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Developing an Enzymatic Film for Sensing Lactate *In Vitro*

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Authorship

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Abstract

Brain tumors, such as glioblastoma multiforme, have extraordinarily low five-year survival rates. Currently, it is difficult to monitor in real-time the progression of a tumor and how it responds to therapy. Biosensors been used to measure key metabolites, such as lactate and glutamate, at shorter time intervals to detect abnormal blood levels. In this study, an enzymatic coating to detect lactate was developed *in vitro*. Chitosan was used to immobilize lactate enzyme. The chitosan with the enzyme was coated a glassy carbon electrode and tested at different concentrations of aqueous lactic acid as a proof of concept. The results from the amperometry indicate that the absolute value of the current output increases when the lactic concentration increases. A lactate biosensor utilizing chitosan film has potential for future applications diagnosing brain cancers such as glioblasoma by detecting blood lactate levels both *in vitro* and possibly *in vivo*.

Chapter 1: Introduction

Brain cancer and brain diseases affect millions of people and families worldwide. In 2010, there were an estimated 22,000 new cases of brain and nervous system cancer diagnosed in the United States alone (Jemal, 2010). Both males and females in the age range of 15-44 years of age have 5-year survival rates of approximately 50-60% (Saikai, 2015). One kind of brain cancer, glioblastoma, is especially aggressive and only has a 5-year survival rate fewer than 2% (Pirzkall, 2009). No treatment options for glioblastoma are curative; early detection is an area of focus for increasing lifespan and quality of life (Tran, 2010).

Tumors can also develop in the brain as a result of cancer in another part of the body. If a patient's cancer is determined to be metastatic, it means the cancer has developed enough that the cancer cells from a tumor can break off and migrate to the brain through the blood from their point of origin (such as the lungs, breast, etc.). This is very common; approximately 20-40% of people with cancer will develop metastatic cancer spread to the brain (Serres, 2012). Unfortunately, many of these tumors are not diagnosed earlier as they are small and often asymptomatic. In most patients the metastatic brain cancer is not diagnosed until the patient becomes symptomatic, and the prognosis for the patients is much worse than those where tumors were detected early on (Sun-Young, 2005).

One of the current methods for diagnosing and monitoring brain tumors is by using magnetic resonance imaging (MRI). While MRI can be a useful tool to detect these tumors, they do have limitations. An MRI does not monitor a patient very frequently, and months can go by before a patient has another scan to check for tumors. This is not ideal due to the high mortality rate of patients whose tumors were not detected early. A patient may also have a physical limitation that limits their ability to be scanned in an MRI machine. Patients that have

pacemakers, heart valve replacements, aneurysm clips, cochlear implants, and those taking neuro-stimulators can be unable to be scanned in an MRI due to the way the machine uses magnets to create the images (‘Magnetic’, 2013). MRI results are not always obtained quickly due to the fact that test results must pass through many hands for interpretation. After a technician has performed the MRI, they then have to pass on the resulting scans to a doctor, and then the doctor must read and interpret the results before speaking with the patient. This ultimately becomes a time consuming and expensive process.

An MRI is also expensive; it can cost thousands of dollars, and can be especially expensive if contrast is needed or the patient requires drugs due to claustrophobia. It can also be time consuming as a scan of the brain can take anywhere from 20-45 minutes or longer if the patient moves during the imaging (Meng, 2011).

Because of the limitations of current imaging systems, there is a need for a way to monitor a patient for brain tumors early and often. One way to achieve this is through the monitoring of lactate levels in the blood. Increased lactate in the blood can indicate an abnormality in cell replication as a byproduct of respiration (Goodwin, 2007).

The principal goal of this project is to create an effective technique for the early detection of tumor growth by the *in vitro* measuring of lactate, using a noninvasive method. The technique in the proposed project can be performed more frequently and efficiently than the current standard of screening. This will allow for early tumor detection and an increased chance of treatability. Patient limitations such as pacemakers would be avoided. In addition, this project could also aid in the diagnosis of other neurological disorders that can cause changes in lactate levels due to damage. Testing for lactate levels in the blood could not only be effective for small brain tumors but also may be promising for other conditions.

One area that shows enormous potential for monitoring metabolite levels is biosensors. These are devices where a molecule, such as lactate, binds and initiates an electrochemical reaction that becomes an electrical current. The output of current corresponds to the concentration of substrate in the initial binding. Currently, there are many various types of biosensors being tested, but their clinical use is limited because of their inaccuracies causing electrical interference with the electrical environment in the tissue and/or cells (Fracchiolla, 2004). Developing a biosensor to work *in vitro* will allow the enzymatic reaction to be optimized for continuous use as an improvement upon current detection methods and as a steppingstone for possible future real time monitoring applications *in vivo*.

This project is to develop a biosensor capable of detecting lactate for early cancer detection. The first step is to determine the different methods in which concentrations of lactate can be converted into a measurable electrical signal by the biosensor. The best materials will then be selected so there will not be impediment or false enhancement of the signal. Finally, the biosensor will be tested with a known static lactate solution as well as several different known concentrations consecutively in order to determine the effectiveness of the film in real time testing. In order for the design to be considered a success, the biosensor must be capable of accurately detecting lactate in an *in vitro* setting without the sensitivity of the sensor decreasing rapidly.

The following report contains literature review, methodology, data collection, analysis, and conclusion sections, to describe in more detail, the process put into designing and testing the lactate biosensor, for improved brain tumor diagnostics.

Chapter 2: Background and Literature Review

Brain Cancers

Tumors in the brain can be extremely hazardous to the health of humans, whether they originate in the brain or the result of metastatic cancer from other locations in the body. Tumors are divided into two different categories, benign or malignant. If a tumor is categorized as benign, this means it is not harmful while a malignant tumor is cancerous and has the potential to spread to other parts of the body. Within the category of malignant tumors, there are primary and secondary brain tumors. Primary brain tumors originate and proliferate in one site, while secondary brain tumors are the result of migrating tumor cells that find a new location to proliferate. Glioblastoma is one of the most aggressive types of malignant primary brain tumors. The average survival rate for a person with glioblastoma is 5 years and under 2% (Pirzkall, 2009). Secondary brain tumors are very prevalent in cancer patients, and 20-40% will develop them (Serres, 2012). It is also important to note that constant monitoring for these tumors is difficult, as well as costly because the current standard of monitoring is MRI.

Several methods of treatment are currently available for tumors. Chemotherapy is the treatment of cancer using drugs, while radiation therapy is uses radiation to treat cancerous tumors (Lombardi, 2014). A third tumor treatment method is surgery, which entails physically removing the tumor via an invasive procedure. The most commonly used method of tumor treatment is combination therapy, which combines the aforementioned treatments used in conjunction with one another to attempt to maximize the chances of successful treatment (Lombardi, 2014).

These methods can be invasive and painful, however, earlier detection may catch the tumor before significant metastasis, may require less invasive treatment. Early detection also

improves the probability of successful treatment and the patient will require less intense treatment.

