Lactate Biosensor to Monitor Glioblastoma Multiforme

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Abstract

Lactate Biosensor to Monitor Glioblastoma Multiforme

***Abstract* — A lactate biosensor was designed to monitor tumor size in patients with Glioblastoma Multiforme, an aggressive form of brain tumor. Current monitoring methods are expensive and not conducive to frequent use, creating a need for a biosensor to frequently monitor GBM tumor size. This biosensor was composed of polypyrrole (PPY) and polyaniline (PANI) layers with lactate oxidase (LOX) cross-linked to the surface using glutaraldehyde. The components of the biosensor were tested iteratively. The biosensor is able to detect increases in lactate concentration. It is has a higher maximum current than other designs and is able to detect greater presence of lactate. More testing is needed in order to continue validation of the biosensor and extend the technology to other metabolites.**

I. Introduction

Glioblastoma Multiforme (GBM) is an extremely aggressive form of brain cancer that has a three-year survival rate of less than two percent [1]. Treatment options for GBM patients are surgery, chemotherapy and radiotherapy. Current methods used to monitor tumor progression are Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) scans. These methods have limitations and are not conducive for frequent use. Therefore, there is a critical need to develop a method to frequently monitor tumor size in GBM patients to determine treatment efficacy.

This project exploits differences in cellular metabolism found in tumor cells. In healthy cells, glucose is broken down into pyruvate and enters the mitochondria and causes the production of 36 molecules of adenosine triphosphate (ATP) per molecule of glucose. However, in cancerous cells, pyruvate is broken down into lactate to generate four molecules of ATP [2][3][4]. The increase in lactate production of tumor cells can be used to monitor tumor size, as more tumor cells will create an elevated level of lactate.

A biosensor was designed to monitor the progression of GBM via lactate concentration. In order to measure lactate concentration, an enzyme, lactate oxidase (LOX), is required to break down the lactate into hydrogen peroxide and then into free electrons.

$$Lactate + O_2 \xrightarrow{LOx} Pyruvate + H_2O_2$$

$$H_2O_2 \xrightarrow{Voltage} 2H^+ + O_2 + 2e^-$$

The signal measured by the biosensor is the current created by these electrons at the electrode surface. The biosensor must be able to sense lactate levels without interference from other molecules. It must also be sensitive enough to detect the levels of lactate created by a GBM tumor. The biosensor must be biocompatible so it does not cause any inflammation that will isolate the biosensor from the metabolite of interest.

In order to ensure that our biosensor has the desired properties, certain materials were selected. The basis of the biosensor is a glassy carbon electrode (GCE). Conductive polymers were used to create a film on the GCE surface and decrease the biosensor response time by aiding electrons to reach the GCE surface more rapidly. In particular, polypyrrole (PPY) was chosen because it has small pores which prevent interference from other molecules. Polyaniline (PANI) was chosen for its high conductivity. LOX was used to break down lactate to create the electrical signal for the biosensor to measure. Glutaraldehyde was used to crosslink lactate oxidase to PANI to prevent diffusion of the enzyme out of the polymer film. The final design consists of a PPY layer deposited onto a GCE followed by a layer of PANI and LOX (Fig. 1).

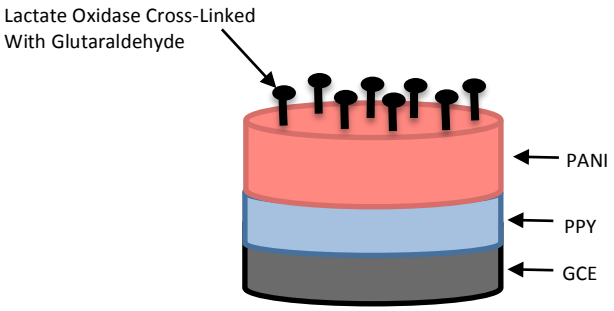


Figure 1. Schematic of final biosensor design

II. METHODS

Three millimeter GCE were polished as described by Anglin et al. [5]. For all electrochemical experiments, the GCE (working electrode) was used with an Ag/AgCl reference electrode and a platinum counter electrode. An AUTOLAB potentiostat (Metrohm) and GPES software were used to control the current and voltage running through the working electrode and to record data.

Biosensor Fabrication

To create the PPY film on the surface of the GCE, 7 µL of pyrrole (Sigma-Aldrich) was added to 10 mL of 0.01 M phosphate buffered saline (PBS) that had been degassed by nitrogen bubbling for 15 minutes [6]. The pyrrole was polymerized to form PPY on the surface of the GCE by using galvanostatic deposition at a current of 25 mA/cm^2 for 120 seconds. Then, a PANI film was created on top of the PPY film by polymerizing 45 µL of aniline (Sigma-Aldrich) in degassed 0.1M PBS of pH 3.5. Nine cycles of cyclic voltammetry between 0.00 and 0.95 V were used to polymerize the aniline. 5 µL of 0.1% glutaraldehyde (Sigma-Aldrich) was placed on top of the polymer films and dried at room temperature for 30 minutes. 5 µL of LOX (2.9 mg/mL, Sigma-Aldrich) was added on top of the PPY-PANI-glutaraldehyde electrode. The glutaraldehyde cross-linked the LOX to the polymer.

Electrochemical Experiments

For each test, a degassed solution of lithium lactate in PBS was placed into the electrochemical cell. To measure the current output of the biosensor in response to lactate, Autolab was used to perform amperometry, in which the voltage is held constant and the current changes as a function of lactate concentration. Cyclic voltammetry (cycling between -0.4 and +0.6V for 10 cycles) was used to gauge biosensor performance as a function of lactate concentration. The biosensor's response to changing lactate concentrations over time was measured.

Cell Culture

Human glioblastoma cells (U87mg, ATCC) were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 1% L-glutamate, 1% penicillin-streptomycin, 1% non-essential amino acids, for 48 hours.

Biosensor Accuracy Testing

To determine the lactate concentration secreted into the media of cultured U87MG cells, ELISA was performed according to manufacturer's protocol (Sigma Aldrich) This was compared to the concentration calculated based on the biosensor reading in order to determine biosensor accuracy.

III. RESULTS

Analysis of PPY and PANI Films

SEM images of the PPY and PPY-PANI films are shown in Figure 2. The PPY film thickness was calculated to be 100 nm.

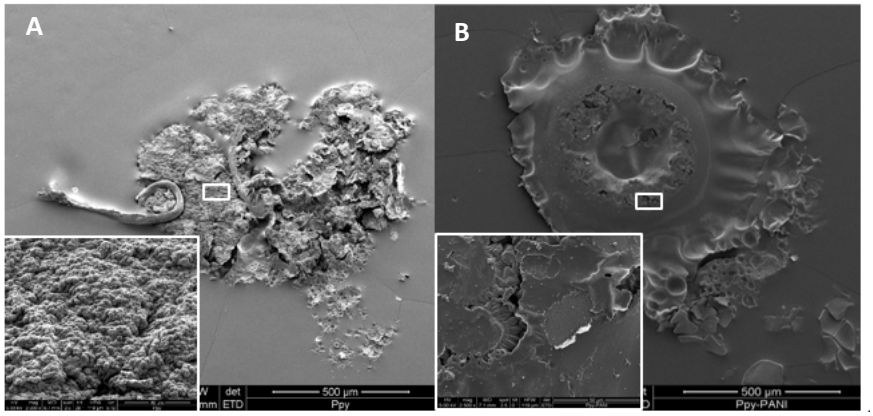


Figure 2. SEM images of A) PPY coating and B) PPY-PANI coating

Electrochemical Testing

Different variations and combinations of the design components were tested iteratively in order to optimize the biosensor. Amperometry results of the maximum current detected at different lactate concentrations are shown in Figure 4. The biosensor is able to detect the decrease in current with increasing lactate concentration.

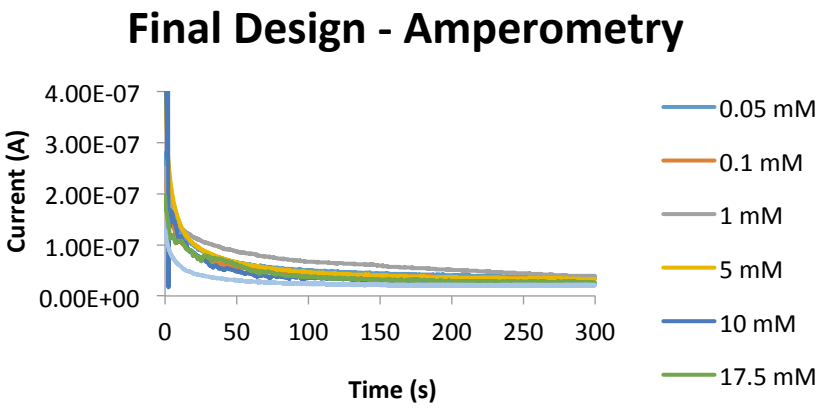


Figure 4. Decreasing current with increasing lactate concentration

Increasing Lactate Levels

Amperometry results of the maximum current detected as lactate was added to the system are shown in Figure 5. The biosensor is able to detect the addition of lactate but over time the maximum current decreases.

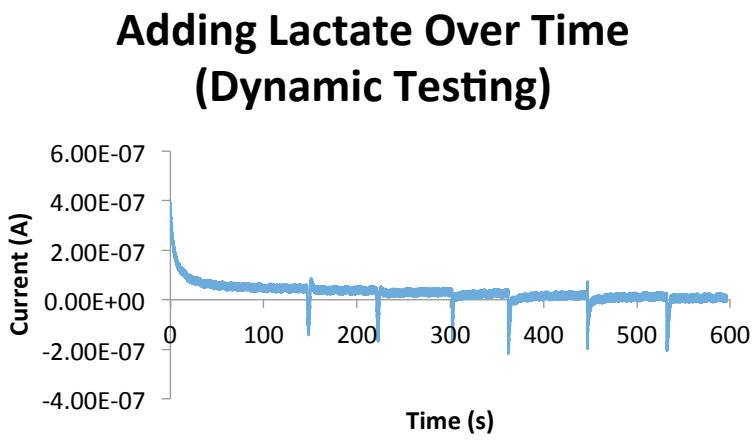


Figure 5. Current response with increasing lactate concentration

Lactate Concentrations in Cell Media

Media collected from U87mg cells cultured over 48 hours was tested with an ELISA and the biosensor to measure lactate concentration. The concentration of lactate based on the current measured by the biosensor was calculated by using a standard curve gathered from the dynamic testing. Unfortunately, our results were unreasonable.

Table 3: The average current read by the biosensor, the average lactate concentration calculated using the standard curve, and the actual lactate concentration measured with an ELISA for media samples containing 1,000,000, 500,000, or 100,000 cells.

Cell Number	Average Lactate Concentration (ELISA) (mM)	Average Current (A)	Average Lactate Concentration Calculated (mM)
1,000,000	0.93 ± 0.25	2.36E-07 ± 5.5E-8	-46.5
500,000	1.24 ± 0.07	2.70E-07 ± 4.4E-8	-55.1
100,000	0.31± 0.008	1.35E-07± 3.4E-8	-21.2

V. DISCUSSION

The PPY-PANI-LOX biosensor designed in this MQP is able to detect changes in lactate concentration with a change in its current output. However, we had expected to see an *increase* in current as lactate concentration increased, not a decrease. Because we were only able to do testing once on properly calibrated equipment, we were unable to explain why this trend of current vs concentration was negative. However, the biosensor was shown to have a very quick response time in dynamic testing. When tested with cell media, the current output of the biosensor was higher than during testing in PBS, which suggests that there might be non-specific reactions from other molecules in the media that are leading to an increased current response.

Although the biosensor is able to measure increased current with increased lactate concentration, continued testing and validation are needed. Before the biosensor becomes an implantable medical device, more research and testing must be completed. The biosensor must be tested over a period of weeks and months at physiological conditions in order to determine how long it can remain effective. Biocompatibility tests must be performed to ensure that the biosensor does not trigger an inflammatory response. The biosensor must be miniaturized before it can successfully be used *in vivo.*

REFERENCES

[1] Pelletier, G., et al., Quality of life in brain tumor patients: the relative contributions of depressions, fatigue, emotional distress and existential issues, Journal of Neuro-Oncology (2002) 57:41-49
[2] Vander Heiden, M., et al. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009. 324(5930): 1029-1033
[3] Marie, S. Shinjo, S. Metabolism and Brain Cancer. Clinics. 2011. 66(S1): 33-43.
[4] Wolf, A., et al. Targeting Metabolic Remodeling in Glioblastoma Multiforme. Oncotarget. 2010. 1(7): 567-577
[5] Anglin, N. et al. Development of a Biosensor to Monitor Glioblastoma. Worcester Polytechnic Institute, 2013.
[6] Li, C.M. et al. Electrochemical Thin Film Deposition of Polypyrrole on Different Substrates. Surface & Coating Technology. 2005. 198:474-477

1. Introduction

Glioblastoma Multiforme (GBM) is an extremely aggressive form of brain cancer, with a three-year survival rate of less than two percent (Pelletier et al., 2002). Because of GBM's aggressive nature, it is essential to find an effective treatment and be able to closely monitor the progression of the tumor. Current monitoring methods, such as magnetic resonance imaging (MRI) and computed tomography scans (CT scans) are not appropriate for frequent use. These tests are typically performed every six months, during which time a GBM tumor can develop significantly. Additionally, these imaging modalities can only detect large, non-diffuse tumors. A small, implantable biosensor could allow for low-risk monitoring as needed of the progression of GBM. This biosensor could be inserted minimally invasively and transmit data to the patient or physician's mobile device, allowing for real-time monitoring of the patient's condition. Daily monitoring of tumor progression will allow a medical team to determine the efficacy of a particular treatment on a patient and tailor the patient's treatment accordingly.

The biosensor designed in this MQP would function by exploiting the increased concentrations of lactate produced by GBM tumors as a byproduct of the altered metabolism in tumor cells. We hypothesize that the local increase in lactate production by glioblastoma tumor cells is significant and detectable by a continuous, real-time implanted biosensor. To this end, we developed a lactate biosensor based on lactate oxidase, an enzyme that catalyzes the degradation of lactate into pyruvate, and conductive polymers (polypyrrole and polyaniline). This project was based on a previous Major Qualifying Project (MQP), completed in May 2013, which began the development of a lactate biosensor (Anglin et al., 2013). The overall goal of the current project was to design, fabricate, and validate a film for a biosensor that could detect lactate sensitively and reproducibly, with a short response time. Desired characteristics for the biosensor include sensitivity, selectivity, reliability, biocompatibility, and the ability to remain stable in the body for at least two weeks.

To create the biosensor, we created design alternatives utilizing different conductive polymers to carry the charge produced from the enzymatic degradation of lactate by lactate oxidase to the electrode surface. An iterative testing process allowed us to validate each component of the final design. Although our biosensor still needs further testing to improve its sensitivity and specificity, we believe that it is a promising start to an implantable biosensor that could eventually be used to monitor all types of cancer, not just glioblastoma multiforme.

2. Literature Review

2.1 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is one of the most common forms of brain cancer in adults, affecting 3 out of 100,000 people in the United States each year (Marucci, 2011). These malignant brain tumors are classified as astrocytomas, which arise from astrocytes, the cells that support the function of neurons. (St. Jude Children's Research Hospital, 2013). GBM is classified as a grade IV astrocytoma, which is the highest and most serious grade. Typically, high-grade astrocytoma patients receive a variety of treatments beginning with surgery. Because surgery rarely removes the entire tumor, it is usually followed by radiation, chemotherapy, or a combination of the two (National Brain Tumor Society, 2013). Generally, by the time symptoms appear, a tumor has spread across a large portion of the brain. The median survival time after diagnosis is 15 months (St. Jude Children's Research Hospital, 2013), with only two percent of patients surviving three years past diagnosis (Pelletier et al., 2002). There are two types of GBM, primary and secondary. Primary GBM is more common and aggressive than secondary GBM, which can arise from a lower grade astrocytoma (Reardon et al., 2006). GBM is more common in adults than children and more common in men than women (St. Jude Children's Research Hospital, 2013).

