is essential to both determine the results of experimentation on the system and to validate the model system in comparison to known behavior of the *in vivo* system being modelled. The analytical demands for a biological model are steep, requiring means to monitor both the immediate health of the model to determine the stresses placed on it during experimentation while also identifying and quantitating the diverse biomolecules including proteins, hormones, and cytokines which provide insight to the interactions of the system by their disturbance. Methods must be able to meet this demand through the analysis of the complex mixture of cell media, preferable in real-time.

Electrochemistry techniques have consistently demonstrated utility in the analysis of the small molecules in complex biological environments with a high degree of specificity and selectivity. Electrodes treated with enzymatic coatings can be made selective to particular molecules of interest[33,34] while other applications can provide sensitivity to environmental conditions of interest such as solution pH[35], conductivity[36], and temperature[36]. Electrode sensors are capable of continuous rapid sampling and when appropriately designed are minimally disruptive to the environment being sampled[37].

Energy metabolism provides substantial insight into the health of a biological system, with shifts from aerobic to anaerobic processes indicative of stress on the system and net changes

in respiration indicative of cell reproduction or death[38]. Glucose and oxygen are consumed as result of energy metabolism and changes in their concentration provide a means to measure the rate of cellular respiration[38,39]. Likewise, the production of lactic acid provides an indication of anaerobic respiration[39], while acidification is a result of all respiration as a consequence of dissolved CO_2 produced[38]. The properties of electrochemical techniques are well suited to measure changes in these analytes in order to observe the health of biological models.

Interest in monitoring cell behavior in this way led to the development of the Multianalyte Microphysiometer (MAMP) by the laboratory of Dr. David Cliffel. Acting as an extension of the previous Cytosensor™ Microphysiometer, the MAMP utilized a variety of specialized electrodes to monitor the respiration of cell cultures as they were subjected various experimental protocols. The MAMP's utility has been demonstrated in a number of studies including evaluation of neuronal stress responses in simulated stroke conditions[40,41], quantitation of cancer cell metabolism[42], evaluation of the effects common fluorescent dyes used in cellular staining on metabolism[43], and the mechanisms of action of cholera toxin[30]. These results have shown that the MAMP can not only quantify the effects of compounds of interest but also identify specific mechanisms of action and intracellular processes by measuring despite measuring only a few key analytes. This is possible through the administration of compounds which activate and inhibit specific cellular processes alongside the compound of interest, a process somewhat analogous to the use of gene knockout to study metabolic pathways.

The MAMP quantifies analytes by a variety of means. Some electrode treatments rely on enzyme films. Within these films, analytes such as glucose[33] or lactate[44] are degraded by appropriate oxidase enzymes in a process that produces hydrogen peroxide. By holding the electrode at a sufficiently high potential to oxidize this peroxide, the rate of oxidation at the

platinum surface of the electrode can be measured amperometrically to quantify the analyte. Oxygen is detected through the use of an unmodified platinum electrode held at a sufficiently negative potential to reduce oxygen[45]. Evaluation of solution pH, the original function of the Cytosensor, is performed by open-circuit potential measurement using light-addressable potentiometric sensor.

Applications of the MAMP's detection principles to system such as bioreactor organ models or multi-organ systems has led to the development of a more flexible detector in the form of a Multianalyte Screen-Printed Electrode (SPE), which sets aside the dedicated pumping systems and cell chambers of the MAMP in order to achieve concise design that is easily integrated into both new and existing cell culturing systems. The SPE constitutes a series of five platinum electrodes deposited on a ceramic base and insulted by an additional layer of clear ceramic which covers the whole of the electrodes except for an exposed working area and contact points for connection to a potentiostat. With one silver-plated electrode acting as a Ag/AgCl reference, each of the four remaining electrode can be independently modified by the application of films to target desired analytes. Sampling is conducting on fluid pumped over the electrode surface from either an upstream population of cells or from a culture inside the SPE housing.

Evaluation of the SPE with various electrode treatments and conditions have allowed validation and optimization of the device and associated procedures to prepare for its use as a next-generation MAMP in a variety of biochemical applications. The results of these experiments contributed to others which have concluded in the success reproduction of former MAMP experiments and the preparation of the SPE for instrumentation of model biological systems[46].

2.2 Experimental Procedures

2.2.1 Reagents and Instrumentation

50 mM pH 7 phosphate buffer solution, silver nitrate, sulfuric acid, potassium ferricyanide, and sodium lactate were obtained from Fisher Scientific. 30% hydrogen peroxide, oxalic acid, bovine serum albumin, and gluteradehyde were obtained from Sigma-Aldrich. Iridium tetrachloride, iron trichloride, platinum used in counter electrodes, and Nafion were obtained from Alfa Aesar. 20% glucose solution was obtained from Life Technologies. Stabilized glucose oxidase and lactate oxidase were obtained from Applied Enzyme Company. Electrochemical treatments were applied using a Ag/AgCl electrode, a 660A Electrochemical Workstation, and a Picoamp Booster Faraday Cage from CH Instruments. Electrochemical measurements were performed on a multichamber multipotentiostat capable of two amperometric and one potentiometric measurement against a reference electrode, controlled by LabVIEW^{TM} software and described in existing literature[47]. Fluidic pumping was performed with Harvard Apparatus PicoPumps.

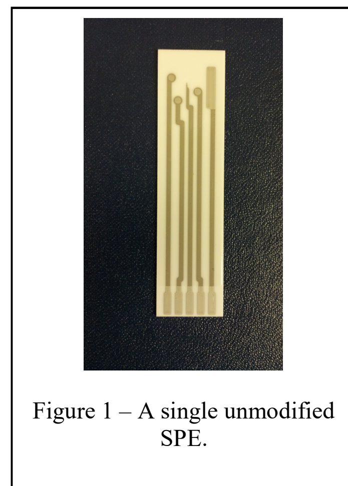

Figure 1 – A single unmodified SPE.

Screen-printed electrodes and accompanying housing were designed by Dr. Jennifer McKenzie and fabricated by Pine Research Instrumentation.