Lactate Levels

Because of the limitations of current imaging systems, there is a need for a way to monitor a patient for brain tumors early and often. Lactate levels in the blood are measured in order to indicate whether or not the body is processing this cellular byproduct correctly, or if there is some type of disease within the body causing an excess buildup of this material. Test results indicating an excess buildup could be indicative of a number of problems, particularly excessive cell growth. Normal lactate levels in the blood of the human body are between 0.5mmol/L lactic acid and 1mmol/L of blood. When lactate is out of the normal range and rises and rises above 2mmol/L (sometimes rising as high as 5mmol/L) this is often indicative of some type of disease within the body (Goodwin, 2007). For this reason, it is important to measure lactate levels in the blood in order to look for signs of illness. When an increase in lactate levels is observed, this often indicates that the body is beginning to undergo anaerobic metabolism, and there may be an abnormal and sharp increase in cellular activity. When the body cannot process this lactate quick enough or correctly, lactate levels will rise. It is important to note that there is a proper and improper time to test for lactate levels in the blood. A proper test time is when the body has been at rest and not exerted. Post-exercise or exertion, the body may have higher levels of lactic acid in the blood, which may produce an inaccurate test result and a false reading (Goodwin, 2007).

Current Methods of Cancer Detection

MRI

Brain tumors are currently detected by imaging. There are different ways to image the brain to check tumor formation, including Magnetic Resonance Imaging (MRI) or Computerized Tomography (CT) scans.

An MRI is the most common method of imaging tumors (Levy, 2014). Patients who have been diagnosed with a brain tumor (or are at high risk for a tumor) are scanned to determine the extent and location of a tumor(s). This kind of imaging works by utilizing a strong magnetic field to align the axes of hydrogen atoms found throughout the body. When the magnetic field is removed, different tissues take different amounts of time to realign normally. By measuring the difference in these times, an image can be created. This image includes tumors (Berger, 2002). The advantage of this kind of imaging is that it is non-invasive, and the risk of complication is low, especially if there is no contrast material injected. MRIs are effective for tissue imaging because the hydrogen atoms used for the imaging are found in water. Soft tissues contain a lot of water and therefore the detail and clarity of the image can detect around 4mm in diameter (Yokoi, 1999). While this kind of imaging has many advantages, there are some limitations that leave room for improvement. The scans require a patient to come into the hospital or imaging center and stay very still for a scan that can take around 20-45 minutes at minimum (Meng, 2011). When trying to detect small tumors, contrast material, typically delivered by nanoparticles, is injected to enhance the difference in the image of the tumor from the surrounding tissue (Mortiz, 2003). While this process increases the visibility of tumor cells, it does increase the time the scan takes. This process can be time consuming for the patient and the doctors as well as relatively expensive. Before insurance, it is estimated that a scan can cost thousands of dollars (Meng, 2011). This cost is due to the size of the machine and the space

needed to house it, the length of the procedure, the possible contrasts or dye, and the number of people required to take a scan, analyze it, and provide the patient with the results (Meng, 2011). In a study of cost analysis for MRI compared to mammography for detecting breast cancer, the cost of the MRI ended up being $18,167 over 25 years for the patient, compared to mammography which cost $4,760 (Moore, 2009). These scans can be a financial burden on the patient as well as on the hospital and physicians. Usually when monitoring a patient for a recurring condition, these scans occur about once every six months. This leaves time in between scans for tumors to originate and develop. These tumors are then able to grow larger undetected, which can increase the probability that the tumor will be more difficult and take longer to treat.

Sometimes a patient's medical condition can also limit their ability to be imaged. An MRI utilizes magnets, meaning it can be very dangerous for patients with metal objects implanted in them, such as pacemakers (Berger, 2002). The pacemaker could stop functioning properly, or a metal implant could shift. These patients are then unable to be monitored for any brain tumors or abnormalities using this kind of imaging.

CT

A different method of imaging is the CT (computed tomography) scan. During a CT scan X-rays are applied from many different angles to create a 2-D image of each section, or slice, of the brain. An advantage of a CT scan is that it generally takes less time than an MRI, and this can be somewhat helpful for a claustrophobic patient. The images produced by CT scans can be used for the imaging of soft tissues as well as bones and blood vessels ('Mayo Clinic', 2015). Therefore by looking at soft tissues, differences in the normal makeups that are found can be used to locate tumors. However, these scans have a significantly lower resolution than the state of the art MRI scans can provide. This means that there is a greater possibility that small tumors

can be missed. In Figure 1, a CT scan is shown on the left compared to an MRI scan on the right. The CT scan also comes with the additional radiation due to the number of X-rays taken. This is not a preferred method of imaging because of this radiation exposure. The average conventional x-ray of the head will expose a person to 0.07mSv of radiation, whereas the same CT scan will have an exposure of 2.0 mSv (Smelka, 2007).

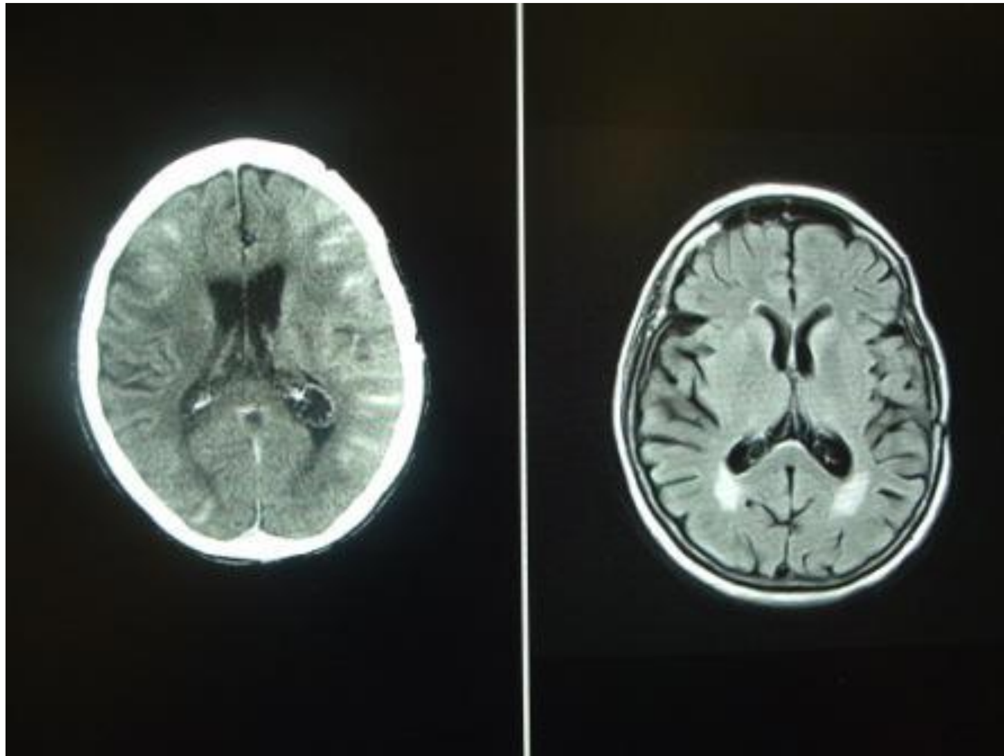


Figure 1: CT Scan Alongside MRI Scan ("Is MRI", 2007)

Imaging Limitations

While the MRI is the current gold standard in screening for tumors in high-risk patients, its limitations leave room for advancement in the way of constant monitoring. While an MRI is able to detect a tumor that is only around 4mm in diameter, this is usually only done every 6 months. Any way to more frequently monitor the patient for any indication of tumor growth would be an improvement, and allow scans to confirm a possible diagnosis rather than be the only method of detection with such a large time gap in between scans.