2.1.1 Symptoms

Both forms of GBM display similar symptoms. Symptoms can vary between patients and depend on where the tumor is located in the brain. The most common symptoms that patients exhibit are a result of increased pressure in the brain and can include headache, nausea, vomiting, and drowsiness. Headaches are usually the first symptom and often increase in frequency as the tumor grows. Other common symptoms include seizures, body weakness, memory loss, speech difficulties and vision changes, such as blurred or double vision (American Brain Tumor Association, 2013). Some patients also exhibit changes in mood, mental function, or personality.

2.1.2 Treatments

GBM is an extremely difficult form of cancer to treat due to its rapid progression and the proximity of tumors to important areas of the brain, which makes surgery difficult. There are a variety of treatments available for GBM, with many more treatments currently in development.

Generally, the first step in the treatment process for GBM is surgery to collect a biopsy and remove as much of the tumor as possible. The biopsy is used to confirm the diagnosis. Physicians can also examine these tissue samples and gather information that can aid in creating an effective treatment plan. Often, it is impossible to remove the tumor entirely because it is able to easily maneuver into the crevasses in the brain. Tumor re-sectioning is when abnormal tissue is surgically removed. Removing this abnormal tissue has many benefits including reduced symptoms, improving function and decreasing pressure within the skull. These benefits help to improve the quality and length of life for patients. There are also risks involved in re-sectioning and it is more difficult when the tumor grows near parts of the brain that control important functions such as speech and motor ability. During re-sectioning, nerves and blood vessels that control vital functions can be damaged and cause loss of sensation, sight, memory, movement and other functions. Other serious risks include infection, seizures, coma and even death (American Brain Tumor Association, 2013).

Radiotherapy, also known as radiation therapy or simply radiation, is when high-energy rays, such as x-rays, are used to destroy diseased tissue. Radiation can also damage healthy tissue. The healthy tissue is often able to repair itself, whereas the diseased tissue cannot. It is often used in conjunction with surgery and chemotherapy, and has been shown to increase the survival time after diagnosis by 4-12 months in elderly patients (Bruce, 2013). Radiation is most often chosen as the initial postoperative treatment for GBM. Responsiveness to radiotherapy varies, but in most cases the tumor size is reduced dramatically. Radiotherapy can induce remission, although it is often brief. Tumors typically reappear within two centimeters of the original tumor site and increase in size within one year after radiotherapy (Bruce, 2013).

For many patients with GBM, chemotherapy is part of their treatment regimen. Studies have shown that chemotherapy is beneficial for over 25% of patients who receive it. For example, 6-10% of patients have an increased survival time after chemotherapy (Bruce, 2013). The most popular chemotherapy drug is temozolomide, an oral drug used in the treatment of GBM. Other forms of chemotherapy include implanting biodegradable polymer wafers that release carmustine (Gliadel). Controlled release systems are also being developed for other chemotherapy drugs including doxorubicin and paclitaxel (Ong et al., 2009).

2.1.3 Monitoring GBM Progression

There are various methods to monitor the progression of cancer including Magnetic Resonance Imaging (MRI), Computed Tomography Scans (CT Scans) and Magnetic Resonance Spectroscopy (MRS). These procedures can sometimes be ineffective at detecting the tumors and can cause detrimental effects to the patient's health. Due to these adverse effects, a more effective and less harmful method to assessing the progression of GBM is needed.

Magnetic Resonance Imaging (MRI) is considered the gold standard of neuroimaging and is able to determine the size and location of the tumor. An MRI uses magnetic fields and radio waves to take images primarily of soft tissues inside of the body (MRI Scan, 2013). This form of imaging is effective at recording images of soft tissue. Although this technology is beneficial in monitoring the location and size of a tumor, it cannot be used to diagnose GBM (Armstrong, 2009). A biopsy can be performed once the location of a suspected tumor is pinpointed using an MRI.

Computed Tomography (CT) scans use X-rays and digital computing technology to create two- and three-dimensional images of the brain. These scans can create an image of every tissue type, including bone, blood vessels, soft tissue, and cancerous tissue. While CT scans can be very beneficial, they require a dye or contrast agent to be injected into patients to enhance the image and expose patients to radiation (Roberts, H. et al., 2002). CT scans can also be ineffective in detecting small tumors.

Magnetic Resonance Spectroscopy (MRS) is a developing technology that can be used to determine biochemical changes in tissue or fluid samples. MRS can detect the concentration of metabolites in the tissue, which can make a cancerous area apparent when compared to the healthy brain. MRS can also be used to monitor a tumor site and differentiate between recurring tumor tissue and damage from radiotherapy treatments (Brandes, 2008). MRS has some limitations, most notably inaccuracy when performed in or near certain tissues, such as bone or fat, air, or hemorrhages. This is especially true in areas of the brain near the sinus pathways, calvarial bone, and skull base (Gujar et al., 2005).

2.2 Cellular Metabolism

Cellular metabolism in GBM is inherently different from the metabolism of healthy brain tissue (Marie and Shinjo, 2011, Vander Heiden et al., 2009, Wolf et al., 2010), as we will discuss further in the sections below. Our biosensor will exploit these differences to identify the presence of a tumor and quantify the progression of GBM.

2.2.1 Non-cancerous Cells

Cells break down carbohydrates, lipids, and proteins in order to produce energy by a variety of chemical processes, collectively known as cellular metabolism. In healthy, non-proliferative tissue, intracellular glucose undergoes glycolysis to form pyruvate. If oxygen is present in sufficient quantities, the pyruvate enters the mitochondria, undergoes the tricarboxylic acid (TCA) cycle, and causes the production of 36 molecules of adenosine triphosphate (ATP) per molecule of glucose by the process of oxidative phosphorylation. In the absence of oxygen, such as in times of heavy exercise, the pyruvate is broken down to lactate and produces two molecules of ATP, in a process known as anaerobic glycolysis (Marie and Shinjo, 2011).

2.2.2 Warburg Effect

Cancerous cells, including those of GBM, do not follow typical cellular metabolism. Figure 1 compares the metabolism of healthy cells to that of cancerous tissue.

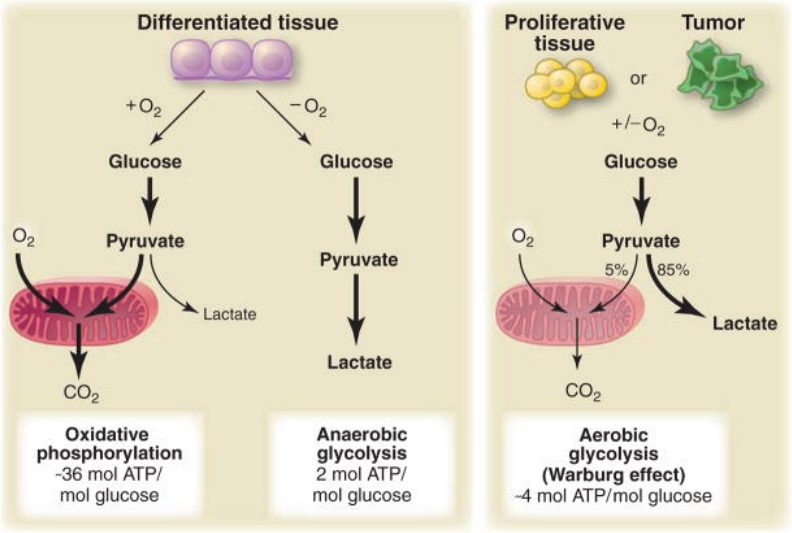


Figure 1: Glucose Metabolism Diagram

Glucose metabolism of healthy differentiated tissue compared to tumor and proliferative tissue (Vander Heiden et al., 2009).

In cancer cells, pyruvate is broken down into lactate to generate four molecules of ATP, even if oxygen is present. This is known as aerobic glycolysis, and was first noted by Otto Warburg in 1924. The difference in cellular metabolism in cancerous cells is therefore named the "Warburg Effect" (Vander Heiden et al., 2009, Marie and Shinjo, 2011, Wolf et al., 2010). The Warburg Effect extends to other aspects of metabolism besides glucose, resulting in the production of more lipids, amino acids, and nucleotides. Aerobic glycolysis is energetically inefficient – one molecule of glucose only produces four molecules of ATP, instead of the 36 molecules produced in oxidative phosphorylation. However, this altered metabolism has many benefits for the survival of the cancer. The shift from energy production to the production of lipids and nucleotides allows the cell to quickly create all of the materials necessary for it to duplicate. This enables the fast proliferation of the cancerous cells (Vander Heiden et al., 2009). The increase in lactate production creates an acidic environment surrounding the tumor, which encourages tumor cell invasion (Marie and Shinjo, 2011). Glutaminolysis, a process in which glutamine is broken down into glutamate and ammonia, is dramatically increased in cancer cells. This process provides a nitrogen source for the cell, allowing it to create amino acids and nucleotides (Dang, 2010). The altered metabolism in cancer cells results in increased levels of lactate, alanine, glutamate, and

amino acids, but a decreased level of glucose (Vander Heiden et al., 2009). Therefore, by monitoring metabolite concentration, cancer progression can be monitored.

2.3 Metabolites found in Glioblastoma Multiforme Cells

Elevated levels of certain metabolites due to the Warburg Effect could help with determining the progression of brain cancers such as GBM. Lactate is one of several metabolites that have statistically significant changes in concentrations in GBM cells compared to normal brain cells (Roslin et al., 2003)**.** An increase in lactate concentration is a common feature of cancer cells. The average normal plasma lactate concentration ranges from 0.3-1.3 mM for healthy adults, during heavy exercise, these levels increase above 5 mM (Phypers, B. and Pierce, J. 2006). Lactate levels above 20 mM have been observed in GBM patients (Cheng, L. et al., 2000). Lactate is an important metabolite to be studied due to the capability of lactate to predict the survival period of patients. Lactate concentrations have been shown to be inversely correlated to patient survival. Increased lactate concentrations have also been shown to have a positive correlation with the increase in resistance to radiotherapy in tumor cells. Lactate concentrations generally decrease in animals after both chemotherapy and radiotherapy. Monitoring of lactate concentrations is a good determinant for the progression of cancer because lactate concentration is proportional to tumor size (Hirschhaeuser et. al, 2011).

2.4 Biosensors

A biosensor is a device made of a biological component (enzymes, cells, DNA) and a physical transducer that converts the concentration of a biological analyte into an electrical signal (Keusgen, 2002). This biological analyte can be in the form of proteins, metabolites, drugs, or other biological chemicals. Many biosensors are enzymatic. An enzyme on the biosensor surface causes the analyte of interest to break down in such a way that a physico-chemical signal is produced. This is converted by the transducer into an electrical signal, which is then interpreted by a computer. The electrical signal read by the computer is directly proportional to the amount of analyte present (Sassolas et al, 2012). Figure 2

explains this process.

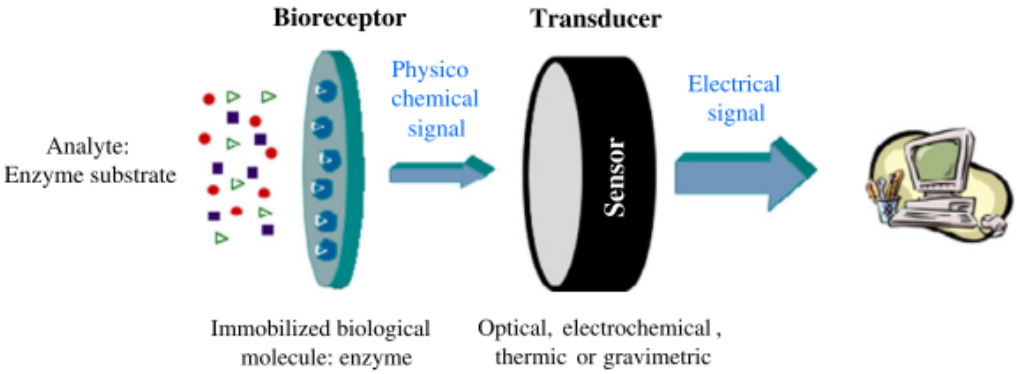


Figure 2: Scheme of a Biosensor
Diagram of how biosensors work (Sassolas et al, 2012).

2.4.1 History of Biosensors

The first biosensor was created by Clark and Lyons in 1962 at the Cincinnati Children's Hospital (Anglin et al., 2013, Wang, 2008, Keusgen, 2002, Wilson and Gifford, 2005). This biosensor detected glucose by using glucose oxidase to break down glucose into hydrogen peroxide, which subsequently broke down into oxygen and electrons. The electrons created an electrical signal that could be quantified. This allowed doctors to monitor the blood sugar levels of patients for the first time (Wang, 2008, Ronkainen et al., 2010). The main challenge faced by early biosensors is the poor aqueous solubility of oxygen, which was needed as a cofactor to make the enzyme work effectively. The second generation of biosensors used mediators to shuttle electrons from the redox site of the enzyme to the surface of the electrode, replacing the function of oxygen. Examples of mediators include ferrocene derivatives, conducting organic salts, and transition metal complexes. Commercially available glucose biosensors used in the monitoring of diabetes mellitus use this second generation technology (Wang, 2008). The field of biosensors is now moving into the third generation, in which mediators will be eliminated and biosensors will be designed so that there is direct electron transfer between the redox site of the enzyme and the surface of the electrode (Wang, 2008).

Biosensors allow for frequent monitoring of the concentrations of biomolecules in applications such as diagnostic testing, development of new pharmaceuticals, and monitoring of patients in a clinical setting. Glucose biosensors, perhaps the best-known example of a biosensor, are used multiple times a day by patients with diabetes mellitus in order to track blood sugar (Wang, 2008). In the future, as more is understood about diseases and as biosensor technology develops, a biosensor could be implanted to detect the concentrations of multiple metabolites or analytes and inform medical personnel of changes in a patient's condition. This project aims to detect the concentration of lactate and glutamate in order to monitor tumor size and progression in GBM patients.

2.4.2 Biosensor Failure

There are several factors that can cause biosensor failure. The most important challenge to overcome when designing a biosensor is to make it compatible *in vivo*. When foreign materials such as implantable biosensors enter the body, they activate the foreign body response (Wang, 2008, Kotanen et al., 2012, Wilson and Gifford, 2005, Bannish et al., 2013). The immune response becomes activated, leading to inflammation and the activation of macrophages. These macrophages release cytokines that recruit more macrophages to the implantation site. Within two to three weeks, these macrophages form a fibrous encapsulation of the biosensor, leading to loss of function. This process is known as bio-fouling (Bannish et al., 2013, Kotanen et al., 2012, Wisniewski and Reichert, 2000) and can be seen more clearly in Figure 3. Titanium oxide may be used in conjunction with anti-inflammatory drugs to slow this process and increase biocompatibility (Bannish et al., 2013). Biosensors that use enzymes may fail due to enzyme unfolding in an unfavorable chemical environment or enzymatic degradation caused by proteases released by neighboring cells. Failure of the biosensor's materials can be due to mechanical stresses or time and temperature related changes of the membrane covering the electrode surface (Kotanen et al., 2012).

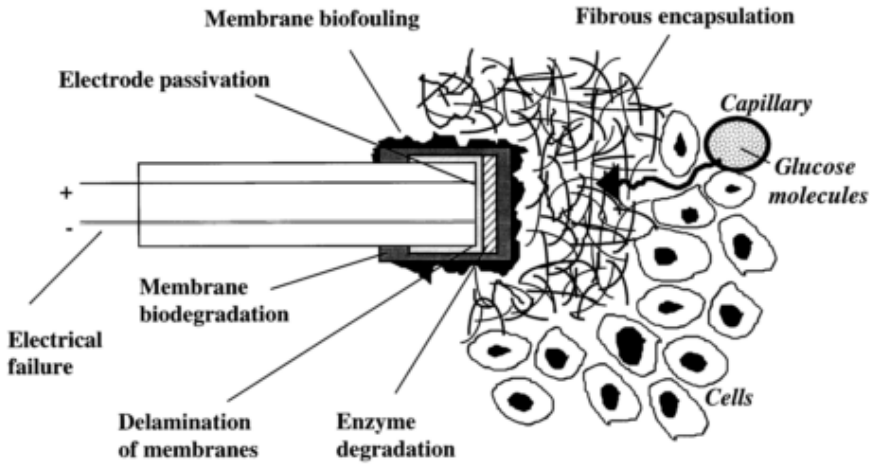


Figure 3: Fibrous Encapsulation of a Biosensor
Fibrous encapsulation of a biosensor due to bio-fouling (Wisniewski and Reichert, 2000).