2.2.2 Electrode Film Preparations

Prior to treatment, all electrodes were cleaned by immersion in 1 M H_2SO_4 accompanied by cyclic voltammetry. An applied potential swept from 0.15V to 0.8V and back repeatedly at a scan rate of 0.5 V/s. Cleaning continued until no substantial changes were observed between subsequent cyclic voltammagrams. Care was taken to ensure that the final step was always from less to more positive voltages to prevent redeposition of hydroxides and oxides.

Silver/silver chloride quasi-reference electrodes were prepared by constant-current electrodeposition of silver and subsequent conditioning according to a previously developed procedure[48]. A solution of 0.3 M $AgNO_3$ in 1 M ammonia was prepared. An electrode placed in this solution was preconditioned for plating by the application of +0.95V for 30 seconds in 3M KCL solution. The electrode was then subjected to an applied current of 6.5 mA/cm^2 for 450 seconds. The resulting silver film was submerged in 50 mM $FeCl_3$ for 60 seconds to create a surface layer of AgCl. All solutions were agitated with a stir bar during treatment.

pH-sensitive IrO_2 electrodes were prepared by constant-potential electrodeposition in an iridium oxalate solution according to the method described by Ges[49]. The iridium oxalate solution was prepared by dissolution of 37.5 mg of $IrCl_4$ in 25 mL DI water aided by stir-bar mixing. After 15 minutes, 30% H_2O_2 was added and the solution was allowed to continue to mix. 10 minutes thereafter 125 mg oxalic acid was added. After an additional 10 minutes, the solution was brought to pH 10.5 by the addition of potassium carbonate. The resulting solution was allowed to develop at room temperature in darkness for a minimum of five days and was then stored at 4° C. Electrodes to be treated were immersed in developed iridium oxalate solution and preconditioned by the application of -0.6 V for 30s. Iridium was plated by the application of +0.6 V for seven minutes, resulting in a visible purple film on the plated electrode. The iridium oxalate solution was agitated with a stir bar during treatment.

Glucose, lactate, and oxygen sensors were prepared by application of enzyme and polymer films. All enzyme films were prepared in a base mixture composed produced by the mixture of 800 μL of phosphate buffer and 50 mg of bovine serum albumin. Glucose-sensitive films were prepared by the dissolution by vortexing of 1.5 mg of stabilized glucose oxidase in 300 μL of buffer-serum mixture. 3 μL of glutaraldehyde were added to the solution which was

again vortexed for 5 seconds. Lactate-sensitive films were prepared by the same process, using 2.5 mg lactate oxidase, 100 uL of buffer-serum mixture, and 0.8 µL glutaraldehyde. Enzyme films were hand-cast using 200 µL pipette tips. Enzyme-film electrodes and oxygen-sensing electrodes were coated with a hand-cast film of Nafion polymer to improve electrode selectivity. All films were allowed to dry for 30 minutes prior to further treatment or storage.

After any treatment, SPEs were stored in buffer solution at 4° C. Enzyme electrodes were typically allowed to age 1-2 days prior to improve the stability of their response.

2.2.3 Electrochemical Measurements

Electrochemical quantitation of analytes in solution was performed using the multichamber multipotentiostat to monitor the response of modified SPEs. Glucose and lactate were measured amperometrically by the application of +0.6 V to the corresponding working electrode relative to the quasi-reference electrode. Oxygen was measured amperometrically by the application of -0.45V relative to the quasi-reference electrode to the corresponding working electrode. Changes in pH were quantified by changes in the open-circuit potential between the corresponding working electrode and the quasi-reference electrode.

Calibration solutions were prepared by dilution from previously prepared glucose and lactate solution. Oxygen and pH were measured but not calibrated throughout the experimental process.

2.2.4 Electrode Reclamation

A protocol for the attempted reclamation of treated electrodes which had lost effectiveness or for which a new treatment was desired was developed. All tested protocols consisted of the immersion of modified electrodes into solutions of buffer or nitric acid and the application of a constant voltage vs a Ag/AgCl reference electrode.

2.3 Results and Discussion

2.3.1 Calibration of Optimized Sensors

Initial experiments focused on validating the abilities of the SPE device while determining areas for improvement. A series of calibrations identified frequent points of failure which were addressed by either structural modifications or procedural changes in the SPE and its use. These modifications improved the consistency and ease of use of the SPE and provided benchmarks as to the device's dynamic range for various analytes.

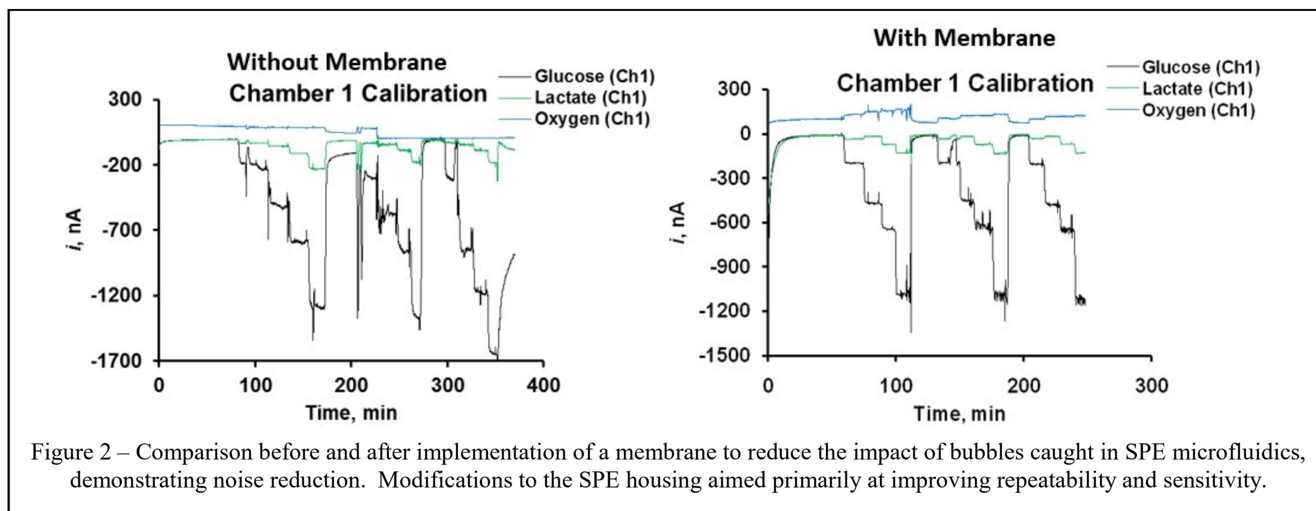

Figure 2 – Comparison before and after implementation of a membrane to reduce the impact of bubbles caught in SPE microfluidics, demonstrating noise reduction. Modifications to the SPE housing aimed primarily at improving repeatability and sensitivity.