Biosensors

Biosensors show great promise for future applications in this area. Biosensors are used in many fields including environmental pollutants, food quality, and glucose monitoring (Bãnicã, 2012). Clearly, biosensors are very versatile and their potential uses are growing. In a very basic sense, a biosensor detects levels of a certain molecule, protein, enzyme, element, etc. and converts that into an electric current through a reaction on the surface of the biosensor. This current is then used to determine concentration of the substance being tested for. Early models of biosensors were known as "enzyme electrodes" (Palchetti, 2010).

This kind of technology already is utilized for the treatment of diabetes. A glucose monitor is a biosensor for glucose levels in the bloodstream (Heinemann, 2013). This is an example of consistent monitoring by the patient without having to go into the hospital or care center and disrupt their life. The monitoring allows the patient to know if there is an abnormal glucose level and to either treat themselves or contact their doctor. This kind of constant monitoring is desired for monitoring patients at risk for a recurring cancer.

Medical Applications

As mentioned before, one of the major current medical applications of biosensors is their use in glucose detection, which accounted for roughly 85% of the world's biosensor market in 2004 (Yoo, 2010). Glucose biosensors can be used *in vivo* and *in vitro*. Applications of biosensors *in vivo* are somewhat invasive, as they require the sensor to be implanted within the body of the patient to detect the glucose. A glucose biosensor *in vitro* is used on a biological sample, in this case blood, to the sensor outside of the body to get a glucose reading (Mandal, 2010). Glucose biosensors are primarily used for the monitoring and treatment of diabetes mellitus, commonly just referred to as diabetes. By using these glucose biosensors, diabetes patients can monitor their blood glucose levels, using the *in vitro* type of sensor, without the need

to go to the doctor's office for every reading. This allows for the treatments of insulin to be tailored to the whether or not high levels of glucose are detected (Yoo, 2010). As glucose levels can vary in the blood based on several factors, it is preferable to an alternative taking a constant amount of insulin per day. Additionally, it may give the patient and doctor a different perspective on the severity of the diabetes (Yoo, 2010). A hypothetical example is that the patient could have had abnormal glucose levels show up during a lab test, but when the glucose levels are constantly monitored day to day, they usually stay at normal levels or are borderline high. This revelation may change the treatments to noninsulin and nutrition therapies rather than constant insulin injections (Yoo, 2010).

Metabolite Biosensor

Lactate Reaction

Lactate is from an oxidation-reduction reaction, specifically when lactate is converted to pyruvate. Lactate dehydrogenase is the enzyme that catalyzes the reaction. Lactate becomes oxidized when it loses two electrons and becomes converted to pyruvate. As this occurs, lactate and NAD^+ bind to lactate dehydrogenase, which creates NADH. The full reaction scheme is shown in Figure 2.

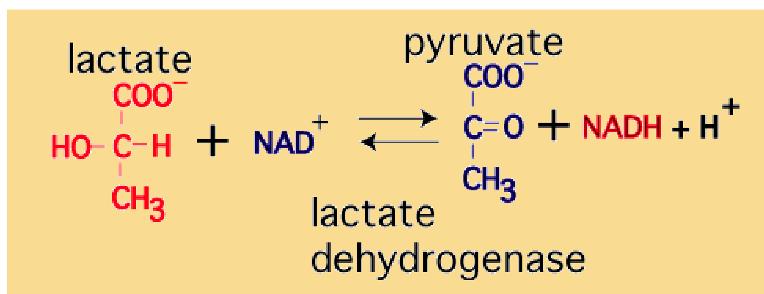
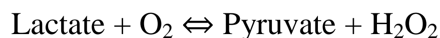


Figure 2: Lactate Dehydrogenase Reaction ("Coupled", n.d.)

As this reaction runs in both directions, pyruvate can also produce lactate, and depending upon electron strength and currents from the reaction, this will indicate the concentration of lactate that must be detected by the biosensor ("Lactate", 2016).

Another enzyme that can be used in this film to detect lactate is lactate oxidase. When in the presence of lactate and oxygen, the lactate oxidase catalyzes the reaction:



$$\text{Lactate} + O_2 \Leftrightarrow \text{Pyruvate} + H_2O_2$$

Lactate will still cause the gaining of 2 electrons by the system when it is converted into pyruvate, and hydrogen peroxide is also formed (“Enzymatic”, 1999). A reaction schematic for lactate oxidase is shown in Figure 3.

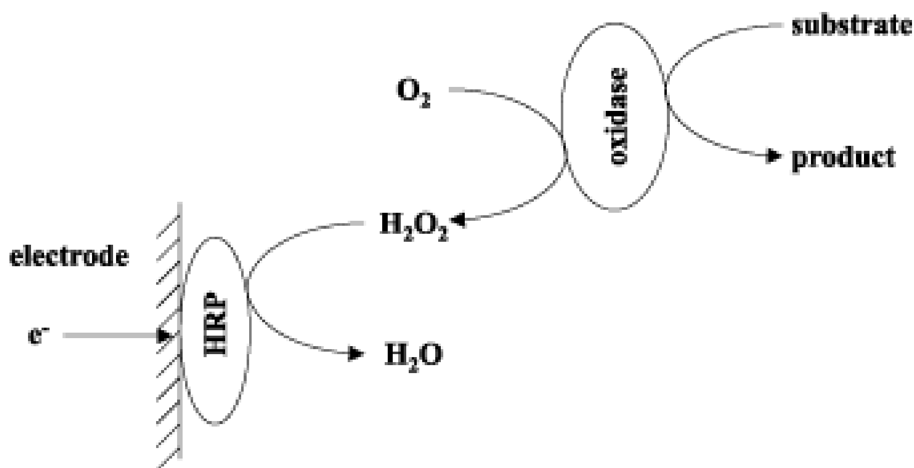


Figure 3: Redox Reaction with HRP (Freire, 2003)

Nanoparticles

Nanoparticles are materials that are smaller than 100 nm. They have been used in such applications as medical imaging, as well as gene therapy and drug delivery (Murthy, 2007). Nanoparticles can also be used to enhance the sensitivity of electrochemical biosensors. For biosensor applications a class of materials known as noble metals are used due to their exceptional properties. Gold and platinum nanoparticles are noble metals that are heavily documented in biosensor applications. This is due to their great catalytic properties that can facilitate chemical reactions and helping to increase the ability of the nanotubes to transport electrons (Kang, 2008).

Enzyme Immobilization

In many cases, the enzymes are bound to nanomaterial (nanotubes, nanoparticles). These nanoparticles carry the charge (the electrons) to the electrode. There are different methods to immobilize the enzymes on the surface of nanoparticles including adsorption, covalent bonding, encapsulation, entrapment, and cross-linking (Datta, 2013)(Figure 4). Adsorption is physically binding the enzyme to the surface of the nanomaterial. Mixing the enzyme into a solution and allowing the nanomaterial to incubate with the solution typically does this. Then the enzyme that did not bind is rinsed off. This method is simple, however the bonds that are made between the enzyme and the surface are not particularly strong. This is a problem because the biosensor will not be stable over time when the enzyme breaks off of the surface; the detecting ability will be hindered. The enzymes can break these weak bonds with slight changes in temperature, pH, or even mechanical force.