2.4.3 Desired Qualities of a Biosensor

There are many qualities desired when designing a biosensor. The biosensor must be sensitive enough to detect small changes in analyte concentration and be capable of detecting analyte concentrations within the physiological range. A biosensor should be selective, in that it measures the analyte of interest without any interference from other molecules. It must be stable in appropriate conditions for extended periods of time and not undergo degradation within the body. Finally, a biosensor must be biocompatible and not induce the foreign body response (Anglin et al., 2013).

2.4.4 Sensors for GBM

Some biosensors have been developed for use in GBM, although none have achieved clinical use. Interestingly, they all approach the problem of monitoring the progression of GBM in different ways. Hirata et al. (2012) have developed a biosensor that employs genetically altered genes that promote tumor invasion and express green fluorescent protein. By using Fluorescent Resonance Energy Transfer (FRET) imaging, they can monitor the invasion of GBM into surrounding tissue. In 2013, Zadran et al. created a GBM biosensor to detect ATP in GBM cell lines. This was also based on fluorescence, but instead of FRET, Enhanced Acceptor Fluorescence (EAF) was used. This is a process in which fluorescent proteins are only activated when they bind to ATP. Baraket et al. (2010) used a formaldehyde

dehydrogenase enzyme coupled with carbon nanotubes to monitor the release of formaldehyde by GBM cells being treated with a formaldehyde-based anti-cancer drug. Castillo et al. (2005) developed a biosensor to detect levels of glutamate and nitric oxide in C6-glioma cells, but only implemented this technology on *in vitro* drug screening systems. Most recently, a previous Major Qualifying Project team developed a biosensor to detect the concentration of lactate produced by GBM cells. This biosensor was based on lactate oxidase and polypyrrole (Anglin et al., 2013). This project continues in their footsteps by redesigning a lactate biosensor for better performance.

2.4.5 Materials Used in Biosensors

A variety of biomaterials can be used in the fabrication of biosensors in order to optimize function and prevent adverse effects. Enzymatic biosensors are typically composed of an electrode, an enzyme, and a conductive entity that speeds the transfer of electrons from the enzyme redox site to the electrode surface. Immunosensors are similar to enzymatic biosensors but use antibodies as their sensing element. Other types of biosensors include microbe-based biosensors and potentiometric biosensors. Different materials may be used for the purpose of improving the biocompatibility or other properties of the biosensor. In this section, we will review some of the materials commonly used in biosensors.

2.4.5.1 Electrode Material

There are many different options for electrode material including glassy carbon, gold, platinum and palladium. Platinum has the shortest response time, most efficiently breaking down hydrogen peroxide into electrons, but can be mechanically unstable when electrochemical layers are deposited onto it. Palladium is more stable for electrochemical deposition (O'Neill, R., et al., 2004). Gold electrodes are unstable at potentials above 700 mV (O'Neill, R., et al., 2004). Glassy carbon (GC) electrodes are used very commonly in biosensors because of their low cost and sufficient mechanical and electrical properties (De Benedetto, G.E., et al., 1996).

2.4.5.2 Carbon nanotubes

Carbon nanotubes (CNTs) are single-walled or multi-walled hollow structures composed entirely of carbon. They have many interesting properties, including an extremely high elastic modulus, good surface area to volume ratio, a hollow core that can store other molecules, simple fabrication, and metallic and semiconducting electron transport. CNTs can also be functionalized for further biocompatibility or other properties. It has been found that CNTs enhance the electrochemical activity of biomolecules and improve the electron-transfer reaction in proteins (Wang, 2005, Balasubramanian and Burghard, 2006). These properties have led to an interest in using CNTs for a variety of novel technologies, including electrochemical actuators, batteries, tips for scanning probe microscopy, and sensors (Sotiropoulou and Chaniotakis, 2003). CNTs have been used in biosensors to facilitate the transfer of electrons between the enzyme redox site and the surface of the electrode. The addition of CNTs can increase the sensitivity of the biosensor. In particular, CNTs are particularly attractive for dehydrogenase and oxidase based biosensors (Wang, 2005). The CNTs can form part of the electrode itself, form a direct attachment with the enzyme, or be incorporated as part of an electroconductive polymer film such as polyaniline or polypyrrole (Wang, 2005, Balasubramanian and Burghard, 2006). CNT based biosensors have been used for a variety of analytes, including glucose, dopamine, and glycerol. (Balasubramanian and Burghard, 2006).

2.4.6 Enzyme Entrapment on Biosensor

There are a variety of different ways that enzymes can be immobilized on the surface of a biosensor. Each method has different advantages and disadvantages. The main methods of enzyme immobilization are described below.

2.4.6.1 Adsorption

Physical adsorption is when the enzyme is attached directly to the electrode. The electrode is brought into contact with the enzyme dissolved in solution, which forms weak bonds between the enzyme and the electrode surface. Excess enzyme is then washed off. Although this method is simple

and does not denature the proteins, the weak bonds make it easy for the enzyme to detach from the electrode, decreasing the stability (Sassolas et al., 2012).

2.4.6.2 Entrapment

In the entrapment method, enzymes are trapped within a polymer or gel scaffold and deposited onto the electrode surface. Immobilizing enzymes within the polymer or gel matrix is simple and multiple enzymes, mediators and other additives can all be entrapped at once. The size of the pores in the scaffold has a significant effect on the sensitivity of the biosensor. If the pores are too small, the metabolite of interest cannot reach the enzyme and the biosensor will be unable to measure the metabolite level. If the pores are too large, it can decrease the selectivity of the biosensor, allowing multiples metabolites to reach the enzyme and electrode. A drawback of using this method is that the enzyme can diffuse out of the scaffold, rendering the biosensor useless (Sassolas et al., 2012).

2.4.6.3 Crosslinking

In the crosslinking method, enzymes are bonded with itself or inert molecules. This is a simple process and forms strong bonds between molecules. This method has been used in a variety of biosensors including detecting blood glucose levels (Pei et al., 2004) and cholesterol (Basu et al. 2007). The main drawback of using this form of enzyme immobilization is the loss of activity due to distortion or chemical alterations due to the bonds created in the crosslinking process (Sassolas et al., 2012). Glutaraldehyde is a cross linker that is commonly used to immobilize a variety of enzymes including lactate oxidase and glucose oxidase (Moser et al., 2002). For example, lactate oxidase can be immobilized via glutaraldehyde crosslinking onto a polypyrrole-coated electrode. One end of the glutaraldehyde molecule binds to the amine groups of the polypyrrole film. The other end of glutaraldehyde binds to amine groups of lysine and arginine residues within the lactate oxidase molecule.

2.5 Electroconductive Polymers

Electroconductive polymers are able to conduct an electric charge due to the presence of one unpaired electron per atom in the carbon backbone of the structure. This allows electrons to travel through the polymer with little resistance. An important application of electroconductive polymers is in biosensors. Electroconductive polymers can transfer electrical charge from the enzyme to the surface of the electrode. This increases the sensitivity and decreases the response time of the biosensor (Malhotra et al, 2006).

2.5.1 Polyaniline

Polyaniline (PANI) is an electroconductive polymer that is frequently used in biosensors due to its high conductivity. PANI can be fabricated using a variety of methods; however, electrochemical deposition is typically used for biosensor applications. In this method, a cyclic range of electric potentials are applied by an electrode to a solution containing the aniline monomer. The potential causes the aniline to polymerize and form a film on the surface of the electrode. The advantages of PANI over other polymers include a higher thermal stability and a relatively low cost (Thomas, 2012). In a study that examined the electrochemical properties of PANI/CNT, polypyrrole/CNT, and PEDOT/CNT composites, it was found that the PANI/CNT composite had the highest conductivity and largest electrode specific capacitance (Peng et al, 2007). It has also been suggested that unlike other electroconductive polymers, PANI can have a synergistic relationship with carbon nanotubes that improves electrical performance (Gajendran and Saraswathi, 2008).

2.5.2 Polypyrrole

Polypyrrole (PPY) is an electroconductive polymer that is biocompatible and easily manipulated. Like PANI, it can also be electropolymerized at low oxidation potentials to form a film on the surface of an electrode. It has small pores, which allow for the entrapment of enzymes or other molecules. These pores also increase specificity by preventing larger molecules from interfering with the biosensor. PPY is often used with CNT and has been shown to effectively wire the enzymes to the CNT and the electrode

surface. This technique has been used in biosensors to monitor many analytes, including glucose (Peng et al, 2007, Gajendran and Saraswathi, 2008, Balasubramanian and Burghard, 2006).

2.5.3 PANI/Polypyrrole Composites

In recent years, researchers have begun to examine the possibility of using PANI/PPY composites for the use in biosensors, in the hopes that they will work better together than either does separately. This composite can be created by mixing the two monomers together in aqueous solution and then electropolymerizing a film onto the surface of an electrode, or by polymerizing first one monomer and then the other. PANI/PPY composites were used successfully to create biosensors for organophosphates, uric acid, and hydrogen peroxide. In all cases, these composites allowed for the creation of a highly sensitive, stable, and reproducible biosensor (Arslan, 2008, Du et al., 2010, Cheng et al, 2007). When analyzed using scanning electron microscopy (SEM), Du et al. (2010) found that the composite formed a well-packed and homogenous layer with a porous nanostructure of an ideal size to entrap enzymes. Cheng et al. (2007) noted that the composite creates a nanostructure with a large surface area. Since PPY is limited by a relatively flat surface area, the addition of PANI corrects this disadvantage.

2.5.4 Chitosan

Chitosan is a non-conductive, natural polymer found in the exoskeleton shells of shrimp, crabs and other crustaceans. Chitosan is non-toxic, biocompatible and biodegradable and has excellent film forming properties (Sun, et al., 2009). Chitosan also has the potential for anti-microbial properties. Solutions and gel-forms made of chitosan are antimicrobial, where as thin-films were not effective in preventing the growth of *Escherichia coli, Staphylococcus aureus* or *Staphylococcus epidermidis* (Foster, et al. 2011). This non-conducting polymer has been used in biosensors as a way to immobilize enzymes. Chitosan embodies many properties that will enhance the function of the biosensor.

2.5.5 Nafion

Nafion is a synthetic co-polymer with stable thermal and mechanical properties. Nafion films have been widely used in biosensors because of its ion exchange and biocompatible properties. A Nafion-coated electrode allows for amperometric detection at low potentials. Nafion has antifouling properties and works especially well with carbon nanotubes. A drawback to using this polymer is that it does not eliminate interference from other molecules such as ascorbic acid (Wang et al., 2002).

2.5.6 Layer-By-Layer Polymer Deposition

There are a variety of different techniques that can be used to deposit polymer films onto the surface of the electrode. The layer-by-layer (LBL) polymer deposition method includes fabricating layers of alternatively charged polymers on the electrode surface. The surface of the electrode is modified to have either a positive or negative charge and then polymer layers are deposited one after another, alternating charges. To deposit a layer of polymer, the electrode is dipped into polymer solution for a set time period and then washed three times. The process is then repeated for each layer. The charge of a polymer is dependent on its isoelectric point. At a pH above the polymer's isoelectric point, the polymer will be negatively charged whereas at a pH below the polymer's isoelectric point, the polymer will be positively charged (Amy Peterson, personal communication).

2.6 Conclusion

A biosensor to monitor tumor size in GBM patients would allow medical personnel to alter a patient's treatment regimen quickly in response to treatment. It will also allow for monitoring of the patient after remission, in order to ensure that any recurrence is found before the cancer becomes too widespread. A successful project will create a biosensor that can effectively monitor tumor size and aid in extending the lives of patients with GBM. In addition, because increased concentrations of lactate are produced by all types of tumor cells, this technology could be used to monitor the progression of other types of cancer.

3. Project Strategy

To begin the design process for this biosensor, a client statement was created in order to focus the design to meet the desired functions of the biosensor. From this statement, a list of design objectives was created as a way to evaluate our design alternatives based on how well the design alternatives met each objective. Certain constraints were applied to the project, such as a budget of $468 and eight months to complete the project.

3.1 Client Statement

At the beginning of the design process the team was given the initial client statement of:

Create a biosensor that will last long-term to detect multiple metabolites. This biosensor will monitor the progression of Glioblastoma Multiforme to determine the effectiveness of treatment on tumor size.

As the project progressed, the team revised and refined the client statement to be:

Create a biosensor that will last for at least two week, at physiological conditions, to detect concentrations of lactate in patients with Glioblastoma Multiforme. This biosensor will be able to correlate metabolite concentrations with tumor size in order to monitor the progression of GBM. The long-term clinical application of this device is to be used in vivo to monitor the effectiveness of treatment on the tumor.

This client statement was the basis of the design process and guided the team in designing a biosensor to monitor GBM.

3.2 Objectives

The objectives for this project were derived from the client statement and through extensive background research. A list of the objectives, ranked in order of importance, is shown below:

1) Specific

2) Accurate
3) Sensitive
4) Reproducible
5) Manufacturability
6) Biocompatible
7) Long-lasting

The first and foremost objective was that the biosensor must be *specific* and detect a signal only from the metabolites of interest. Interference from other molecules, such as ascorbic acid and acetaminophen (Yoo and Li, 2010), would cause the current produced by the biosensor to be disproportional to the actual metabolite level. Minimizing the noise and interference from other metabolites would ensure that the biosensor reflects the actual metabolite level. The biosensor must also be *accurate* at detecting the concentrations of lactate and translating it into a corresponding current output. This is important because clinicians must be able to use the biosensor as a tool to judge the efficacy of therapies on the tumor. *Sensitivity* is also important in biosensor design. In order to be effective, the biosensor must be sensitive enough to detect physiological levels of the metabolites of interest, which range from 0.79 mM in healthy patients to 0.97mM in patients with GBM (Holroyde, 1979). The biosensor was also designed to produce *reproducible* results so that it can be used multiple times with the same performance outcomes. The electrical current produced from each concentration of lactate should be constant with multiple tests of the biosensor. We would expect the biosensor to have reproducible results over time as well as reproducible results between multiple biosensors with the same fabrication method. The device needs to be *easy to manufacture* so that it can actually be implemented in laboratory and clinical settings. This means that the device must be relatively simple to fabricate. The cost of manufacturing prototypes and the final designs should also remain relatively low so that this project can stay on budget and so that the final cost for patients is reasonable. The biosensor should be *biocompatible* and minimize the foreign body response by the immune system. When the human body detects a foreign object such as a biosensor, the immune system reacts by

forming a capsule of fibrous tissue around the object. It is vital that the biosensor minimize this response to avoid encapsulation. Encapsulation could limit the availability of lactate to the biosensor and decrease the ability of the biosensor to effectively monitor metabolite concentrations for an extended period of time. The biosensor should be *long lasting* once implanted into the body. An ideal biosensor would last for several months to years inside the body. Implanting a biosensor is a somewhat invasive procedure and minimizing the need for this procedure would benefit not only the patient, but also keep the costs of patient monitoring down. In order for the biosensor can remain functional, it should function well at physiological conditions, resist degradation, and maintain enzyme activity.

3.3 Constraints

The design of the lactate biosensor was limited by some constraints. The first major constraint was that the biosensor could not be cytotoxic. This forced us to only select materials that have been proven to be biocompatible. The biosensor design was also constrained by the equipment available. The biosensor had to be easily used with the AUTOLAB potentiostat found in Dr. Susan Zhou's lab. The costs for the materials for all prototype designs were limited to a budget of $156 per person, for a total of $468. The time to complete the design and test prototypes was limited to the 2013-2014 academic year.