Particularly attention was directed towards the behavior of the glucose and oxygen enzyme electrodes during the iterative process and it was observed that quantitation of these analytes noticeably influences the detection of oxygen. The enzyme electrodes demonstrated linear behavior throughout the range of interest without need for correction of Michaelis-Menten kinetics. After the completion of these improves, the electrodes demonstrated a response of $(220\pm20)\frac{nA}{mM}$ for glucose and $(620\pm40)\frac{nA}{mM}$ for lactate. Modifications developed during the optimization process contributed to further improvements by other researchers and to the design of the standardized SPE housing and the standard operating protocols for its use.

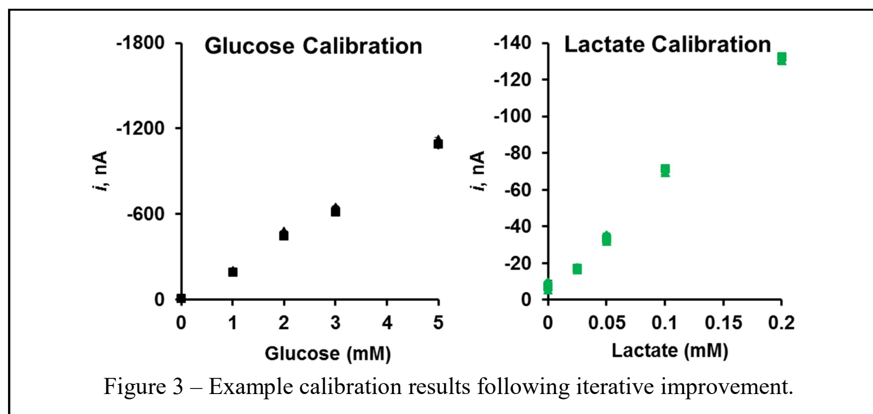

Figure 3 – Example calibration results following iterative improvement.

2.3.2 Electrode Recovery

Treated electrodes were subjected to various environmental conditions and electrochemical treatments in an attempt to remove applied films without damage to the underlying platinum electrodes. Initial efforts utilized mild conditions, specifically 50 mM phosphate buffer solution, and electrical potentials of 1.0, 1.05, and 1.15V, within the electrochemical window of platinum but failed to noticeably degrade either metal or enzyme films. A series of progressively higher potentials (1.2, 1.5, 2.0, 2.5, 5, and 10V) were applied and demonstrated limited success, causing slow stripping of metal films and damaging but not removing enzyme films. Sustained

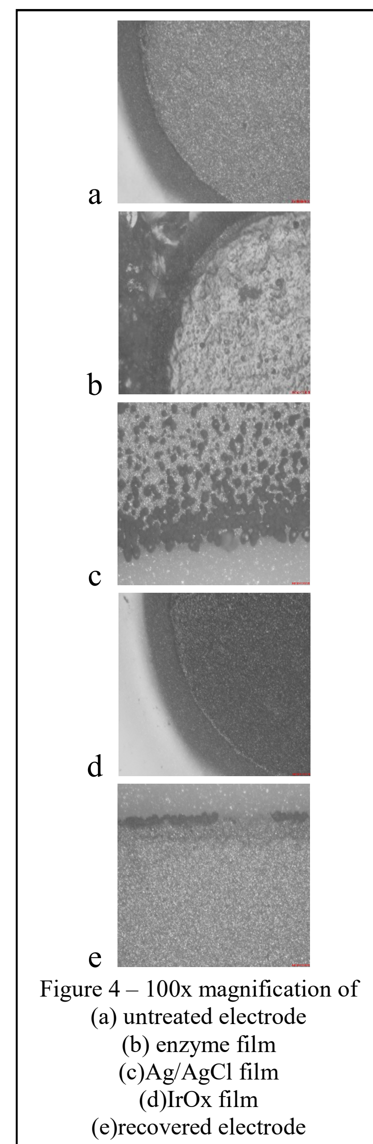

Figure 4 – 100x magnification of
(a) untreated electrode
(b) enzyme film
(c)Ag/AgCl film
(d)IrOx film
(e)recovered electrode

application of high potentials (10V) in a solution of 0.5M sulfuric acid resulted in the rapid (<30 seconds) degradation of enzyme films and eventual (~40 minutes in the case of Ag/AgCl) removal of metal films. Stripped electrode performance was compared to the behavior of fresh, unmodified electrodes by cyclic voltammetry in a solution of 5mM ferricyanide. Compared to the control electrodes, recovered electrodes produced nearly-identically shaped voltammograms with overlapping peaks but exhibited larger current magnitudes. The enhanced electrode response was attributed to the combination of more complete removal of any passivating layers on the electrode and the possible increase of electrode surface area due to surface area. While the conditions of the experiment were sufficient to oxidize the platinum of the electrode, no such damage was observed. The stability of the electrodes may stem from the considerable kinetic favorability of water electrolysis over platinum oxidation.