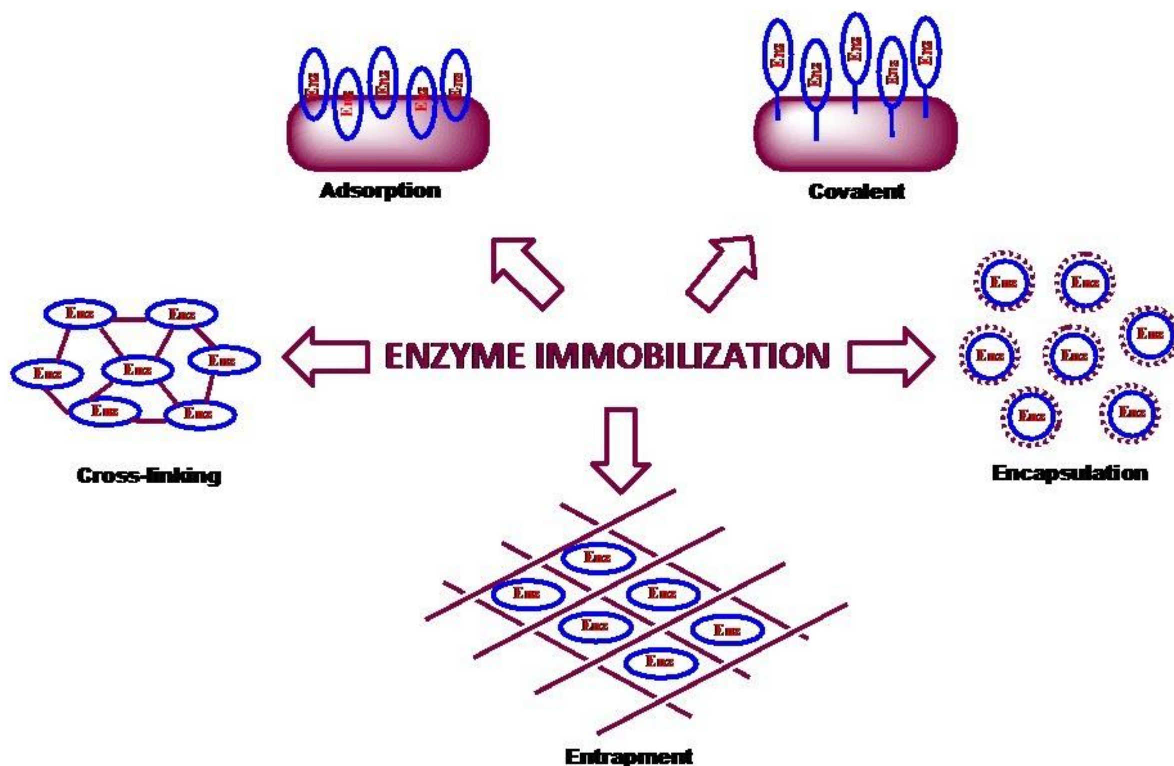


Figure 4: Methods of Enzyme Immobilization (Shomu's Lab, 2015)

One method used to combat this problem is to adhere the enzymes within a polymer film. Electropolymerized films are polymers attached to the electrode with the enzyme in it. One very commonly used electropolymerized film is polypyrrole (PPy). The advantages of this film are that its electrical conductivity is stable, and it can be synthesized in a neutral environment in terms of oxidation potential and pH (Sassolas, 2011). This means that the environment for the enzyme is as stable as possible and enzyme denaturing is limited. Another electropolymerized film is made out of polyaniline. This film is used because the conductivity of the film can be controlled based on how it's formed. Polyaniline is a great electron transfer mediator, as well as polypyrrole.

Another kind of film used to immobilize the enzymes is polysaccharide films. These include materials such as alginate and chitosan. These films are advantageous especially when the biosensor has potential for *in vivo* use because they are non-toxic and biocompatible. These films avoid enzyme leakage while allowing substrate to get to the enzyme (Sassolas, 2011).

Immobilization by crosslinking is when one enzyme is chemically cross-linked to another. While this does keep the enzymes on the surface, enzymes are denatured because of chemical alteration, thus decreasing the sensing capability of the biosensor. Immobilization by covalent bonding can also cause some enzyme denaturing, and the amount of enzyme that is fully immobilized on the surface is relatively low compared to other methods (Datta 2013). Enzyme immobilization by affinity increases enzyme availability by correcting the orientation of all the enzymes. This avoids any blocking or deactivation of the active site of the enzyme. However, the correct orientation of the enzymes is achieved by binding a specific group that has an affinity to the surface to the enzyme. This can be expensive and time-consuming (Sassolas, 2011).

Electrodes and Sensing Technology

The final stage of the biosensor reaction is the electrode. After the lactate reacts with the surface of the biosensor, the resulting electrons are sensed by electrodes near the surface of the electrode and transduced into a signal.

Traditional electrodes that are used in electrochemical biosensors are metal electrodes (ex. gold, copper, platinum, etc.) and glassy carbon electrodes. However, they tend to suffer from issues such as relatively low sensitivity and greater response times (Balasubramanian, 2006).

Materials in the form of nanotubes are another type of electrode used in electrochemical biosensors, either in combination with traditional electrodes, or as a replacement

(Balasubramanian, 2006). Carbon nanotubes (CNT) are relatively commonplace and TiO_2 is a potential competitor, there are other additional materials used for as electrodes in biosensors. These less commonly used materials are silver-based and silicone-based (Higson, 2012; Yudin, 2007).

The most common materials used are carbon nanotubes, which come in many different forms. CNTs are semi-conductors and are therefore useful in the electron transportation utilized in electrochemical biosensors (Balasubramanian, 2006). There are two types of CNTs, the single-wall carbon nanotubes (SWCNT) and the multi-wall carbon nanotubes (MWCNT). SWCNTs are formed from one sheet of graphene, which means that the walls on the nanotube are only one atom thick. The MWCNTs are made from separate graphene sheets being layered over one another.

Two of the most common methods of configuring CNTs are CNT-coating electrodes and CNT-binder composite electrodes. The CNT-coating electrode transducers are usually covered by a Nafion film, which helps prevent fouling of the biosensor, as well as helps to make the sensor more biocompatible, on top of the benefits of CNTs. The transducer is what converts the detected electrons into a signal detailing the information that has been detected. Additionally, the CNTs can be formed in vertically aligned nanotube electrode array, another type of CNT-coating. In this configuration the CNTs are attached perpendicularly to the underlying electrode. CNT-binders are biocomposite electrodes. One type combines paste electrodes with CNT, which is a more usual approach to the composite type. A composite electrode can also be made using a composite of CNT and Teflon. In the case of the CNT and Teflon composite, the nanotubes are used as the transducer, without the need for a secondary electrode (Wang 2004).