3.4 Project Approach

This project is based upon a MQP project completed in the 2012-2013 academic year, which designed and tested a biosensor to monitor lactate levels. The approach for this project was to redesign the existing lactate biosensor. The goal of this project was to create a biosensor that had improved sensitivity, reproducibility, and response time when compared to the previous design. In order to do this, the literature was reviewed to examine which materials would be advantageous for use in this project. In an iterative testing process, each component of the design was tested for effectiveness. Scanning electron microscopy was used to evaluate the uniformity of the films for two of the design

alternatives. The final design was validated by creating a standard curve of current output as a function of lactate concentration, and then using that curve to estimate the concentration of lactate in media that had been cultured with U87mg cells. Finally, the biosensor's response to changing lactate levels over time was tested.

There were some technical challenges that had to be overcome over the duration of this project. The first challenge was the group's inexperience in the field of electrochemistry. The group had to learn and perfect the approach and techniques required to successfully complete electrochemical experiments. Equipment availability due to other researchers and maintenance contributed to some delays. The major technical issue the team faced was electrochemical equipment errors. During testing the equipment was outputting noisy and inconsistent data. When a clean glassy carbon electrode was tested in PBS, the three trials conducted were very inconsistent. As a result of the issue, the group traveled to the University of Connecticut to perform testing. The ability to complete experiments was hindered by travel time and the availability of the researcher the team worked with. These technical issues prevented the team from progressing as far as the team had hoped.

4. Design

4.1 Need Analysis

Current methods for the treatment of Glioblastoma Multiforme such as MRI and CT scans are expensive, inconvenient, and do not detect small or diffuse tumors. This creates a need for an effective method to monitor GBM that can be used frequently and long-term.

In order to create an effective biosensor to monitor lactate in GBM patients, many different design alternatives were created and evaluated. In order to evaluate these design alternatives, the functions and specifications were analyzed to determine the most effective design. The biosensor must be able to determine the level of lactate in order to be able to accurately monitor the progression of GBM.

In order for the biosensor to be considered effective, it will need to satisfy the needs of the client, which are as follows:

- **Accurate:** The biosensor should be able to produce accurate results for the analysis of metabolite concentration.
- **Specific:** The device must be able to recognize lactate concentrations without being affected by any other metabolite levels in the tissue.
- **Reproducible:** The biosensor should be able to be reproduced and the same results should be able to be replicated.
- **Sensitive:** The biosensor readings should be able to adjust with increasing or decreasing levels of lactate within the tissue.
- **Biocompatible:** The system needs to be able to be implanted into the body in future studies and not induce a foreign body response.

In addition to the design needs given by the client, desired properties must be considered, which are:

- **Long-lasting:** The biosensor should be able to work in the body for weeks to months.
- **Implantable:** The system should be able to be implanted into the body for future *in vivo* studies with minimally invasive techniques.

- **Quick response time:** The biosensor should be able to detect the metabolite concentration within seconds of a change in lactate concentration.
- **Easy to manufacture:** Eventually, this design should be easy to manufacture in order to produce the device in large volume.

Through understanding and prioritizing the needs and wants of the client we can rank which objectives should be satisfied first and the objectives that we can attempt to satisfy in future designs.

4.2 Functions

The overall function of the biosensor is to frequently measure lactate concentration in order to monitor the progression of GBM. Sub-functions are necessary for our biosensor in order to satisfy the main function of our design. Using a black box diagram, the sub-functions necessary for our biosensor to be considered effective are shown. The black box diagram shown in Figure 4 displays the initial input of unknown metabolite concentration and the final output as the current progression status of the GBM cells that are being monitored. The black box contains sub-functions which are needed to convert the input into the final output.

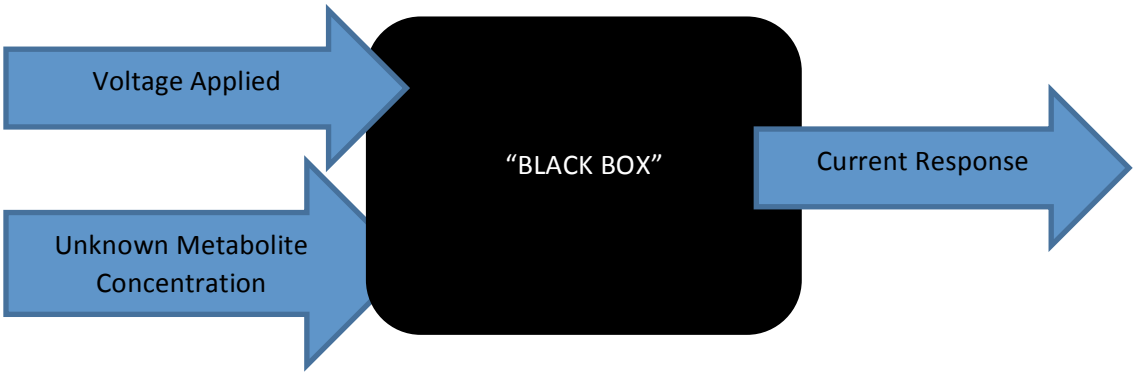


Figure 4: Black Box of Biosensor Functions

A black box diagram displaying all functions and sub-functions necessary for our device to be effective

As shown previously, the black box diagram has two steps. The sub-functions of the biosensor take in the voltage applied and the unknown metabolite concentration and convert this to an electrical signal output that is then sent to a processor.

4.3 Specifications

Specifications that the design must meet are discussed below.

4.3.1 Metabolites

The biosensor needs to be able to measure the concentration of lactate in order to effectively monitor the progression of GBM. GBM tumor cells release excess lactate that enters into the blood stream and becomes systemic. The amount of lactate released is proportional to GBM tumor size, which makes the analysis of lactate a suitable way to determine the progression of GBM. The concentration of lactate in a healthy adult is approximately 0.79 mM and the concentration of lactate is 0.97 mM in a patient with cancer (Holroyde, 1979).

4.3.2 Enzymes

In order to enhance biosensor function, the enzyme lactate oxidase (LOX) will be used. Lactate oxidase is used to catalyze the breakdown of lactate into hydrogen peroxide and then free electrons, as shown in the equation below, which reduces response time.

$$Lactate + O_2 \xrightarrow{LOx} Pyruvate + H_2O_2$$

$$H_2O_2 \xrightarrow{Voltage} 2H^+ + O_2 + 2e^-$$

Using lactate oxidase increases the specificity of the biosensor. Other analytes such as glucose and glutamate follow the same breakdown pathway; however lactate oxidase only catalyzes the breakdown of lactate.

4.3.3 Electroconductive Polymers

Conductive polymers can enhance biosensor function by increasing selectivity and decreasing response time. The pore size of a polymer film determines what size molecules are able to pass through and reach the electrode surface. Adding a polymer film to the electrode decreases interference by filtering out any molecules that are too large to pass through its pores. Conductive polymers, such as polypyrrole, polyaniline and Nafion, allow electrons to easily pass through and reach the electrode

surface, decreasing biosensor response time. Lactate oxidase was crosslinked with glutaraldehyde to the polymer films, because preliminary testing revealed that the lactate oxidase leached out of the biosensor if it was not crosslinked.

4.3.4 Sensitivity and Range

The sensitivity and range of the biosensor is based on physiological lactate levels found in healthy adults and GBM patients. The biosensor must detect the concentration range of 0.79mM lactate found in healthy patients to 0.97mM lactate which is found in patients with GBM. An ideal range for this biosensor would be between 0-10 mM. The biosensor also needs to be sensitive enough to determine the minute changes in the concentration of lactate over time.

4.3.5 Dimensions

The glassy carbon electrodes provided by the client have a diameter of 3mm. The area of contact between the electrode and the film determines the amount of polymer and enzyme used in creating the film.

4.3.6 Stability

Enzymatic biosensors are generally stable for approximately 21 days, based on a literature review, but the length of stability of the biosensor is highly dependent on the storage temperature. For our biosensor, we hope to achieve stability of the biosensor for 21 days. Ideally, this biosensor will be able to function on the scale of months to years, to minimize the number of implantations of the device.

4.4 Design Alternatives

Design alternatives were created involving different combinations of conductive polymers, along with the enzyme and a crosslinking agent. Preliminary testing was performed on the design alternatives in order to determine the design components that were the most efficient in detecting the changes in the concentration of lactate.

4.4.1 Single Electroconductive Polymer

The team used both polypyrrole and polyaniline as the chosen polymers for the biosensor film. For a film layer of polypyrrole, the team used a clean glassy carbon electrode and polymerized pyrrole onto the surface of the electrode. To create the layer of polyaniline, the team used a clean glassy carbon electrode and polymerized aniline onto the surface. On both of these designs, as seen in Figure 5, the polymer was crosslinked, using glutaraldehyde, with the enzyme.

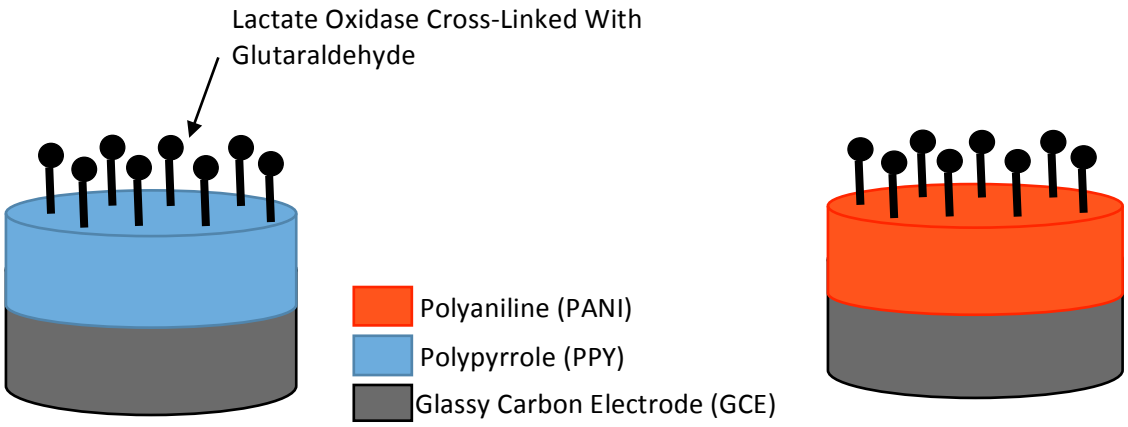


Figure 5: Single Layer Polymer Deposition
Single layer of PPY or PANI with the lactate oxidase crosslinked with glutaraldehyde

4.4.2 Layered Electroconductive Polymers

Electroconductive polymers were layered one on top of the other. For the first design, pyrrole was polymerized onto the surface of the clean electrode. The same electrode was then used to polymerize aniline onto the surface for a film consisting of a polypyrrole and polyaniline layered film coating. The top coating, polyaniline, was then crosslinked, using glutaraldehyde, with the enzyme. The second design used the same logic, but polymerized aniline onto the clean surface of the electrode before then polymerizing pyrrole onto the surface. The top coating of polypyrrole was then crosslinked, using glutaraldehyde, with the enzyme as seen in Figure 6.

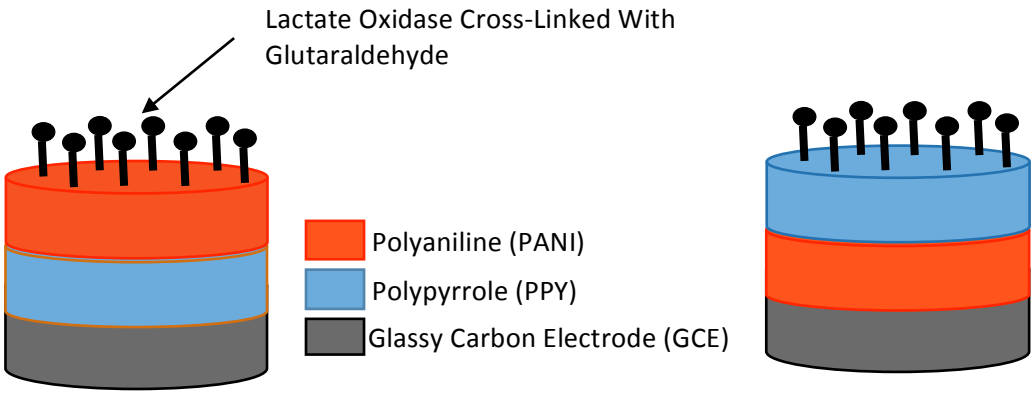


Figure 6: Dual Layer-by-Layer Polymer Deposition
Layered PPY and PANI with the lactate oxidase crosslinked with glutaraldehyde

4.4.3 Co-Polymer Blend

Based on the literature, both polypyrrole and polyaniline showed an increased current response because of their conductive properties. Blending the two polymers together may have an increased current response; therefore this design was considered to be an alternative as well, as seen in Figure 7. The pyrrole and aniline were polymerized onto the electrode surface as a co-polymer blend. The co-polymer was then crosslinked with the enzyme using glutaraldehyde.

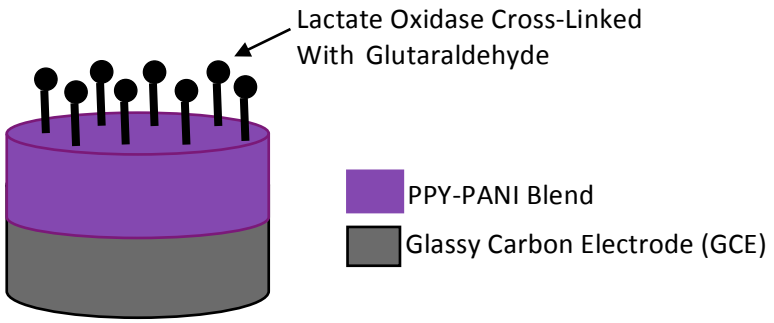


Figure 7: Co-polymer Blend Deposition
A co-polymer blend of PPY and PANI with the lactate oxidase crosslinked with glutaraldehyde

4.5 Design Alternative Evaluation

To evaluate the design alternatives, the team performed preliminary testing on the designs to see which design had the greatest sensitivity to the different lactate concentrations. This preliminary testing consisted of amperometry and cyclic voltammetry tests with multiple known concentrations of

lactate. After testing, the final design was chosen due to the highest current output in the amperometry and cyclic voltammetry for a given lactate concentration.

4.6 Final Design

The final design that was chosen was the PPY-PANI-LOX on the glassy carbon electrode. This design, which layered the polymers by polymerizing the pyrrole onto the electrode followed by the addition of polyaniline with lactate oxidase crosslinked to surface using glutaraldehyde, proved to be the most responsive in the preliminary testing. After the preliminary testing, the final design, PPY-PANI-LOX was evaluated by testing with various cell counts of GBM cells in media and through the addition of lactate over time to determine the response time of the biosensor.

5. Methods

An iterative process was used to add and test components of our lactate biosensor. The glassy carbon electrode, polypyrrole deposition, polyaniline deposition, and addition of enzymes were all tested separately to determine the effects that each new material had on overall biosensor functionality. This section describes the fabrication of the different biosensor components, cell culture, testing and validation procedures, and methods of data analysis.

5.1 Fabrication of Biosensor

Glassy carbon electrodes (GCE) with a diameter of 3mm were polished in sequential steps of 1 μm and 0.3 μm alumina slurry, followed by sonication for 15 minutes in deionized water. For all electrochemical experiments, the glassy carbon electrode (working electrode) was used with an Ag/AgCl reference electrode and a platinum counter electrode. An AUTOLAB potentiostat (Metrohm) and GPES software were used to control the electrical current and voltage running through the working electrode and to record data. The same experimental set-up, seen in Figure 8, was used for all experiments. After the working electrode became coated with the PPY, PANI, LOX, and glutaraldehyde, it was referred to as the biosensor.

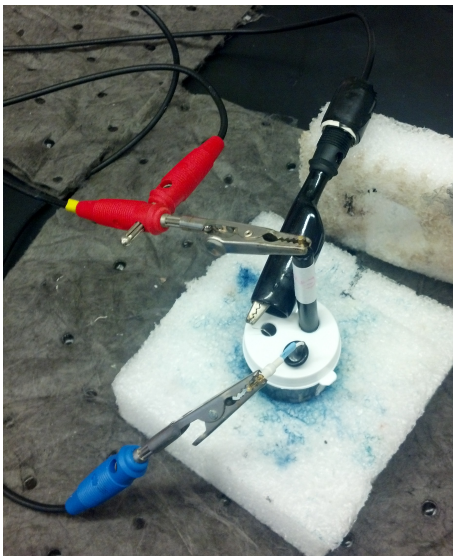


Figure 8: Biosensor Set-up

Set-up of biosensor/working electrode (red), platinum counter electrode (black), Ag/AgCl reference electrode (blue).