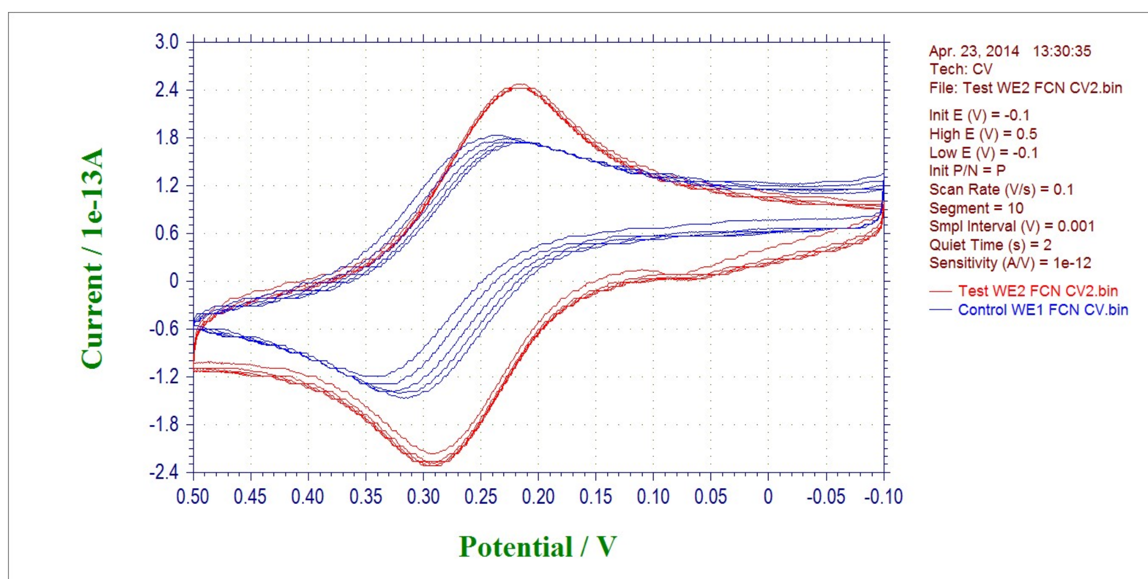

Figure 5 – Comparison of cyclic voltammograms from a recovered electrode previously treated by application of an enzyme film (red) to and unmodified control (blue). CVs were conducted in 5mM ferricyanide over a range of 0.5 to -0.1V, at a 0.1V/s scan rate. Additional CVs showing comparisons for enzyme, silver/silver chloride, and iridium oxide films both prior and following recovery are included in the appendix of this document.

2.4 Conclusions

Protocols and device modifications developed during testing and in response to problems observed during testing contributed significantly to the preparation of the SPE platform for wider use as an analytical platform, contributing to its reliability and ease of use. A version of the SPE utilizing these and other improvements is described in literature[46]. A refined version of the electrode recovery procedure is included in the standard operating procedures available to Vanderbilt Institute for Integrative Biosystems Research (VIIBRE) members and their collaborators for integration of the SPE platform in their experiments. The development of a reliable standard protocol for the SPE device is a necessary step in the electrochemical instrumentation of model biological systems, efforts towards which are described in the following chapter.

CHAPTER III

Towards Real-Time Electrochemical Monitoring of Metabolism in Hollow Fiber Bioreactor Cellular Cultures

3.1 Introduction

A substantial motivation in the development of model biological systems is to provide reliable testing platforms to study the effects of potential therapeutic compounds without risk to living patients. Models of the blood-brain barrier (BBB) are a matter of particular interest in this field as, depending on its application, a candidate compound's ability or inability to cross the barrier can be an overwhelming obstacle to its clinical application. State of the art BBB models recapitulate the *in vivo* environment in considerable detail, recreating the cellular interactions and the physical environment of the BBB[24]. One such barrier model, the FloCel Hollow Fiber Bioreactor (HFBx), has served as a test candidate for integration of the Multianalyte Screen-Printed Electrode developed by the Cliffel lab as a means to monitor the metabolic activity of cell cultures in the bioreactor.

The HFBx's design consists of three chambers, with the outer chambers on each side connected to one another by a series of porous hollow fibers that pass through the central chamber. Two ports connect to the central chamber to allow for the filling of the chamber and while another port connects to each of the end chambers to allow for cell loading and fluid flow through the device. Four gold electrodes allow measurement of the conductivity between the inner and outer chambers. Fluid is pumped through the device by a pulsatile pump which allows independent control of flow and pulse rates. In BBB experiments, the insides of the hollow

fibers are seeded with endothelial cells while astrocytes are cultured in the central chamber. Other cell lines such as neuronal or immune cells may also be co-cultured in the central chamber as the needs of individual experiments dictate. The pores of the fibers allow chemical contact between the cell populations. By allowing interaction between the endothelial and astrocytic populations consistent with human anatomy[22] and reproducing the shear strain pumped blood would exert through the pulsatile flow of media through the hollow fibers[25], the HFBx can induce organization and behavior in the endothelial cells that is substantially closer to *in vivo* BBB endothelium than that of endothelial cells maintained in a traditional 2D culture.

Integration of the SPE to the HFBx presents substantial challenges. As many of the electrodes of the SPE chip utilize enzyme films to achieve their selectivity, processes which would sterilize the chip would also degrade the films. The activity of the enzymes does not survive autoclaving or applications of sterilant solutions. As recirculation of media is necessary in the HFBx in order to allow accumulation of biomolecules in the media, even downstream contamination endangers the bioreactor. As the response of the SPE varies with flow rate, the pulsatile pump of the HFBx introduces substantial noise in analyte quantitation. Diffusion between the outer and central chambers of the HFBx, while desirable during the maintenance and monitoring of a culture in recirculating media which will come to equilibrium, presents a complication to SPE calibration. The effects of these and other challenges have been considered and possible solutions are proposed to many of them.

3.2 Experimental Procedures

2.2.1 Reagents and Instrumentation

F-12K medium, gentamicin-sulfate, and type I collagen were obtained from Life Technologies. Dulbecco's modified Eagle's medium, PC-12 adh cells (CRL-1721.1), and SK-N-MC cells (HTB-10) were obtained from the American Type Culture Collection. Heat-inactivated horse serum and fetal bovine serum were obtained from Atlanta Biologicals. FloCel hollow-fiber bioreactors and accompanying TEER platform, pulsatile pump, and assorted fluidics materials were provided by FloCel Inc. along with permission to reproduce existing representations of their materials or their capabilities. Fluidic adaptors and tubing were obtained from IDEX Health & Science, with the exception of three-way stopcocks which were obtained from SAI Infusion Technologies and 0.20 μm filters which were obtained from Fisher Scientific. Additional pumping needs were met by a MasterFlex peristaltic pump from Cole-Palmer and by a rotary planar peristaltic micropump provided by VIIBRE. A VWR water-jacketed CO_2 incubator was used for the maintenance of cell cultures in the bioreactor. Supplies and devices for the SPE are identical to those described in the previous chapter.