Titanium (Ti) is a biocompatible material that has many biomedical applications in its pure metal, alloy, and TiO_2 forms. Titanium has a very high biocompatibility, and is therefore very suitable for implantation within the body in orthopedic applications, as well as several others. Titanium dioxide, TiO_2, is a particularly important form of titanium, with qualities such as being a semi-conductor and having a high level of biocompatibility (Li, 2005). TiO_2 can be used as a film on the surface of other materials to increase the biocompatibility of implants. Additionally, nanotubes and nanoparticles made from TiO_2 have started to come on to the technology scene as an alternative to carbon nanotubes. Multi-walled carbon nanotubes have been composited with TiO_2, which was shown to increase the photoactivity of the nanotubes (Cendrowski, 2014). TiO_2 also show stability and little to no degradation when exposed to a wide range of pH levels from 2-12, unlike porous silicon which starts to degrade significantly at pH's exceeding 8 (Mun, 2010).

Biosensor Limitations

As mentioned in previous sections, there are two different ways that can be utilized by a biosensor to collect information, *in vivo* and *in vitro*. Both of these classes of biosensors have limitations that may cause them to fail to accurately collect data or not operate correctly.

In vivo only limitations:

The first limitation for biosensors implanted inside the body is known as biofouling. Biofouling is when proteins, cells, and other objects accumulate on the surface of the biosensor and imped its readings. However, there are several ways in which this can be combatted by various methods such hydrogel coatings, diamond like carbons, and covalent attachments (Wisniewski, 2000).

In vivo and *in vitro* limitations:

Interference can be a limitation to both *in vivo* and *in vitro* biosensor. A physical barrier on the biosensor that inhibits the transducer producing a quality signal reception can cause interference. "Road block" interference" is another name for this phenomenon. It usually occurs when the outer layer of the biosensor has hydrophobic properties (Van Antwerp, 1998).

Interference

A further interference limitation of biosensors is specific to the enzymatic biosensor subset. As stated previously, enzymatic biosensors use the oxidation of peroxide to determine the quantity of the substrate present. This is problematic because the biosensor requires a relatively high current input to oxidize the hydrogen peroxide. This current can have a side effect of also oxidizing other electroactive substances that may be contained in biological samples, such as ascorbate and urate. This would cause the measurement reading to be higher than the true substance level (Rad, 2012). Additionally, there are relatively significant levels of hydrogen peroxide in human tissue that also has a potential to be oxidized by the biosensor (Halliwell, 2000). In summary, not all of the peroxide oxidized by the biosensor is the result of the targeted substance for measurement, causing a need for an established baseline.

Patents

In addition to the published findings that researchers have discovered with respect to how biosensors and the materials that make the up work, a great many patents have also been filed. The following two patents are examples of different ways that have been used to develop a biosensor that can detect glutamate.

A patent was filed in 2003, and issued in 2008 in the United States for a nanobiosensor that utilized carbon nanotubes. The patent for this biosensor, US Patent No 7399400 B2 details that this biosensor was originally designed to sense glucose. However, in the claims section of

the patent, it states that the biosensor could be modified to use glutamate oxidase instead of glucose oxidase in its redox reaction, which should allow the biosensor to detect glutamate. The patent also states that the usage of the carbon nanotubes as part of the biosensor caused the biosensor to have an enhanced sensitivity (Soundarrajan, 2008).

Another patent discovered was for a biosensor that is meant to detect glutamate in food, sometimes known as MSG. This patent WO Patent No 2007114650 A1 was filed and issued in 2007 with the World Organization. This biosensor was designed to utilize nanowires made from either silicone or zinc oxide to detect the electrons given off from an enzymatic reaction without using additional nanoparticles (Hong, 2007).

One lactate biosensor patent was also discovered, where the design was to detect levels of lactate in whole blood. This design utilized a layer-by-layer technique with carboxymethylcellulose (CMC), carbon nanotubes and lactate oxidase. These components were used successfully in a disposable biosensor for lactate in blood. This design improved upon a carbon electrode, which the authors acknowledged does have poor surface redox properties when compared to other electrodes. Utilizing the carbon nanotubes helped to amplify the signal from the reaction (“Biosensor”, 2003).

Chapter 3: Project Strategy

Overall Client Statement

For this project it is important to cater to the needs and desires of the client, while making sure the design solves the problem at hand and is possible in the time or budgetary constraints.

At the beginning of this project, the client statement was very broad. This client statement was: "Develop a practical biosensor enzyme coating to measure levels of extracellular glutamate consistently and accurately". While this gives a basic idea of the project, a more specific statement was developed after further research and discussion with the client.

From discussions with the client the important and novel component of the biosensor was specified to be the titanium dioxide electrode that would be used. Titanium dioxide is advantageous because of its great electron transport and sensitivity (Cendrowski, 2014). The project was also specified so that the biosensor would be designed to be used *in vitro*. This means non-invasive procedures for the patient with potential for future real-time monitoring of metabolite levels. It was also specified that metal nanoparticles would be used to further increase the sensitivity of the biosensor. These nanoparticles can be bound to the inside of the nanotubes.

In the next meeting with the client, the project was split between this group and a MQP group from the Chemical Engineering department. This led to the responsibility for the titanium dioxide nanotubes to be given to the Chemical Engineering group. Therefore, the focus of this project narrowed to characterizing and optimizing the enzymatic reaction. So, this led to an updated client statement: "Characterize, develop, and optimize an enzymatic reaction on nanoparticles for a glutamate and/or lactate biosensor. The biosensor will utilize titanium dioxide nanotubes and detecting levels of glutamate and/or lactate *in vitro"*. Detecting lactate was added

to the client statement because the Chemical Engineering group was originally going to develop a biosensor for lactate. After further research and discussing the options with the client, lactate was selected as the project's focus due to project limitations such as timeline and funds, leading to the final client statement: "Characterize, develop and optimize and enzymatic film for a lactate biosensor. The biosensor will utilize titanium dioxide nanotubes and detect levels of lactate *in vitro*".

Objectives

Primary Objectives

The primary objectives of the project are to make sure the biosensor: (1) can detect lactate, (2) is reliable, (3) is user friendly, (4) is adaptable, and (5) is non invasive. These objectives can be seen in Figure 5.