To create the polypyrrole film on the surface of the GCE, 7 μL (0.01M) of pyrrole (Sigma-Aldrich) was added to 10 mL of 0.01 M phosphate buffered saline (PBS) that had previously been degassed by nitrogen bubbling for 15 minutes (Li et al., 2005). The pyrrole was polymerized to form polypyrrole on the surface of the GCE by using galvanostatic deposition. Polymerization occurred at a current of 25 mA/cm^2 for 120 seconds.

After pyrrole polymerization on the electrode surface, a polyaniline (PANI) film was created on top of the pyrrole film by polymerizing 45 μL of aniline monomer in degassed 0.1M PBS of pH 3.5 (Gaikwad et al., 2006). Nine cycles of cyclic voltammetry between 0.00 and 0.95 V were used to polymerize the aniline.

5 μL of 0.1% glutaraldehyde (Sigma-Aldrich) was placed on top of the layered polymers and left to dry at room temperature for 30 minutes. 5 μL of lactate oxidase enzyme(LOX) (2.9 mg/mL, Sigma-Aldrich) was added on top of the pyrrole-glutaraldehyde electrode. The glutaraldehyde served to crosslink the pyrrole to the enzyme.

5.2 Cell Culture

Human glioblastoma cells (U87mg), harvested from a 47 year old male patient and stabilized for cell culture, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin, 1% Non-Essential Amino Acids for 48 hours.

5.3 Testing Procedures

The individual components of our biosensors (GCE, PANI, PPY, LOX) were added in an iterative process to ensure that each new design component improved the functionality of the biosensors. Therefore, we tested the effects of different concentrations of lactate (0.05 mM, 0.1 mM, 1 mM, 10 mM, 25 mM) on the plain GCE, GCE + PPY, GCE + PANI, GCE+PPY+LOX, GCE+PANI+LOX, GCE+PANI+PPY, GCE+PPY+PANI, and GCE+PANI+PPY+LOX. To measure the effects of lactate on these biosensor design iterations, we used the electrode setup, AUTOLAB, and GPES software as described in Section 5.1.

For each test, degassed PBS containing 0.05mM, 0.1mM, 1mM, 10mM, or 25mM of lactate was placed into the electrochemical cell. A new solution was used for each test. To measure the maximum current output of the biosensors in response to lactate, we used the Autolab to perform amperometry, in which the voltage is held constant at 0.2 V and the current changes as a function of analyte concentration. We also used cyclic voltammetry (cycling between -0.4 and +0.6V, 10 cycles) to gauge overall biosensor performance as a function of analyte concentration. The biosensors' response to increasing lactate concentrations over time was performed by adding different volumes of 25 mM lithium lactate solution in PBS over time (see Table 2).

5.4 Validation of Biosensor

Lactate assays (Sigma-Aldrich) were used to determine the concentration of lactate present in the cell media. This was compared to the concentration calculated based on the biosensor reading in order to determine biosensor accuracy.

The three dimensional structure of the PPY and PPY-PANI films were examined using scanning electron microscopy.

5.5 Data Analysis

Three separate trials were completed for all experiments. All graphs shown represent the average of the absolute values for the three trials for each condition. Standard curves were created in excel by fitting a linear regression to collected data points. The standard deviations for comparative ELISA and amperometry results were also calculated using excel.

6. Results

In order to tailor our biosensor to be sensitive to the *in vitro* concentrations of lactate typically produced by glioblastoma cells, U87mg human glioblastoma cells were cultured for 48 hours. The media was then collected. An ELISA measured the different lactate concentrations produced by different numbers of U87mg cells seeded, as seen in Table 1. These concentrations were calculated by using an equation derived from a standard curve, seen in Appendix 2.

Table 1: Concentration of Lactate Produced by Glioblastoma Cells

The concentration of lactate produced by different numbers of glioblastoma cells after 48 hours of culture.

Cell Count (seeded)	Lactate Concentration (mM)
1,000,000	3.29
100,000	2.70
10,000	2.43
Media (control)	2.11

Multiple design iterations were successfully fabricated and tested. In order to make a PPY film with a strong current response, we tested four different electropolymerization methods. Based on the results, seen in Appendix 3, it was decided that polymerizing a solution of 0.01M pyrrole by holding the current constant at 25mA/cm^2 for 120 seconds would produce a PPY film with the highest current output in response to lactate. Li et al. (2005) had stated that this methodology would lead to a PPY film thickness of 100 nm. The methodology for creating the PANI film was based on the protocol from Viswanathan et al. 2009, in which the voltage applied to the working electrode was cycled between +0.0 and +0.95 V for multiple cycles. Robberg et al. (1998) state that the thickness of the PANI film formed is dependent on the total charge/area, where 1 Coulomb/cm^2 will result in a film thickness of 50 μm. Using this equation and the oxidation and reduction charges for each cycle given by GPES, we calculated that

for nine cycles, the thickness of PANI was calculated to be between 273 nm – 2.2 µm. Unfortunately, due to human error or equipment malfunction, the calculated film thickness was different each time that the biosensor was fabricated.

To characterize the polymer films, scanning electron microscopy (SEM) was performed. The images of the PPY and PPY-PANI films, shown below in Figure 9, show that the PPY film is uniformly composed of many small bumps, with pores present that are capable of holding LOX. Our results match SEM images in the literature. The PPY-PANI film had a very thin smooth layer on top, with more bumpy topography underneath this thin layer. The thickness of both films was found to be greater than expected, with a thickness between 10-40 µm (data not shown) that varied in different regions of each film. More SEM images can be seen in Appendix 4.

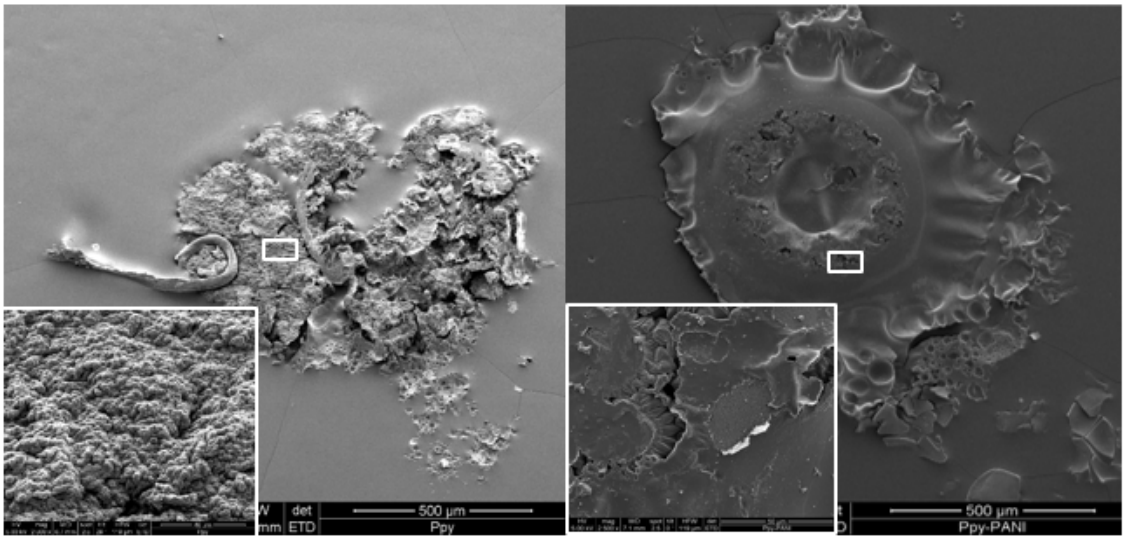


Figure 9: SEM Images of Polymer Coatings
SEM images of PPY (left) and PPY-PANI films (right). Magnification=2500x.

To select a final design from our design alternatives, different polymer films were tested with 1 mM lactate to test their current output. It was found that the PPY-PANI film (PPY on bottom, PANI on

top) had the highest current output, as seen in Figures 10 and 11. Believing that this would result in a more sensitive and responsive biosensor, we chose to do all further tests with a PPY-PANI film.

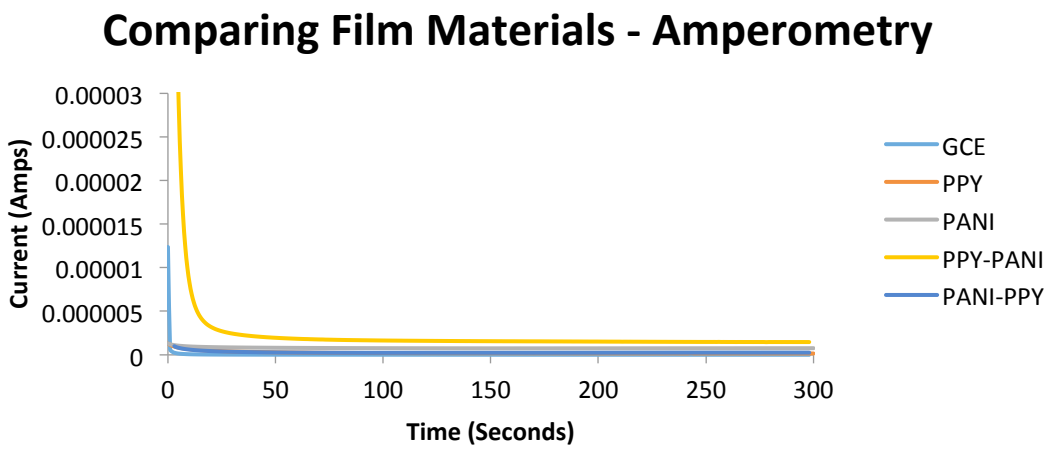


Figure 10: Amperometry Results

Amperometry curves produced from testing the GCE, PPY film, PANI film, PPY-PANI film, and PANI-PPY film in 1 mM lactate.

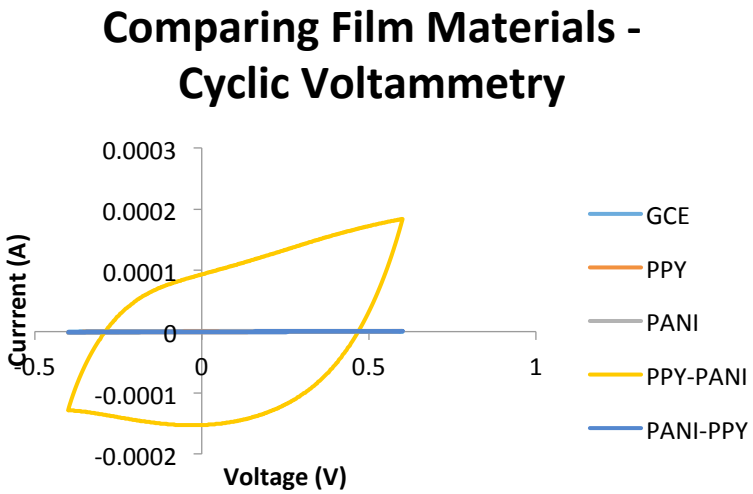


Figure 11: Cyclic Voltammetry Results

Cyclic Voltammetry curves produced from testing the GCE, PPY film, PANI film, PPY-PANI film, and PANI-PPY film in 1 mM lactate as the voltage was cycled from -0.4V to +0.6V.

Another step in optimizing the biosensor was to attach the enzyme, lactate oxidase (LOX), to the biosensor. Early results showed that when the lactate oxidase was simply left to dry and adsorb onto a polymer film, the current response of the biosensor to lactate decreased dramatically after every test.

This was most likely due to the LOX leaching off of the biosensor into the lactate solution. Because the biosensor needs to work repeatedly for a long period of time, we chose to crosslink the lactate oxidase to the polymer film with glutaraldehyde. Glutaraldehyde has the ability to bind to two amine groups, which allows it to crosslink the LOX to PPY and PANI, both of which contain amine groups. Not only did crosslinking significantly prevent the decrease in current over time, but it also improved the current response as a whole, because more of the LOX stayed on the biosensor after the initial rinse step. Figures 12 and 13 show the difference that crosslinking made on a PPY-LOX iteration of the biosensor.

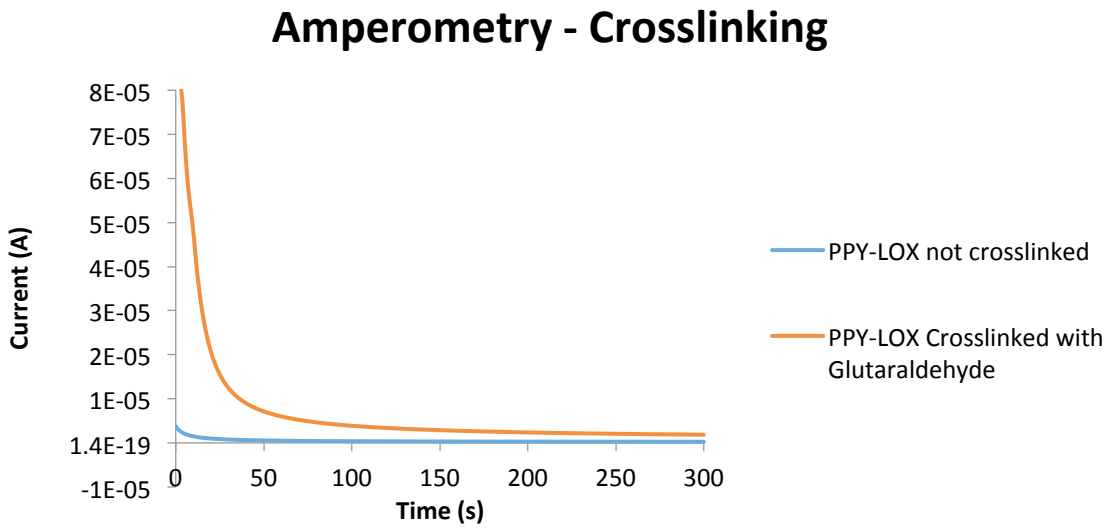


Figure 12: Amperometry Curves Comparing Addition of Glutaraldehyde
Amperometry curves for PPY-LOX with and without glutaraldehyde crosslinking in 1 mM lactate.

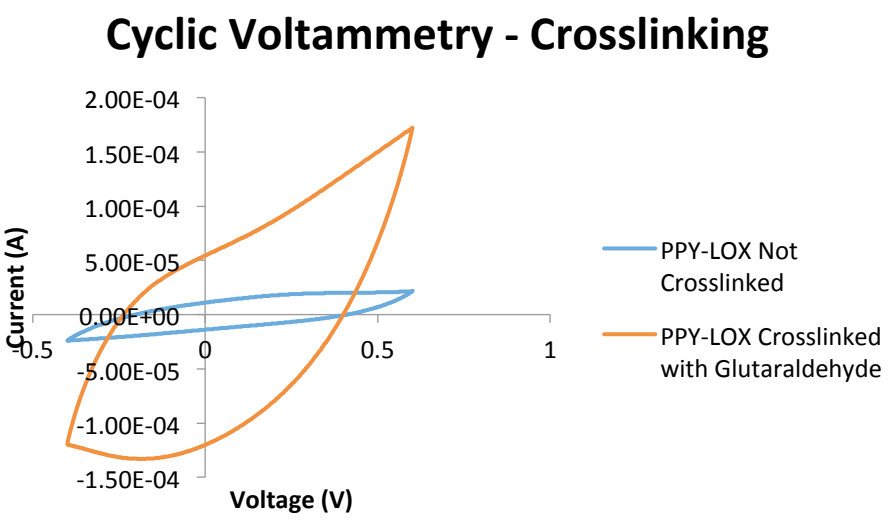


Figure 13: Cyclic Voltammetry Curves Comparing Addition of Glutaraldehyde
Cyclic Voltammetry curves for PPY-LOX with and without glutaraldehyde crosslinking in 1 mM lactate.