2.2.2 SPE Protocols

Measurements with the SPE were conducted as described in the previous chapter with minor alterations. Fluid flow to the SPE was regulated by the use of a peristaltic pump to allow a degree of isolation from the action of the pulsatile pump. The interface between the SPE and the bioreactor is described in section 2.2.4.

2.2.3 Cell Culturing

Cell lines were maintained under the conditions and according the protocols specified by the ATCC for each cell line. All cell handling was performed inside a Class II biosafety cabinet

2.2.4 Bioreactor Loading and Maintenance

The FloCel HFBx and associated components were sterilized prior to assembly and cell loading. The media reservoir bottle and its cap along with the bioreactor cartridge and its caps were sterilized by autoclaving at 121° C for 45 minutes, while the remaining fluidic components were sterilized by immersion in or pumping through of AquaMax® sterilant (1000 parts sterilant stock: 1 part bleach mixed immediately prior to application) for a minimum of 24 hours prior to assembly. All solutions used in the cabinet which contacted the interior of the bioreactor system were filtered by use of a syringe filter or filter flask prior to use. All sterile components were transferred into a biosafety cabinet still sealed. The outer surfaces of tubing were disinfected by spraying with 70% isopropyl alcohol (IPA) during the transfer process.

The fibers of the bioreactor were wetted by administration of 10 mL through one outer chamber port into an empty syringe at the other outer chamber port. The fibers were allowed to wet for at least one minute after which that ethanol which could not removed by the action of a syringe was displaced by deionized water (DI) administered according to the same procedure.

In cases where the fibers of the bioreactor were treated with collagen, a collagen film was applied by the passage of a filtered portion of 1mg/mL collagen solution administered from a syringe attached at outer chamber port to the opposite inner chamber port and back, and then repeated using the remaining outer and inner chamber ports. The fibers were allowed to soak in the collagen solution for one hour. This procedure results in the collagen treatment of both the interior and exterior surfaces of the hollow fibers.

Cells were loaded into the bioreactor by gentle passage of 10 mL of cell suspension between an inner and opposite outer port pair and then repeated by the remaining port pair. For loading of cells to the interior of the hollow fibers, cell suspension should be passed from outer to inner ports, while the loading of cells in the central chamber is accomplished by passage of cell

suspension from inner to outer ports. Following application of cells to the inner surfaces of the fibers, a process not utilized in this work, the bioreactor should be allowed to rest in a sealed state for one hour within an incubator, being rotated 180° around the axis parallel to the fibers after thirty minutes to ensure complete surface coverage.

Assembly of the bioreactor and fluidic systems followed cell loading. Sterilant solution was displaced from the fluidics by manual pumping of deionized water followed by cell media. Prior to the opening of any fluidic link the link was sprayed with 70% IPA. A three-way stopcock and a 0.20 μM sterile filter were attached to the outer port which would be downstream relative to pulsatile pump flow. The bioreactor was connected by attachment of appropriate fluidic links to its remaining outer port and to the remaining port of the three-way stopcock, which should be configured to allow recirculating flow while sealing the filter. The media reservoir was filled with 25 mL of the appropriate media and attached to the fluidics. The assembly was removed from the biosafety cabinet, attached to its TEER platform and pulsatile pump, and placed in an incubator. Following cell loading, the bioreactor was maintained by the circulating action of the pulsatile pump and by media replacement when acidification or glucose depletion of the media became significant.

2.2.5 Bioreactor Configuration and Sampling

For sampling, appropriate instrumentation was connected to the 0.20 μM sterile filter following application of 70% IPA to both the filter and the fluidic being attached. Instrumentation consisted of a peristaltic pump, optional inclusion of an SPE chamber, and either a collection vessel or biological waste receptacle. No effort was made to sterilize the attached fluidics downstream of the filter to avoid potential contamination of collected samples or degradation of the enzyme films of the SPE. Sampling of the running media in the bioreactor

was sampled by the complete opening of the three-way stopcock to allow media to be pulled through the filter by the action of the peristaltic pump.

3.3 Results and Discussion

3.3.1 Evaluation of Integrated SPE Sensitivity

Three-point calibration experiments using the SPE in monitoring the HFBx revealed a substantial reduction in sensitivity compared to previous calibration experiments using the SPE platform. The observed response for these experiments was (94±1)nA/mM+7±2nA for glucose and (275±86)nA/mM±11nA for lactate, under half that observed in previous calibrations.