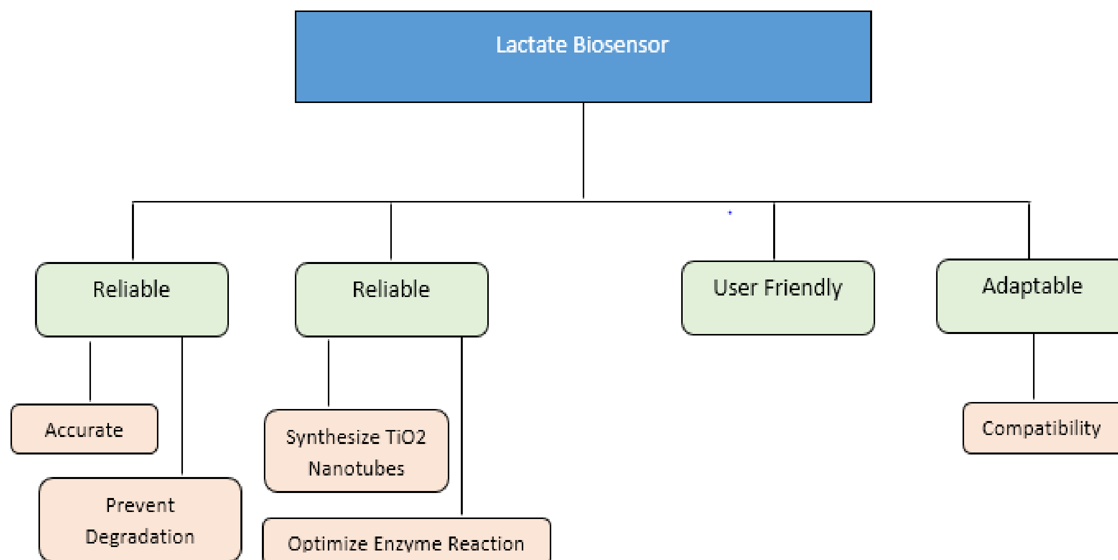


Figure 5: Objective Tree

Detect Lactate: It is important for the purposes of this project for the sensor to detect lactate. The different sources that lactate will be detected from are known and unknown lactate solutions, and from *in vitro* cell cultures. This will be the same for lactate. However, future applications of this kind of biosensor may be *in vivo*, and therefore invasiveness and biocompatibility are

significant concerns. Methods and designs for this project should be done in consideration of this. Using the biosensor *in vitro* is ideal because it is non-invasive and this can warn the patient of any lactate system irregularities in their brain.

Reliability of lactate detection: Our next objective is to validate the reliability of the biosensor. This biosensor should detect any amount of lactate in the blood. For detecting lactate the sensor should be able to distinguish between mmol/L levels of lactate, as anything above 2mmol/L is considered abnormal (Goodwin, 2007).

User Friendly: The next objective of this project is to make sure the biosensor is user friendly. The reason for making the biosensor user friendly is so the product can be distributed without any required specific training. This will save money and resources in potential clinical applications if no staff has to take time to become familiar with the sensor. User-friendliness is also important if future use is by the patient. They will have to safely and properly use the sensor based on a set of written instructions and any suggestions of their physician.

Non invasive: The biosensor is used to help diagnosis illness within people, so it is important that it is not invasive, which may lead to complications that actually cause a problem when the purpose of the procedure was to prevent a problem.

Adaptability: The final objective in the project is to make sure the enzymatic reaction is reproducible and relatively simple as well efficient and stable. The reason for this is so that other molecules that indicate brain disease or cancer, such as glutamate, can be sensed with similar accuracy. The ranked objectives can be seen in Figure 6.

Ranking the Primary Objectives

Objective	Detection	Reliable	Adaptable	User-friendly	Total Score
Detection	X	1	1	1	3
Reliable	0	X	1	1	2
Adaptable	0	0	X	1	1
User-Friendly	0	0	0	X	0

Figure 6: Primary Objective Ranking Chart

The rankings for the objectives are based on what was deemed to be the most important. A table of objectives ranked 1-4 is shown in Figure 7. The most important part of this project is the detection of lactate, which is why it is the top ranked objective. Reliability was the objective that was determined to be second most important. The sensor should not miss any lactate in the solution, cells, or blood that it is tested with. It is equally important that false reading is never given; the biosensor should be very reliable. The third ranked objective was that the device be non-invasive. By being non-invasive, the device will not cause adverse effects in the patient, such as inflammation. Ranked fourth out of five was that the biosensor be potentially adaptable. This is important if any changes need to be made throughout the project or if the sensor has potential for use with different substrate in the future. The final objective is user friendliness. This biosensor should not require any significant amounts of training beyond what a qualified person should already have. In the future this could be expanded to patients.

Objective	Rank
Detection	1
Reliable	2
Adaptable	3
User-Friendly	4

Figure 7: Objective Ranking

Secondary Objectives

The following are the secondary objectives of the project. The secondary objectives are objectives that are consist of what needs to be realized to achieve the primary objectives of the project.

Optimize enzyme reaction: The enzyme reaction is critical to the detection of the lactate, as the reaction is what breaks the enzyme down a form that can be detected (electrons).

Synthesize TiO_2 nanotubes: The TiO_2 nanotubes will act as an electrode, outputting a signal based on the quantity of electrons, or current level, produced by the enzymatic reaction. These nanotubes will by synthesized by the work of the Chemical Engineering team.

Accurate: the biosensor must read the correct level of the lactate within a small percent error in order to be useful.

Prevent Degradation: The materials (enzymes, nanoparticles, nanotubes, etc.) that make up the biosensor must retain their properties for a minimum period of time. The biosensor must be capable of working properly for that minimum time period to be considered useful. Manufacturers of glutamate and lactate biosensors typically guarantee the biosensor to last and perform optimally for 21 days after the shipment of the sensor is received (Pinnacle Technology). Taking this into account, as well as the known degradation rates of the chemicals on the sensor, the longevity of our biosensor should be roughly 25 days. After 25 days, these types of biosensors are not guaranteed to perform correctly and may not necessarily detect trace amounts of glutamate or lactate. However, once the sensor has been used, it will typically only last an additional 5 days while maintaining the expected reading parameters.

Compatibility: For the purposes of this project, this biosensor must be capable of sensing lactate and be able to have its design modified for glutamate. Therefore the enzymatic reactions to detect both glutamate and lactate must both be similar. For example, the reaction of glutamate or

lactate with their respective oxidase enzymes would both need to produce the same amount of hydrogen peroxide per molecule of glutamate or lactate reacted. Otherwise the biosensor readout would have to be recalibrated for what each current level meant if lactate and glutamate produced different amounts of peroxide per molecule reacted.

Ranking of Secondary Objectives

Through discussions with the client, the secondary objectives were ranked as follows in Figure 8:

Objective	Optimize	Sythesize	Accurate	Prevent	Companatability	Total Score
Optimize	X	(1/2)	1	1	1	3(1/2)
Sythesize	(1/2)	X	1	1	1	3(1/2)
Accurate	0	0	X	1	(1/2)	1(1/2)
Prevent	0	0	0	X	1	1
Compatability	0	0	0	0	X	(1/2)

Figure 8: Secondary Objective Ranking Chart

There was a 2-way tie for most important secondary objective. We ranked these objectives for many reasons. First we have optimizing enzyme reaction and the reason is because the enzyme reaction is critical to the detection of the lactate, as the reaction is what breaks the enzyme down a form that can be detected. Also tied for first is synthesizing TiO_2 nanotubes and the reason for this is it will act as an electrode, outputting a signal based on the quantity of electrons, or current level, produced by the enzymatic reaction. We also want it to be accurate so this came in as our third objective. Ranked second to last is "prevent degrading". The last objective with a very low score was compatibility. We did not think this was as critical to our biosensor. The rankings are shown in Figure 9.

Objective	Rank
Optimize	1
Sythesize	2
Accurate	3
Prevent	4
Compatability	5

Figure 9: Secondary Objective Ranking

Project Constraints

In order for a successful completion of the project, it must stay within several constraints as defined by the project team and the client.

Budget: The team is limited to a budget of $450.

Time: The entire project including optimizing the enzyme reaction, data collection, analysis, and completion of the written report must be complete by the end of the 2015-2016 academic year. A separate MQP project will focus on developing the titanium dioxide nanotubes concurrently with the nanoparticle enzyme optimization accomplished in this project. Proof of concept testing cannot occur until both the nanotubes and nanoparticles are complete.