To test the current response of the final biosensor design to different lactate concentrations present in PBS, we performed amperometry for 3 trials of 300 seconds for each of the following lactate concentrations: 0.05 mM, 0.1 mM, 1 mM, 5 mM, 10 mM, 17.5 mM, 25 mM. The results of these tests can be seen in Figure 14. The steady-state current values (taken at t=300s) for each lactate concentration were plotted to create a standard curve of current vs concentration, shown in Figure 15. A linear trendline was fitted to this data, although the R^2 value was only 0.78. The trendline shows that with increasing lactate concentration, there is a decrease in biosensor current. This is different than expected. As more lactate would be broken down by the biosensor, we would expect that more electrons would be produced, resulting in a greater magnitude of current flowing through the biosensor.

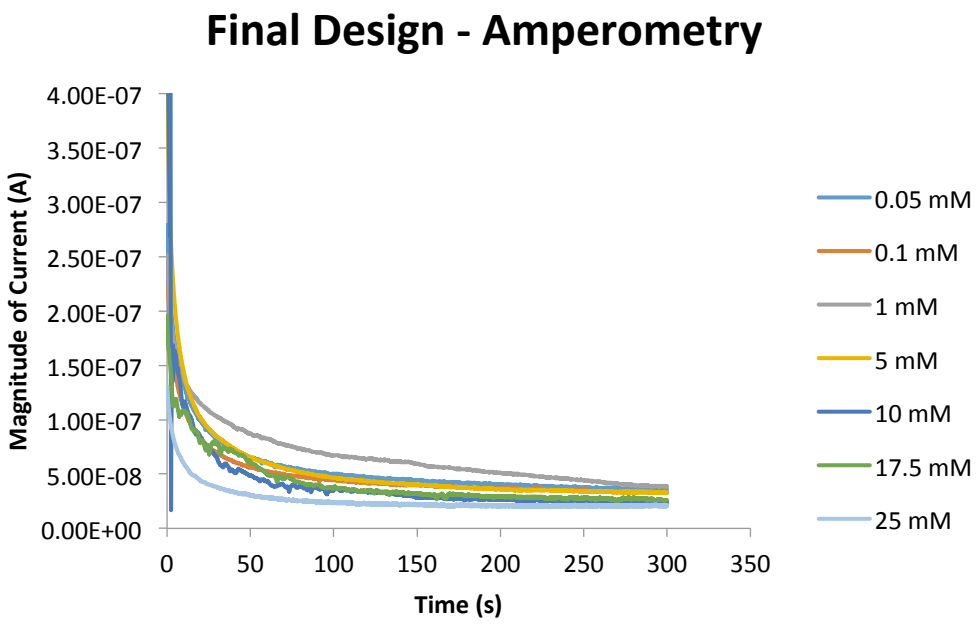


Figure 14: Amperometry Results for PPY-PANI-LOX Crosslinked

Amperometry curves for the final design (PPY-PANI-LOX crosslinked) for 0.05 mM, 0.1 mM, 1 mM, 10 mM, 17.5 mM, 25 mM of lactate.

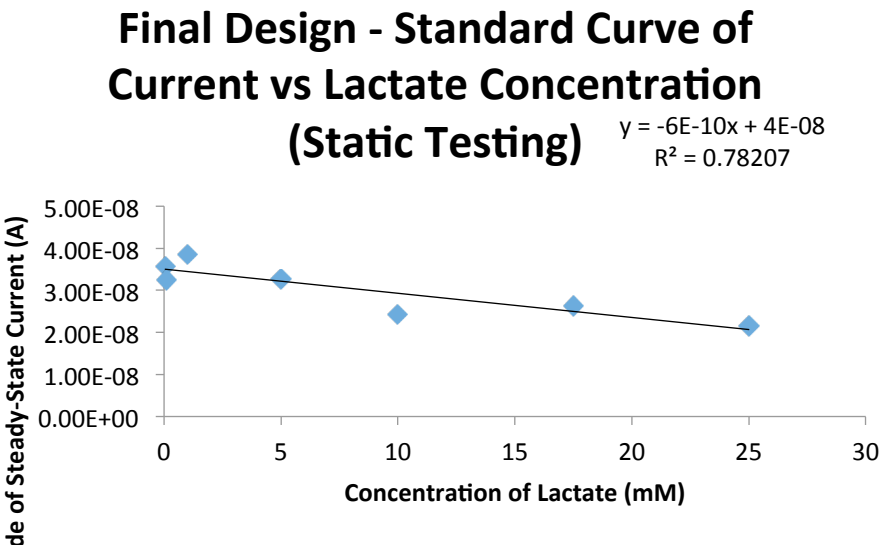


Figure 15: Steady-State Current for Lactate Concentrations

The standard curve of the steady-state current for several different lactate concentrations tested with the final biosensor design.

To test the efficacy of the biosensor in dynamic conditions, as it will be used *in vivo,* different volumes of a 25 mM lactate solution were added every 100s to a solution that initially consisted only of PBS as amperometry was performed. Table 2 shows the addition of lactate over time and the resultant lactate concentration. The data for the dynamic testing is shown in Figure 16 reveals that the biosensor has a fast response time, with changes in current happening immediately after the addition of lactate. A standard curve of steady-state current as a function of lactate concentration was created for this data set, as seen in Figure 17. As was seen with the static testing of various lactate concentrations, the magnitude of the current decreased as a function of increased lactate concentration. Again, this is not what was expected, and further testing should be done to explain why this current trend is observed.

Table 2: Lactate Concentration Test over Time

The volume of 25 mM lactate solution added at each time point, with the resultant lactate concentration.

Interval	Volume of Lactate Added	Total Number of Moles	Total Volume (mL)	New Concentration (mM)
1	--	0	5	0
2	0.2 mL of 25 mM lactate	0.000005	5.2	0.96
3	0.4 mL of 25 mM lactate	0.000015	5.6	2.68
4	0.8 mL of 25 mM lactate	0.000035	6.4	5.47
5	0.8 mL of 25 mM lactate	0.000055	7.2	7.43
6	0.8 mL of 25 mM lactate	0.000075	8	9.38

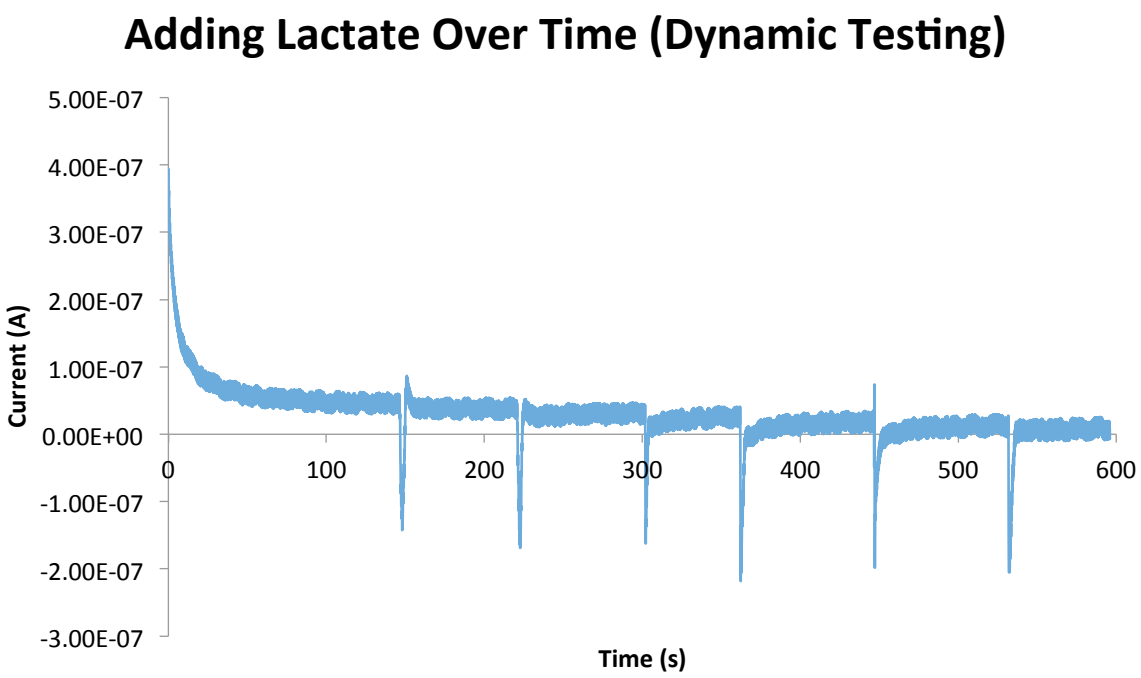


Figure 16: Current Response with Addition of Lactate over Time
The current response to an increase in lactate concentration over time.

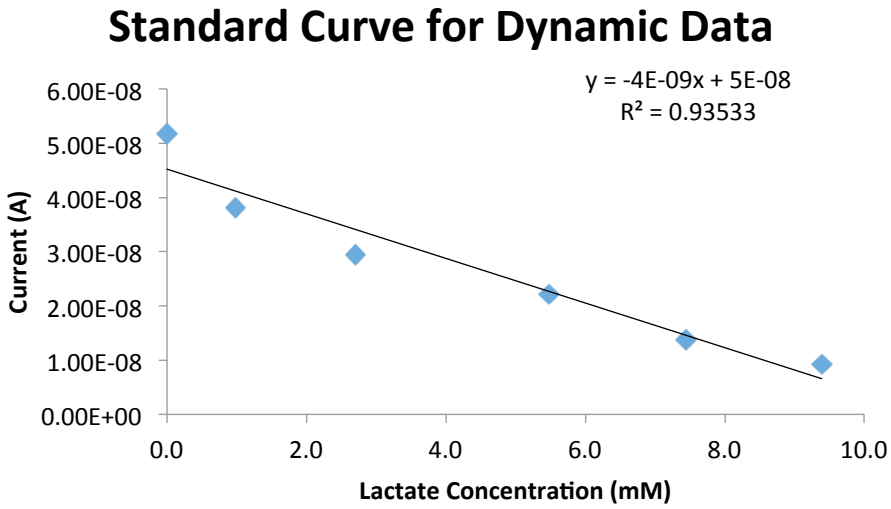


Figure 17: Standard Curve with Addition of Lactate over Time
The standard curve of steady-state current as a function of lactate concentration, tested in a dynamic system.

Media that was collected after culturing with U87mg human glioblastoma cells for 48 hours was tested with both the lactate ELISA and the biosensor in order to compare the biosensor's efficacy at determining lactate levels in media. It was found that the media with 500,000 cells seeded had the

highest lactate concentration at 1.2 mM. Although the plates containing 1 million cells was expected to have the highest lactate concentration, this can be explained by the general unhealthy appearance of the plates containing 1 million cells after 48 hours of culture. Redoing this experiment may lead to healthier cells, and therefore the media containing 1 million cells would have the highest lactate concentration. When amperometry tests were performed with the biosensor of the media (3 samples of media per cell number), it was found that similarly to the ELISA results, that there was a higher current for the media containing 500,000 cells. However, when we input the current values into the equation for the standard curve shown in Figure 17, negative values for lactate concentration were calculated. This is obviously unreasonable. This can be explained by the fact that the magnitudes of the currents for the biosensor tested in media were much higher than those tested in PBS. This would indicate that there are some non-specific interactions with other molecules in the cell media that are causing more electrons to be created and flow through the biosensor.

Table 3: Lactate Concentrations of Glioblastoma Cells

The average current read by the biosensor, the average lactate concentration calculated using the standard curve, and the actual lactate concentration measured with an ELISA for media samples containing 1,000,000, 500,000, or 100,000 cells.

Cell Number	Average Lactate Concentration (ELISA) (mM)	Average Current (A)	Average Lactate Concentration Calculated (mM)
1,000,000	0.93 ± 0.25	2.36E-07 ± 5.5E-8	-46.5
500,000	1.24 ± 0.07	2.70E-07 ± 4.4E-8	-55.1
100,000	0.31± 0.008	1.35E-07± 3.4E-8	-21.2

The performance of the biosensor design developed by this MQP was compared to the performance of a PPY-LOX (not crosslinked) biosensor, which the previous MQP designed, and a Chitosan-LOX biosensor, which uses a non-conductive polymer. Both amperometry (Figure 18) and cyclic voltammetry (Figure 19) tests were performed in order to compare the different biosensors. It was found that the PPY-PANI-LOX (crosslinked) design developed by this MQP had a higher current response than the other biosensor designs. The improvement of the current biosensor over that of the previous MQP suggests that the addition of the PANI and the crosslinking of the enzyme to the polymers were beneficial to biosensor performance. The improvement of the current biosensor over the Chitosan-LOX design shows the importance of conductive polymers in our design.

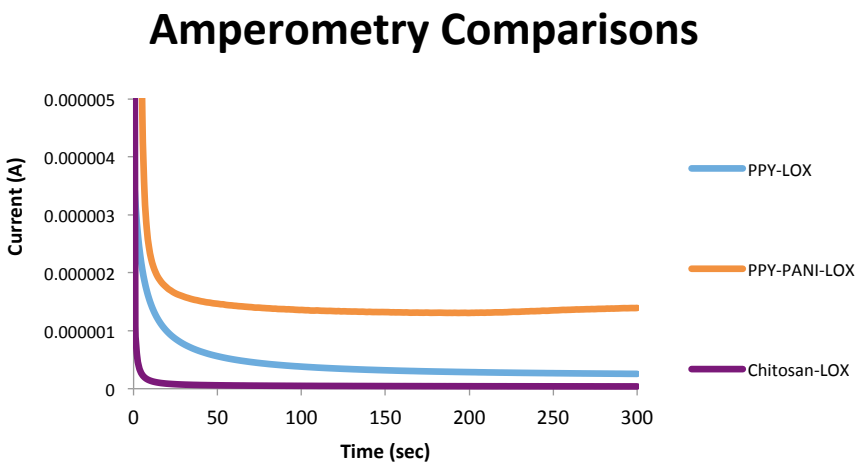


Figure 18: Amperometry Comparisons of Different Biosensor Designs

Amperometry curves for the PPY-LOX (previous MQP's design), PPY-PANI-LOX, and Chitosan-LOX designed biosensors in 1 mM lactate.

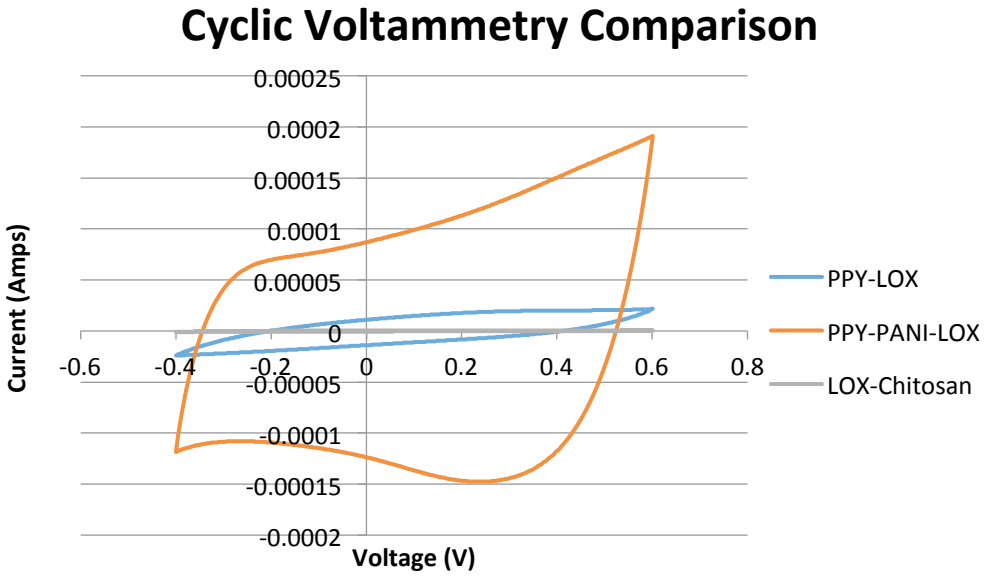


Figure 19: Cyclic Voltammetry Comparisons of Different Biosensor Designs

Cyclic voltammetry curves for the PPY-LOX (previous MQP's design), PPY-PANI-LOX, and Chitosan-LOX designed biosensors in 1 mM lactate.