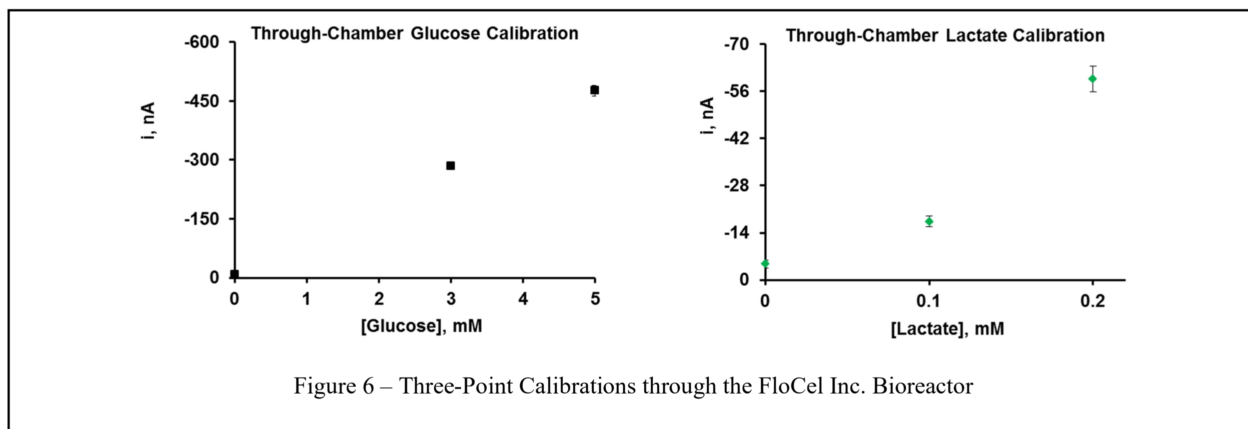

Figure 6 – Three-Point Calibrations through the FloCel Inc. Bioreactor

This loss of sensitivity was attributed to diffusion between the inner and outer chambers of the bioreactor, resulting in each calibrant solution mixing with the previous solution. Measurements performed while glucose- and lactate-free media was circulated through the inner chamber showed less than 25% of the expected signal response compared to previous SPE calibrations. As the HFBx utilizes recirculating media to promote the accumulation of signaling molecules from cultured cells, it may be expected that inner and outer chamber conditions will reach equilibrium given sufficient time. Under sampling conditions these diffusion effects are tolerable (and even desirable, in that they provide insight into the activity of cells cultured in the inner chamber even when sampling from the recirculating line) but during experiments where the

culture is perturbed, such toxicology or drug candidate studies, are likely to suffer from poor time resolution and signal intensity relative to similar experiments in devices like the MAMP. An additional sensitivity study was initiated to examine signal response to prepared cell medium but was abandoned when it became evident that medium mixed with any substantial amount of serum had lactate concentrations sufficient to completely saturate current lactate oxidase enzyme electrodes.

3.3.2 Evaluation of Integrated SPE Effects on System Sterility

Cells were cultured in the HFBx and the system was subjected to simulated sample collection to determine whether the sterile filter and associated procedure were sufficient to preserve the sterility of the HFBx. Initial results utilizing PC-12 cells were inconclusive; after operation for ten days with no apparent depletion of the running media, the HFBx was inspected it was found that setting of applied collagen had entirely obstructed the fibers of the bioreactor and caused the bioreactor media to crystalize. No media could be extracted from the bioreactor chambers. Upon inspection using a hemocytometry microscope, no evidence of contamination or of PC-12 cells was observed in the media reservoir with or without the addition of trypan blue. Inspection by microscopy of media taken from the sampling line revealed clusters of a fibrous red organism in the media.

Sterility experiments were repeated with naturally adherent HTB-10 cells in order to avoid the use of collagen. Media was sampled regularly and changed weekly by replacement of the reservoir bottle. The sampling line showed consistent evidence of contamination as in the previous PC-12 experiment but inspection of the replaced media showed no sign of contamination either from the sampling line or from passage of HTB-10 cells through the pores of the HFBx. After three weeks of operation, the media of the inner chamber was removed and

the remaining contents of the chamber extracted by treatment with trypsin. The removed media contained HTB-10 cells confirmed as dead by universal staining by trypan blue. The trypsin-extracted sample contained primarily living HTB-10 cells. No evidence of contamination by other organisms was observed in either sample. No electrochemical analysis of collected samples was conducted as the lactate concentration in media containing more than 5% fetal bovine serum was known to exceed the detection limit of SPE enzyme film applications available at the time of testing.

3.4 Conclusions

Both fundamental challenges of integrating the SPE to new experimental platforms and specifically obstacles in doing so with the FloCel HFBx or similar devices have been identified. It was seen in some cases, such as preserving the sterility of the HFBx during sampling, that appropriate protocols can resolve obstacles without compromising the function of the HFBx or the SPE. The implementation of printed enzyme film techniques which have since been developed for the SPE address the difficulties in lactate quantitation in media. While the effects of diffusion between the inner and outer chambers introduce a complication in the time resolution of cellular response experiments, it is one that can be accounted for in the analysis of collected data and is unlikely to be observed in experiments that fully recapitulate the BBB and thereby prevent unmediated diffusion through the fibers. These experiments provide a clear path to a more substantial demonstration of the viability of integrated SPE-bioreactor platforms.

APPENDIX

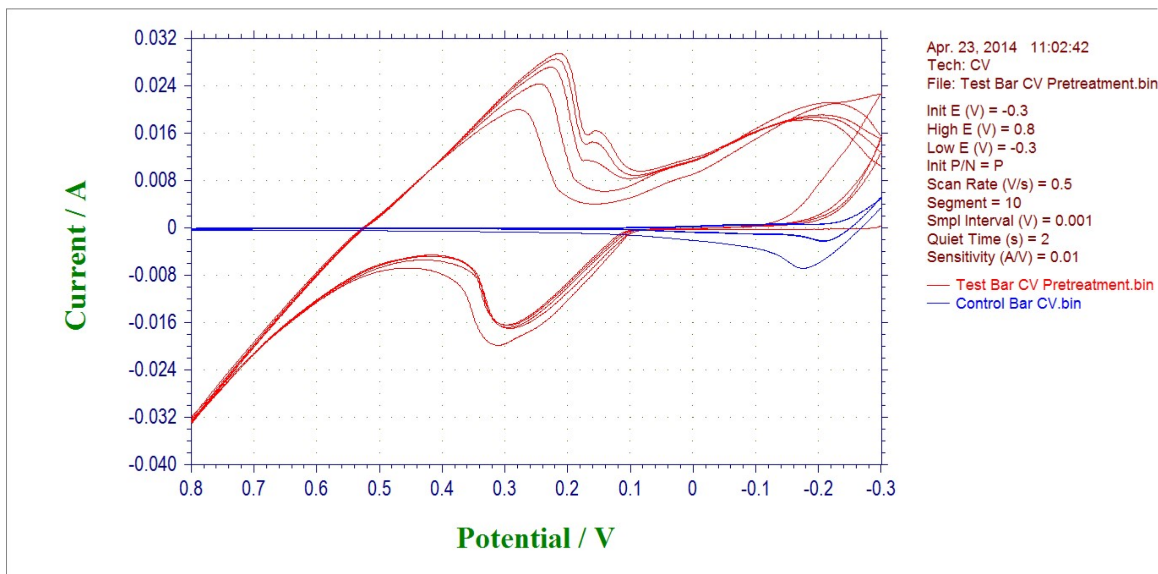

Figure A1 - Comparison of cyclic voltammograms from an electrode with an applied silver/silver chloride film(red) to and unmodified control (blue). CVs were conducted in 5mM ferricyanide over a range of 0.5 to -0.1V, at a 0.1V/s scan rate.