Sensitivity level: The main purpose of this project is to optimizing an enzyme reaction that will be able to detect lactate for differences between mmol/L levels.

Ability to use: The device must be within the capabilities of the members of this project team to operate and analyze.

Equipment: As a result of time and budget constraints the MQP team is limited to the types and models of equipment found in Professors Jain and Zhou's labs for construction and data testing. There was also a limitation with the availability of the AUTOLAB technology in Professor

Zhou’s lab, as our team required someone with access to let us on the system as well as be there to monitor testing.

Project Approach

The purpose of this project was to develop an *in vitro* method of detecting metabolites produced by a growing brain tumor. This was further narrowed down to the utilization of an enzymatic coating with a biosensor to achieve this purpose. Several steps were taken to achieve this goal: research, design, testing, data analysis and design improvement, future direction, and compilation. This project had a time limit of approximately 28 weeks in which to accomplish these steps.

Research

It was important to gain as much knowledge as possible after establishing the goal of the project. There were three major topics of research required to gain a sufficient understanding of the need and requirements. The first topic was brain cancer, with a focus on glioblastoma. It was important to understand the prognosis of those affected by these tumors, how the tumors are currently detected, and the current treatment options. The second topic of research was on enzymatic coatings. This research looked into different enzymes capable of reacting with metabolites produced by brain cancer and different materials capable of immobilizing the enzyme to a biosensor surface without impeding function. The third topic of research was on biosensors. The biosensor research included determining their potential uses, the different types, and methods of testing the functionality of a biosensor, including expected data outputs from these tests.

Design

The next step was the design process. The initial client statement was modified for the first time, based on the increased knowledge gained by the research contained within the

literature review. By interviewing with the clients, Dr. Jain and Dr. Zhou, the objectives, constraints, requirements, and functions of the project were sharply defined. This allowed the development of a final client statement, from which the next part of the design process began.

The next part of the design process was developing several design options for the development of an enzymatic biosensor. This focus was mostly on the materials to use in the immobilization of the enzyme. Due to the limited budget size and time, it was imperative to compare the positive and negative attributes of each design thoroughly prior to building a prototype. A final design would be selected, following consultation with the advisors, by December 17, 2015.

Development of Prototype

The building of the prototype of the chosen biosensor design and utilizing different testing methods for validation were conducted concurrently starting on January 14, 2016. The purpose for approaching the project in this way was due to the materials involved. As an example, it was important to perform material properties testing on the enzymatic immobilization material, to determine if it was prepared properly. This concurrent testing was important to the later step of validating the design, as it provided a system of checks aimed at preventing data becoming tainted by experimental error. The prototype development was concluded by March 14, 2015.

Testing

Following the successful building of the prototype of the selected design, more testing was performed to determine its functionality. The first set of testing was a contact angle analysis, as previously stated, to determine if the developed enzymatic coating formed properly. Secondly, an assay was performed, modified to compare the mixture of enzyme and immobilization

material with the pure enzyme. This would determine if the enzyme could remain active within the biosensor coating.

The next tests were electrochemically based. The first of the electrochemical tests, cyclic voltammetry, would determine if and where there was an optimal voltage at which the biosensor detected the desired substrate. The second test, amperometry, determined the sensitivity of the biosensor to different concentrations of the desired substrate. This was important, as the final client statement specified being able to detect levels of lactate, not just whether or not lactate was present. The final electrochemical test was a real time amperometry test, where the level of lactate was increased at set intervals throughout the test. This was to determine if the data outputs for detected lactate concentrations were set only by the enzymatic reactions occurring at the beginning of the test or if the biosensor was capable of changing its readout accordingly with increased lactate concentration, within the same test.

Data Analysis and Design Improvement

The data produced by each type of test was compared to the expected values and trends found during the literature review. It was expected that any test had the potential for outliers. Therefore, due to the limited duration of the project, the most emphasis was placed upon data that significantly contested the theoretical results. Possible causes for the unexpected results were researched in literature. Following this the prototype was rebuilt and subjected to a second round of the same tests. The new data from each test was then compared to the previous data and literature to determine if there was improvement. This cycle continued until any fixable issues were resolved with the design and construction of the prototype.

Future Works

As previously mentioned, limited time and budget were highly significant factors in the process of completing this project. Therefore the final step was to use the implications of the

analyzed data, along with published literature, to determine possible directions that this technology could take in future work. This is significant as it further solidifies the conclusions formed from the analyzed data and provides a platform for the beginning of a new project to work off of, rather than starting from scratch.

Compilation

All of the previous steps were to be completed by April 18, 2016. They were compiled into a final project report over the entire course of the project. This project report was submitted on April 28, 2016.

Chapter 4: Methods and Alternative Designs

Materials

A simple polypyrrole layer serves the purpose of being a conducting polymer. Advantages of this material as a conducting polymer include its biocompatibility and that it is thermally and environmentally stable. One significant advantage of polypyrrole on its own as a conducting polymer is its ability to directly receive and transduce signals from the enzymes of the substrate (Ramanavičius et al., 2005). In addition, polypyrrole is known for its ability to reduce interference from sources outside of the biosensor film (Ramanavičius et al., 2005). The ability of polypyrrole to form nanoparticles is also advantageous in some biosensor applications. The major limitations associated with polypyrrole as a biosensor material are its physical properties, which can make it difficult to work with in the creation of a biosensor layer. For example, polypyrrole has poor ductility, oftentimes making it difficult to process and implement into the system (Chen, 1995).

Another film option for this biosensor is chitosan. Chitosan functions to entrap enzymes, allowing for less interference and allows for the achievement of a better and more accurate reading. With polypyrrole, this will work well with a conducting polymer because the enzymes will be obtained with little interference, and the polypyrrole will be able to conduct a signal for interpretation of the sample results. The most notable advantage of using chitosan and polypyrrole together in a biosensor is the increased sensitivity and accuracy, making for reproducible and accurate results (Senel, 2015). Together, these materials will also create a porous surface, mostly attributed to the chitosan, and this is good for trapping test enzymes. Limitations of a polypyrrole layer with chitosan include the potentially poor physical properties of polypyrrole, especially in acidic solutions. In fact, chitosan layers have been found to break down in acidic environments over short periods of time (Islam, 2012). While this break down

may be problematic for this material, modifications to chitosan (such as an polymer coating) can be made to decrease the rate of the degradation in an acidic environment (Islam, 2012).

Our biosensor will be detecting levels of lactate. Lactate oxidase (or lactate dehydrogenase) will be in the biosensor film to aid or enhance detection by the electrode of charge proportional to the level of substrate. Adding HRP may help to amplify this reaction, and will be determined through testing whether or not it is an effective addition to the sensor design (Senel, 2010).

Crosslinking, as mentioned in Chapter 2, is a method of enzyme immobilization. There are two common materials used in the immobilization process, Poly(ethylene glycol) diglycidyl ether, also known as PEGDE, and glutaraldehyde. Crosslinking using PEGDE is less harsh on the enzymes than the glutaraldehyde fixation. Therefore the sensitivity of the enzymatic biosensors utilizing PEGDE for glutamate detection is more sensitive than biosensors that use glutaraldehyde (Vasylieva, 2011).