7. Analysis and Discussion

In order to determine what concentration of lactate the biosensor must be able to measure, the amount of lactate produced by human glioblastoma cells was measured using a lactate colorimetric assay (Sigma-Aldrich). GBM tumors will have cell numbers in the millions, so the biosensor must be able to detect lactate levels on the millimolar scale. The biosensor was tested at lactate concentrations ranging from 0.05 mM to 25 mM but was focused in the 1 to 10 mM range, which is an appropriate range to monitor tumor size in GBM patients.

In order to optimize the polymer films of the biosensor, a variety of fabrication methods were tested. For PPY films, the galvanostatic method produced a film with the highest current response and created a uniform film on the surface of the electrode. Because the current measured by the biosensor is so small, it was necessary to choose the design with the highest current response. In the fabrication of the PANI film, there was variation in film thickness. This could be due to equipment/software malfunction or human error. In order to accurately determine the thickness of the PANI layer, SEM was performed.

SEM images were taken of the PPY and PPY-PANI coatings. The PPY film looks similar to other published images of PPY coatings (Liu et al., 2009) and has pores of 0.5 – 5 μm, which can entrap lactate oxidase. The PPY-PANI film has a very thin, smooth layer. Through cracks in this layer, pores and other topographical features can be seen. The SEM images were used to determine film thickness and the films were much thicker than expected. The PPY film was expected to be 100 nm while the PPY-PANI was between 200 nm and 2 μm. The actual thickness of the films was 10-40 μm. The high film thickness can interfere with the ability of electrons to reach the electrode surface and decrease the current measured by the biosensor. In order to optimize the biosensor, the film thickness must be reduced.

Preliminary testing at 1 mM lactate was performed on a variety of design alternatives in order to determine which one had the highest response. Both PPY and PANI individually and PANI-PPY had a current response comparable to a plain GCE. The PPY-PANI film had a much higher current response than the other alternatives, which means that more electrons reached the GCE surface. This suggests that the film promoted electron transfer to the electrode surface rather than into the surrounding solution and will make a more sensitive biosensor with a shorter response time.

The enzyme lactate oxidase catalyzes the breakdown of lactate into hydrogen peroxide and eventually free electrons and shortens the biosensor response time. When lactate oxidase was dropped on the polymer film and allowed to dry, the current response quickly decreased in consecutive trials. This is due to excess lactate oxidase and the enzyme diffusing out of the polymer film. The biosensor was not rinsed sufficiently and excess lactate oxidase on the biosensor surface was reacting in the first trial but was not present in the subsequent trials, causing a decreased current response. Because the enzyme was not bound to the polymers, it was easily able to diffuse from the film, which decreases the biosensor current response in subsequent trials. In order to maintain a constant current response and solve these issues, the lactate oxidase was crosslinked to the polymer surface using glutaraldehyde and the number and length of rinses was increased to three, ten-minute rinses. Once these adjustments were made, the current response was constant in multiple consecutive trials.

The final design was tested at different lactate concentrations ranging from 0.05 to 25 mM lactate. The current response from these lactate concentrations were used to create a standard curve. The standard curve showed a slight trend of decreasing current with increasing lactate concentration. It is expected that the current would increase with increasing lactate concentration. This experiment was only performed once on properly calibrated equipment at the University of Connecticut, so repetition of this test is needed in order to verify this result.

The response of the biosensor was compared to the response of other similar biosensors. The final design, and another design based on PPY had much higher current responses than a similar design with a chitosan and lactate oxidase film. Chitosan is a nonconductive polymer, so electrons will meet more resistance and fewer electrons reached the electrode surface. The final design had a higher current response than the PPY-LOX film, designed and tested by a previous project team (Anglin et al., 2013).

The response of the biosensor was also tested over time in changing lactate concentrations. When more lactate was added to the system, a current spike occurs, meaning the biosensor is able to detect the addition of more lactate. After the initial spike when lactate is added, the current response decreases. The biosensor is successful at detecting the change in lactate concentration in dynamic testing. In the future, when this biosensor is implanted into the body, it will be exposed to a dynamic system and it is important that the biosensor can respond and measure changing lactate concentrations.

The concentration of lactate produced by human glioblastoma cells was measured using an ELISA kit. As expected, the 500,000 cells had produced more lactate than the 100,000 cells; however, 1 million cells produced slightly less lactate than 500,000 cells. When the media was collected from culture after 48 hours, the 1 million cells did not look healthy, explaining why they produced less lactate. The current response of the biosensor in these media samples was also tested. The 500,000 cells had the highest current output because there was the highest lactate concentration. When the equation from the standard curve was used to calculate the lactate concentration, the values were negative, which is not possible. We believe this is due to non-specific reactions between analytes in the media and the biosensor. The culture media contains glutamate and other analytes that can also be broken down into hydrogen peroxide and eventually electrons. This may explain why the current output was an order of magnitude higher when tested in cell

media than when tested in different lactate solutions in PBS. In order to reduce this interference, in the future adding more lactate oxidase to the biosensor will increase the biosensor's specificity.

This biosensor system created will greatly benefit GBM patients economically. Current monitoring methods cost thousands of dollars per test so it is not feasible to frequently monitor lactate levels. Although the biosensor will also be on the order of thousands of dollars, it will allow for frequent monitoring over the period of months to years, reducing the overall cost of monitoring GBM significantly.

The biosensor is feasible to manufacture in large quantities. The process of depositing the film on the electrode surface is fairly simple and does not require excess time or abnormal conditions. However once the lactate oxidase is deposited, the sensor must be stored at $4^{o}C$ or below. Although the design is currently simple to manufacture, more problems may be encountered in the miniaturization process.

Excess lactate is produced by all forms of cancer, so once optimized this technology can be applied to monitoring any form of cancer. Current monitoring methods are done infrequently, usually approximately every six months. During that time, a recurring tumor could grow substantially and become fatal. The biosensor allows physicians and patients to monitor lactate levels constantly and find recurrences right away. While right now, the output of biosensor is measured by a computer, in the future, the output could be sent to a smart watch, a cell phone app or any other device. The biosensor can be used to monitor the efficacy of different treatments and help improve the chance of survival for all cancer patients.

8. Recommendations

In order to optimize the biosensor, the film uniformity must be improved and the thickness decreased. To improve film uniformity, the team recommends that a stir bar be added to the solution during deposition. The pyrrole or aniline is not soluble in PBS and adding a stir bar would ensure complete mixing of the two liquids. In order to decrease the thickness of the polypyrrole film, the polymerization time could be shortened or less pyrrole be added. In order to decrease the thickness of the PANI layer, fewer cycles should be performed during electrochemical deposition or less aniline should be polymerized.

Throughout the project, the team encountered some setbacks. The team researched and completed preliminary testing on many design alternatives. Less preliminary testing would have allowed the team to choose the final design and collect more data to validate this design. In order to avoid setbacks and progress further, it is also recommended that all equipment be properly calibrated. Due to time constraints, some tests were only completed once. These tests should be performed in triplicate in order to ensure the resulting data is validated.

Although the lactate biosensor created for this project was able to detect changing levels of lactate concentration and detect the lactate concentration in media, we were unable to validate the biosensor's effectiveness completely. Biocompatibility testing *in vitro* should be done to ensure that the device is not cytotoxic. Additionally, the biosensor should be kept in aqueous conditions at 37 degrees Celsius for a period of at least 8 weeks to test the overall function of the biosensor over time in physiological conditions. This is important because the biosensor will need to function accurately for a period of several months in order to be effective as a monitoring device. To evaluate the selectivity of the biosensor, lactate solutions that contain molecules such as glucose or ascorbic acid should be tested in order to ensure that the current produced by the biosensor is only a result of lactate breakdown, not the breakdown of other molecules.

This biosensor should also be redesigned with glutamate oxidase replacing the lactate oxidase, so that the concentration of glutamate could also be tested. Glutamate is also produced in higher quantities by glioblastoma cells, and could also serve as an indicator of tumor progression. The lactate and glutamate biosensors could be combined into a dual biosensor system that could be used more effectively to monitor glioblastoma tumor progression as a function of lactate and glutamate production.

In order for this biosensor to be a device that could be used in vivo, miniaturization will be necessary. The design would need to be optimized in order to work most effectively on a smaller electrode surface. A device that could export the biosensor's data, through WiFi or other means, to a physician's computer or mobile device, would be necessary in order for the biosensor to be used as an implantable medical device. Once the addition of a data reporter was validated, the biosensor could be tested *in vivo* on a small animal model.

9. Conclusions

A lactate biosensor was designed and tested to monitor tumor size in GBM patients. Because GBM is so aggressive, it is important to be able to frequently monitor tumor progression to determine if treatments are effective. The current monitoring methods are not appropriate for frequent use because they detect large tumors and are very expensive.

The lactate biosensor is able to detect changes in current with changing lactate concentration and has an almost instantaneous response of less than one second. The low cost will allow GBM patients more constant monitoring of lactate levels and will allow physicians to detect tumor recurrence early.

Although the biosensor shows potential, many issues must be overcome before it can be implemented in GBM patients. Validation of the biosensor was hindered by equipment malfunction, so more testing is needed to ensure that lactate levels are being measured accurately. Further research is

needed to optimize the biosensor for long-term use, on the timeframe of months to years, at physiological conditions and miniaturizing the device to make it implantable. The biosensor is early in the design and validation process but with further research this technology can be applied to all types of cancer.

An early-stage lactate biosensor was created to monitor tumor size in GBM patients. Although this design requires more troubleshooting and validation, it shows promise. Once optimized, this system can be applied to not only monitoring GBM, but also monitoring all forms of cancer. This technology has the potential to revolutionize tumor monitoring for cancer patients and greatly reduce medical costs.

References

Anglin, N. et al. Development of a Biosensor to Monitor Glioblastoma. Worcester Polytechnic Institute, 2013.

Armstrong, Terri. Head's Up on the Treatment of Malignant Glioma Patients. Oncology Nursing Forum. 2009. Vol. 36 (5):233-240.

Arslan, F. An Amperometric Biosensor for Uric Acid Determination Prepared From Uricase Immobilized in Polyaniline-Polypyrrole Film. Sensors. 2008. 8(9): 5492-5500

Balasubramanian, K. Burghard, M. Biosensors based on carbon nanotubes. Analytical and Bioanalytical Chemistry. 2006. 385(3)452-468

Bannish, C., et al. Anti-Inflammatory System for Subcutaneous Biosensors. Worcester Polytechnic Institute, 2013.

Baraket, L., et al. Bioelectrochemistry. Carbon nanotubes based electrochemical biosensor for detection of formaldehyde released from a cancer cell line treated with formaldehyde-releasing anticancer prodrugs. 2010. 77(2): 94-99

Basu A., et al. Development of cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase on oxygen electrode for the determination of total cholesterol in food sample. Bioelectrochem 2007;70:375–9.

Brandes, A., et. al. Disease Progression or Pseudoprogression after Concomitant Radiochemotherapy Treatment: Pitfalls in Neurooncology. Neuro-Oncology. 2008. Vol. 10 (3): 361-367.

Bruce, J. et. al. Glioblastoma Multiforme Treatment and Management. Medscape. 2013.

Castillo, J., et al. Simultaneous detection of the release of glutamate and nitric oxide from adherently growing cells using an array of glutamate and nitric oxide selective electrodes. Biosensors and Bioelectronics. 2005. 20(8): 1559-1565

Cheng, L. et al., Quantification of microheterogeneity in glioblastoma multiforme with ex vivo high-resolution magic-angle spinning (HRMAS) proton magnetic resonance spectroscopy. Neuro Oncology. 2000. 2(2):87-95

Cheng, Y., et al. Electrochemical polyaniline/polypyrrole composite film with novel nanostructure and high biosensitivity. 2007 Asia Optical Fiber Communication and Optoelectronics Conference. 2007.

Dang, C. Glutaminolysis: Supplying carbon or nitrogen, or both for cancer cells? 2010. Cell Cycle. 9(19): 3884-3886.

De Benedetto, G.E., et al., One-step fabrication of a bi-enzyme glucose sensor based on glucose oxidase and peroxidase immobilized onto a poly(pyrrole) modified glassy carbon electrode. Biosensors and Bioelectronics, 1996, 11(10): 1001-1008

Disease Information, Brain Tumor: Astrocytoma/Glioma. St. Jude Children's Research Hospital. 2013.

Du, D., et al. Acetylcholinesterase biosensor design based on carbon nanotube-encapsulated polypyrrole and polyaniline copolymer for amperometric detection of organophosphates. Biosensors and Bioelectronics. 2010. 25(11): 2503-2508

Foster, L., et al., Chitosan films are NOT antimicrobial, Biotechnol Lett (2011) 33:417–421

Gaikwad, P. D., et al.. Immobilization of GOD on Electrochemically Synthesized PANI Film by Cross-linking via Glutaraldehyde for Determination of Glucose, Int. J. Electrochem Sci. 2006. (i), 425–434.

Gajendran, P. and Saraswathi, R. PANI-CNT Composites. Pure and Applied Chemistry. 2008. 80(11): 2377-2395

Glioblastoma. American Brain Tumor Association. 2013.

Glioblastoma Multiforme (GBM). National Brain Tumor Society. 2013.

Gujar, SK. Maheshwari, S. Bjorkman-Burtscher, I. Sundgren, PC. Magnetic Resonance Spectroscopy. Journal of Neuro-opthalmology. 2005. Vol. 25 (3): 217-226

Hirata, E., et al. In vivo fluorescence resonance energy transfer imaging reveals differential activation of Rho-family GTPases in glioblastoma cell invasion. Journal of Cell Science. 2012. 125(4): 858-868

Hirschhaeuser, F. et al, Lactate: A Metabolic Key Player in Cancer, Cancer Research 2011;71:6921-6925

Keusgen M. Biosensors: New approaches in drug discovery. Naturwissenschaften. 2002;89:433–444

Kotanen C., et al. Implantable enzyme amperometric biosensors. Biosensors and Bioelectronics. 2012. 35(1):14-26

Li, C.M. et al. Electrochemical Thin Film Deposition of Polypyrrole on Different Substrates. Surface & Coating Technology. 2005. 198:474-477

Liu, Andréa Santos, Bezerra, Marcia Cristina, & Cho, Liu Yao. (2009). Electrodeposition of polypyrrole films on aluminum surfaces from a p-toluene sulfonic acid medium. *Materials Research*, *12*(4), 503-507

Malhotra, B., et al. Prospects of conducting polymers in biosensors. Analytica Chimica Acta. 2006. 578(1):59-74

Marie, S. and Shinjo, S. Metabolism and Brain Cancer. Clinics. 2011. 66(S1): 33-43.

Marucci, G. The effect of WHO reclassification of necrotic anaplastic oligoastrocytomas on incidence and survival in glioblastoma. The Journal of Neuro-Oncology. 2011. 104(2):621-622.

Moser, I. et al., Biosensor arrays for simultaneous measurement of glucose, lactate, glutamate and glutamine. Biosensors & Bioelectronics. 2002. 17:297-302

MRI Scan. Better Health Channel. Monash University. 2013. www.betterhealth.vic.gov.au

O'Neill, R.D., et al., Comparisons of platinum, gold, palladium and glassy carbon as electrode materials in the design of biosensors for glutamate. Biosensors and Bioelectronics, 2004, 19(11): 1521-1528

Ong, B. et al., Paclitaxel delivery from PLGA foams for controlled release in post-surgical chemotherapy against glioblastoma multiforme. Biomaterials. 2009. 30(18): 3189-3196

Pei J, Tian F, Thundat T. Glucose biosensor based on the microcantilever. Anal Chem 2004;76:292–7.