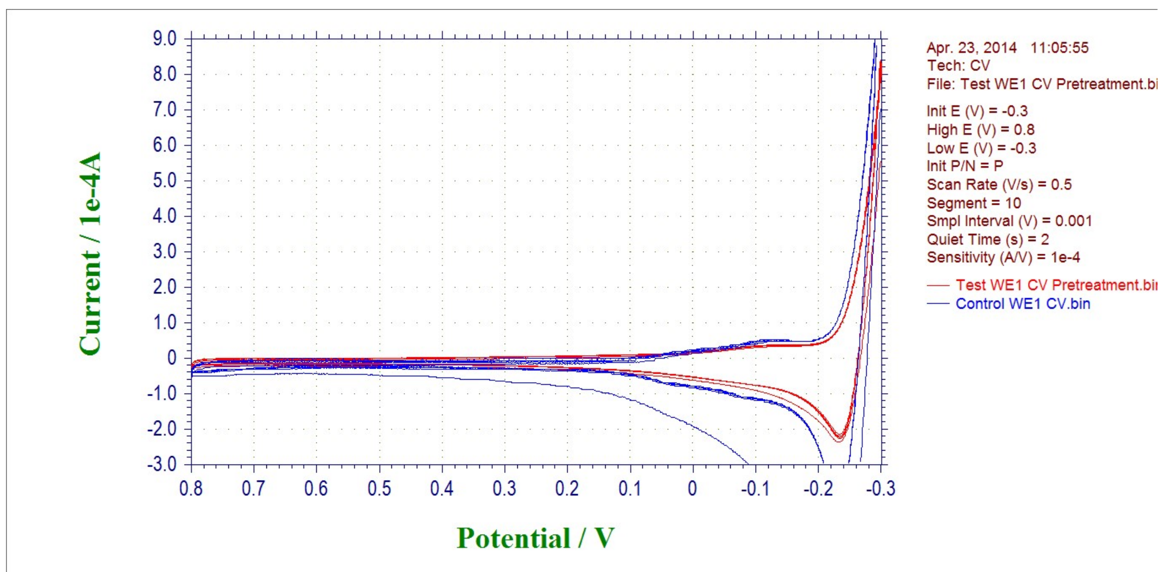

Figure A2 - Comparison of cyclic voltammograms from an electrode with an applied enzyme film (red) to and unmodified control (blue). CVs were conducted in 5mM ferricyanide over a range of 0.5 to -0.1V, at a 0.1V/s scan rate. It is noted that the film substantially reduces the magnitude of current at any given potential.

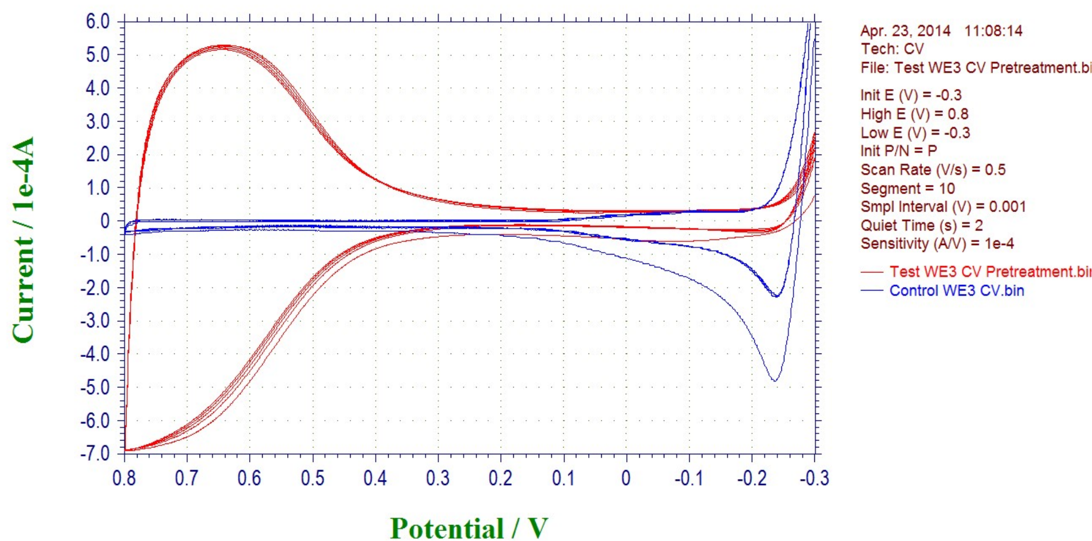

Figure A3 - Comparison of cyclic voltammograms from an electrode with an applied iridium oxide film (red) to and unmodified control (blue). CVs were conducted in 5mM ferricyanide over a range of 0.5 to -0.1V, at a 0.1V/s scan rate.

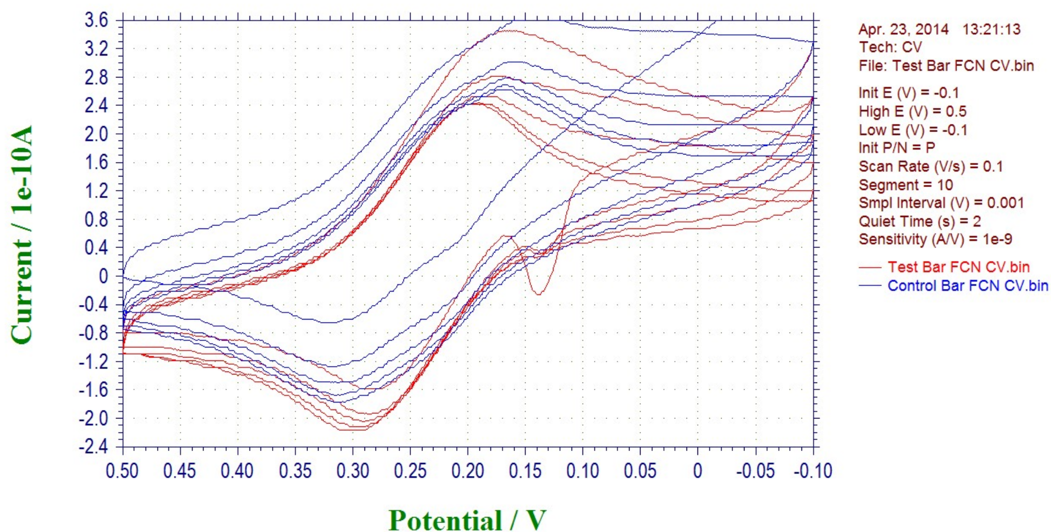

Figure A4 – Comparison of cyclic voltammograms from a recovered electrode previously treated by application of a silver/silver chloride film (red) to and unmodified control (blue). CVs were conducted in 5mM ferricyanide over a range of 0.5 to -0.1V, at a 0.1V/s scan rate.

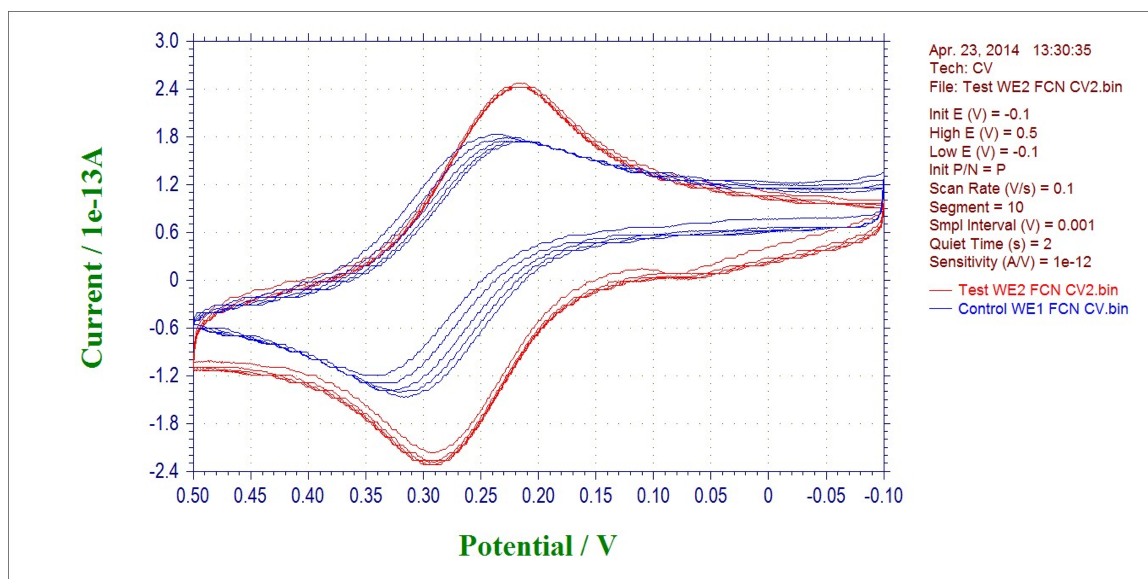

Figure A5 – Comparison of cyclic voltammograms from a recovered electrode previously treated by application of a enzyme film (red) to and unmodified control (blue). CVs were conducted in 5mM ferricyanide over a range of 0.5 to -0.1V, at a 0.1V/s scan rate. This figure is present in the body of the text as Figure X

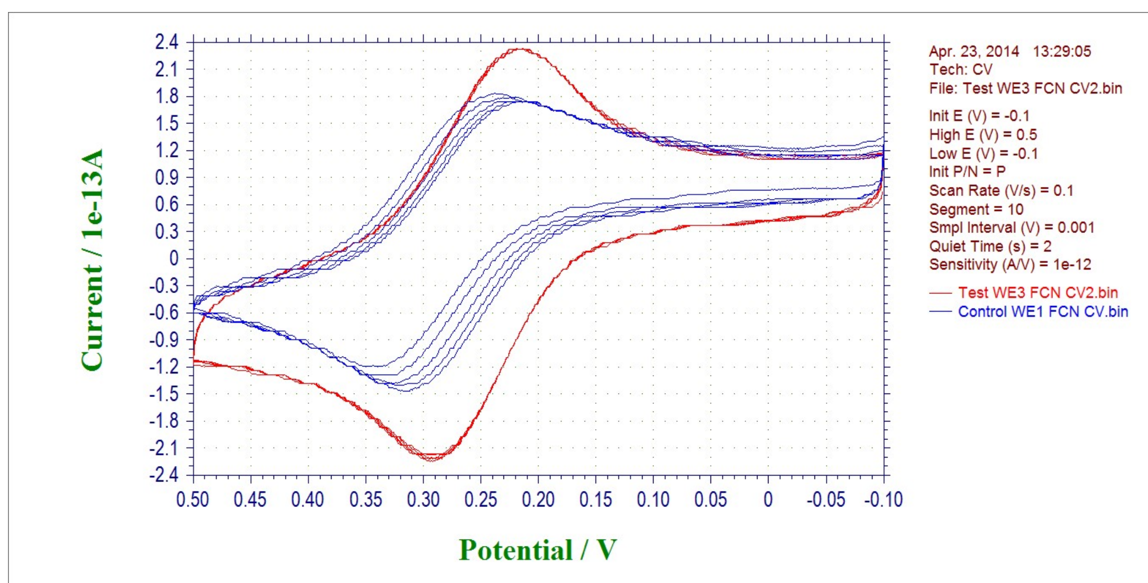

Figure A6 – Comparison of cyclic voltammograms from a recovered electrode previously treated by application of a iridium oxide chloride film (red) to and unmodified control (blue). CVs were conducted in 5mM ferricyanide over a range of 0.5 to -0.1V, at a 0.1V/s scan rate.

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