Peng, C. et al. A comparative study on electrochemical co-deposition and capacitance of composite films

of conducting polymers and carbon nanotubes. Electrochimica Acta. 2007. 53(2):525-537

Pelletier, G., et al., Quality of life in brain tumor patients: the relative contributions of depressions, fatigue, emotional distress and existential issues, Journal of Neuro-Oncology (2002) 57:41-49

Phypers, B. and Pierce, J. Lactate physiology in health and disease. Continuing Educations in Anaesthesia, Critical Care & Pain. 2006. 6(3):128-132

Reardon, D., et al. Recent advances in the treatment of malignant astrocytoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006. 24(8): 1253-1265

Robberg, K., et al. The influence of porosity and the nature of the charge storage capacitance on the impedance behaviour of electropolymerized polyaniline films. *Journal of Electroanalytical Chemistry* 1998. *443*(1), 49–62.

Roberts, H., et al. Dynamic, Contrast-Enhanced CT of Human Brain Tumors: Quantitative Assessment of Blood Volume, Blood Flow, and Microvascular Permeability: Report of Two Cases. American Journal of Neuroradiology. 2002. 23:828-832

Ronkainen N., et al. Electrochemical biosensors. *Chemical Society reviews*.2010;39(5):1747–63

Roslin, M. et al., Baseline levels of glucose metabolites, glutamate and glycerol in malignant glioma assessed by sterotactic microdialysis, Journal of Neuro-Oncology (2003) 61:151-160

Saleh Ahammad, A.J. Hydrogen Peroxide Biosensors Based on Horseradish Peroxidase and Hemoglobin. Biosensors and Bioelectronics. 2013. S9:1-11

Sassolas, A., et al., Immobilization strategies to develop enzymatic biosensors. Biotechnology Advances. 2012. 30:489-511

Sotiropoulou, S. Chaniotakis, N. Carbon nanotube array-based biosensor. Analytical and Bioanalytical Chemistry. 2003. 375(1): 103-105

Sun, W., et al., Electrochemistry of Hemoglobin in the Chitosan and TiO2 Nanoparticles Composite Film Modified Carbon Ionic Liquid Electrode and Its Electrocatalysis. Bull. Korean Chem. Soc. 2009, 30:3

Thomas, S. Wiley-Scrivener: Handbook of Engineering and Speciality Thermoplastics, Volume 4 : Nylons. Wiley. 2012.

Vander Heiden, M., et al. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009. 324(5930): 1029-1033

Viswanathan, S., et al. Electrochemical biosensor for pesticides based on acetylcholinesterase immobilized on polyaniline deposited on vertically assembled carbon nanotubes wrapped with ssDNA. *Biosensors & Bioelectronic.* 2009. *24*(9), 2772–7.

Wang, J. Carbon-Nanotube Based Electrochemical Biosensors: A Review. Electroanalysis. 2005. 17(1):7-14

Wang, J. Electrochemical Glucose Biosensors. Chemical Reviews. 2008. 108(2): 814-825

Wang, J. et. al., Solubilization of Carbon Nanotubes by Nafion toward the Preparation of Amperometric Biosensors. Department of Chemistry and Biochemistry. New Mexico State University, NM. 2002.

Wilson, G. Gifford, R. Biosensors for real-time in vivo measurements. Biosensors and Bioelectronics. 2005. 20(12): 2388-2403

Wisniewski, N. and Reichert, M., Methods for reducing biosensor membrane biofouling. Colloids and Surfaces B: Biointerfaces. 2000. 18:197-219

Wolf, A., et al. Targeting Metabolic Remodeling in Glioblastoma Multiforme. Oncotarget. 2010. 1(7): 567-577

Ye, Z. Sontheimer, H. Glioma Cells Release Excitotoxic Concentrations of Glutamate Glioma Cells. Cancer Research, 1999. 4383-4391

Yoo, E. and Li, S. Glucose Biosensors: An Overview of Use in Clinical Practice. Sensors. 2010. 10:4558-4576

Zadran, S., et al. Enhanced-acceptor fluorescence-based single cell ATP biosensor monitors ATP in heterogeneous cancer populations in real time. Biotechnology Letters. 2013. 35(2): 175-180

Appendix 1 - Experimental Protocols

Culturing Cells

U87mg human glioblastoma cells were cultured in media containing DMEM, 10%Fetal Bovine Serum, 1% Pen-Strep, 1% NEAA in 1-5 mL of media for 48 hours before the media was removed for use in testing.

Assays

Lactate Assay

Using the protocol found on the Sigma-Aldrich website, http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/1/mak064bul.pdf, a standard curve ranging from 0-10 nmol was created. For measuring the concentration of lactate in cell media, 5 μL of media in 45 μL of buffer was used as the sample. After incubation with the reaction mix for 30 minutes at room temperature, the absorbance of each well was measured using a plate reader at 570nm.Based on the equation given by the standard curve (Appendix 2), the concentration of lactate present in the media before dilution could be calculated.

Testing Solutions

All solutions were made of lithium lactate dissolved in 0.01 M PBS. All solutions should be de-gassed by adding nitrogen gas to the solution for 15 minutes prior to testing.

Amperometry

1. Open GPES software.
2. Insert working electrode (glassy carbon), reference electrode (Ag/AgCl), and counter electrode (platinum) through the cap of the electrochemical cell and into the solution being tested.
3. Attach the red wire to the working electrode, the blue wire to the reference electrode, and the black wire to the counter electrode.
4. Under methods, mouse over to chrono methods > 0.1s, and select amperometry.
5. Select the following settings for the amperometry:
    a. Potential = 0.2V
    b. Time=300s

Note: the same solution was tested with both amperometric and voltammetric methods for each trial.

Cyclic Voltammetry

1. Open GPES software.
2. Insert working electrode (glassy carbon), reference electrode (Ag/AgCl), and counter electrode (platinum) through the cap of the electrochemical cell and into the solution being tested.
3. Attach the red wire to the working electrode, the blue wire to the reference electrode, and the black wire to the counter electrode.
4. Under methods, select voltammetry – normal.
5. Select the following settings for the cyclic voltammetry:
    a. Number of cycles=10

b. Start potential= -0.4V
c. Minimum Potential = -0.4V
d. Maximum Potential = +0.6V
e. Step Potential = 0.00244V
f. Scan rate = 0.05V/s

PPY polymerization (Li et al., 2005.)

1. De-gas 10 milliliters of 0.01 M phosphate buffered saline (PBS) for 15 minutes.
2. Add 7 μL of pyrrole (liquid) and stir rapidly with pipet tip.
3. Set up working electrode, reference electrode, and counter electrode (described previously) and open GPES.x 10
4. Under Methods, mouse over to chrono methods > 0.1s, and select potentiometry (galvanostatic.)
5. Settings should be as follows:
    a. Current = 0.007A
    b. Time = 120s

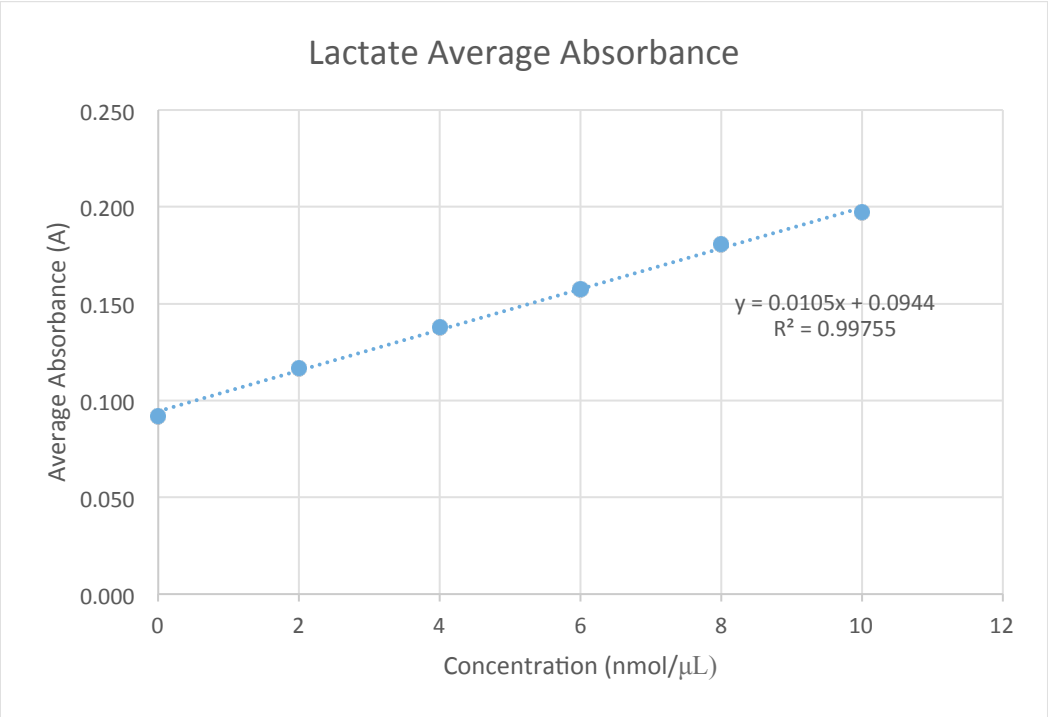
PANI polymerization (Gaikwad et al., 2006)

1. De-gas 10 mL of 0.1 M PBS (pH 3.5).
2. Add 0.5 mL aniline to 10 mL of 0.1 M PBS.
3. Set up working electrode, reference electrode, and counter electrode (described previously) and open GPES.
4. Under Methods, select cyclic voltammetry (normal).
5. Select the following settings for the cyclic voltammetry:
    a. Number of cycles=10
    b. Start potential= 0.0V
    c. Minimum Potential = 0.0V
    d. Maximum Potential = +0.95V
    e. Step Potential = 0.00244V
    f. Scan rate = 0.100 V/s

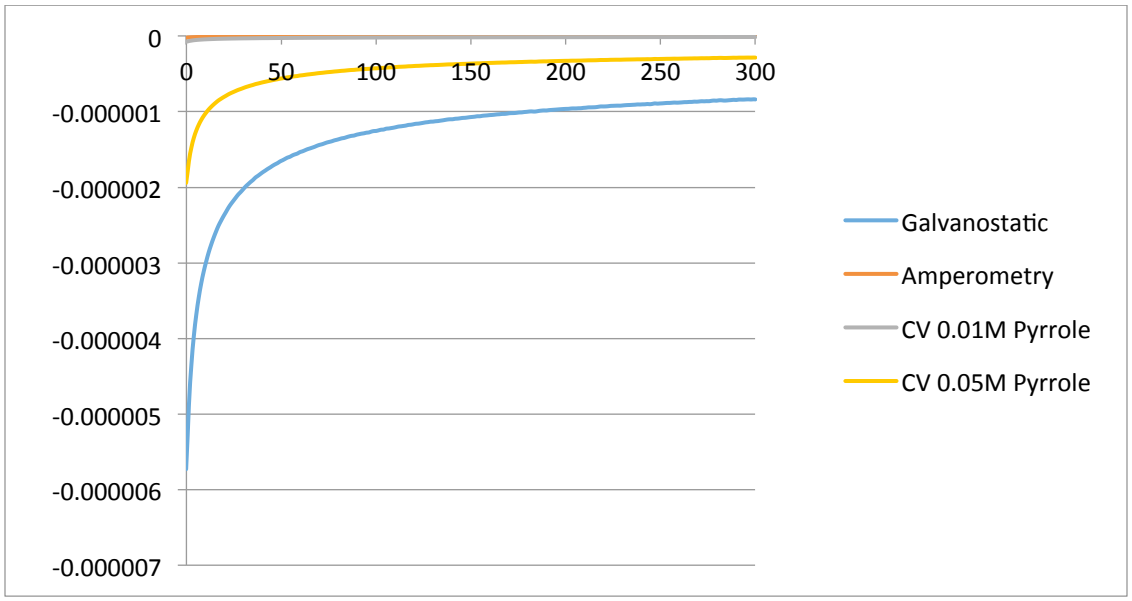
Crosslinking Enzyme to Films

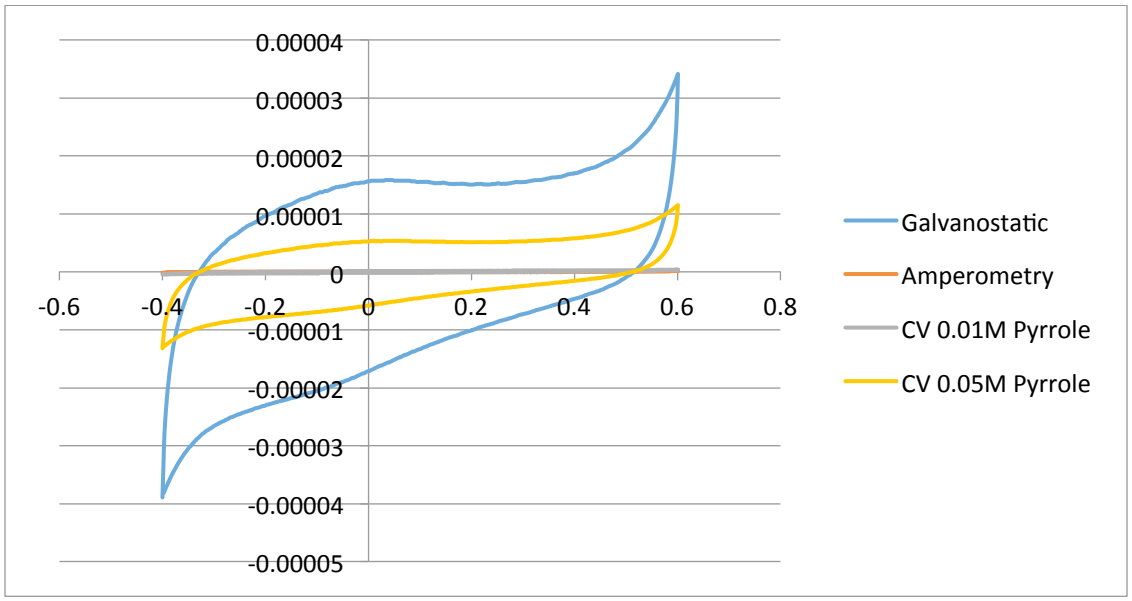
1. After creating the desired polymer films using the protocols described above, add 5 μL of 0.1% glutaraldehyde on top of the polymer film. Let dry at room temperature for 30 minutes.
2. Add 5 μL of 2.9 mg/mL lactate oxidase (Sigma) on top of the polymer-glutaraldehyde film. Let dry at room temperature for 30 minutes.
3. Rinse electrode 3 times for 10 minutes each in 0.01 M PBS.
4. Store in fridge if the biosensor will not be used immediately.

Appendix 2 – Lactate Standard Curve



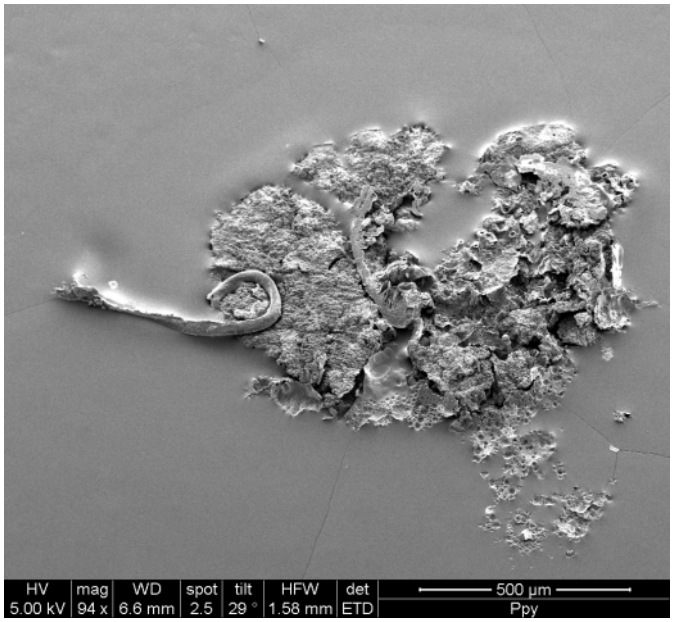
Appendix 3 – Comparing PPY Electropolymerization Methods





Appendix 4 – More SEM Images

PPY Film



PPY-PANI Film