The cost of the materials in film designs is in Appendix C.

Alternative Designs

The first design chosen was a layer of polypyrrole mixed with lactate oxidase (or lactate dehydrogenase) as shown in Figure 10. This was chosen as is the simplest type of polymer-enzyme coating to develop as the lactate oxidase is simply mixed with the. The polypyrrole-enzyme is deposited on top of the TiO_2 nanotubes electrode using electrophoresis (see Appendix A for protocol). Through further research, the layer-by-layer method of coating was determined to be a superior method.

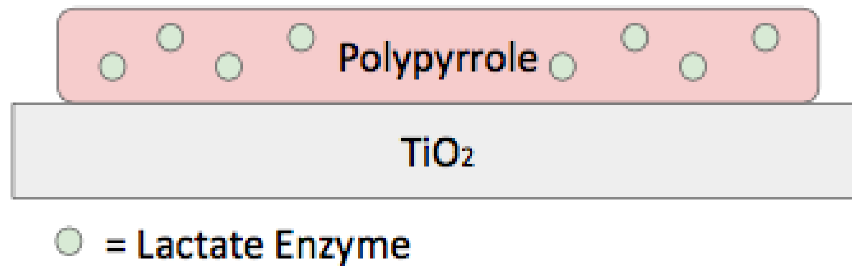


Figure 10: Single Polypyrrole Layer Design

In order to incorporate beneficial properties of different materials into our design, layer-by-layer coating was considered. This coating design would have different materials layered on top of another. In a layer-by-layer coating method, each component of the coating is applied as its own layer to the surface of the biosensor. The main force behind the layer-by-layer method of coating biosensors is the electrostatic force. Electrostatic force utilizes oppositely charged layers of polymer films, alternating from positive to negative in each layer. The opposite charges do not have to be equal for layer-by-layer coating to be effective (Zhao, 2006).

One example of a layer-by-layer coating is on a glucose biosensor utilizing a gold electrode. The enzyme used in this biosensor, glucose oxidase, is a polyanion, meaning that it has a negative charge. The Ferrocene poly(allylamine) (FcPAA) is a polycation, which serves as the positive layer. The surface of gold electrode is coated with a monolayer of mercaptan to give the surface a negative charge, which is attractive to the FcPAA. It was not stated why the mercaptan was used instead of the negatively charged enzyme for the first layer. The ferrocene component of the polycation layer is additionally important, as it is known to be a catalyst for redox reactions. This is the type of reaction that provides the sensor with a reading (Harper, 2010). This article mentioned that the major advantage of using the layer-by-layer is to immobilize the glucose oxidase to the electrode of the biosensor without inhibiting it. The three dimensional structure of the glucose oxidase is unchanged and its overall activity level (rate of

glucose catalyzed) is only slightly reduced, as opposed to other more damaging methods (Harper, 2006). An interesting take on another layer-by-layer biosensor was made using polypyrrole and single-walled carbon nanotubes. The nanotubes and polypyrrole are the layers on a platinum-coated polyvinylidene fluoride (PVDF) membrane. Both the nanotubes and the polypyrrole (PPy) layers were coated to the biosensor while dissolved in different solutions. This biosensor was prepared in two-layer (nanotubes-PPy), three layer (PPy-nanotubes-PPy), and four-layer (PPy-nanotubes-PPy-nanotubes) formations, after which each was soaked in a glucose oxidase solution to adsorb the glucose oxidase to the surface. The major advantages of the layer-by-layer method are that it allows the positive qualities of both the nanotubes and the PPy to enhance the biosensor because of the specific formation. The environment and thermal stability of PPy and the electrical conductivity and chemical stability of the carbon nanotubes may not properly contribute if they are applied in the wrong way (Shirsat, 2007).

To apply these advantages to a film design for our biosensor, layering chitosan and polypyrrole was considered. The next design considered was adding a film of chitosan to the polypyrrole film mixed with lactate enzyme (oxidase or dehydrogenase). In the initial stages of gathering information for the layer-by-layer technique, chitosan was considered as a potential polymer to be used in conjunction with the polypyrrole in a layer-by-layer setup shown in Figure 11.

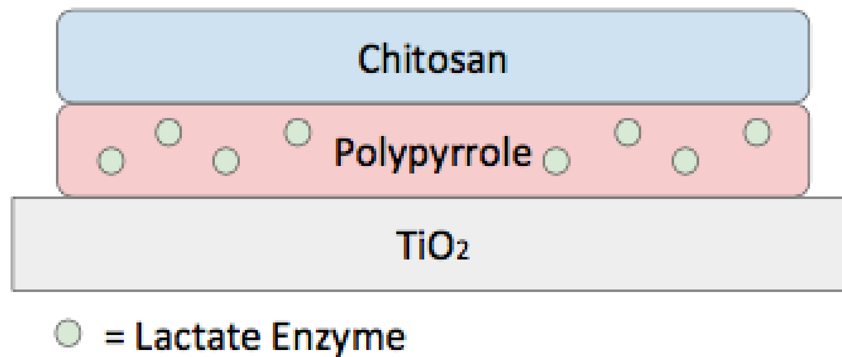


Figure 11: Polypyrrole and Chitosan Layer-by-Layer Design

The intent was for the chitosan to reduce possible interference. However, through further research, it was determined that both the polypyrrole and the chitosan are positively charged. In order for the layer-by-layer technique to work properly, a polycation (positively charged) and polyanion (negatively charged) for electrostatic forces to apply. Therefore, if the layer-by-layer procedure were performed with two positively charged materials, such as polypyrrole and chitosan, no electrostatic binding would occur between the layers. Subsequently, it is likely the layers will separate.

Because the layer-by-layer design with chitosan and polypyrrole was not feasible as described above, the next inclination was to design a film using a polypyrrole and chitosan composite material. This is shown in purple in Figure 12. Through further research, it was found that a composite of these two materials would be difficult to fabricate and optimize for our design in the time restraint we have (Huang, 2013). Making the composite would result in a hydrogel with exceptional water absorbency, but for our applications this composite is not ideal or practical (Huang, 2013).

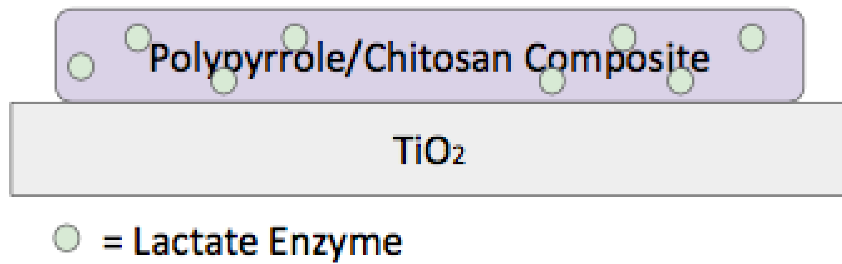


Figure 12: Polypyrrole and Chitosan Composite Design

The next film designs are each variations on one another. These designs are chosen as the "ideal" designs because they all utilize cross-linking the enzyme (lactate oxidase or lactate dehydrogenase) to increase the time before the enzymes are desorbed, and therefore increasing the life of the biosensor. PEGDE was chosen as the crosslinking agent because it is less denaturing to the enzyme as commonly used glutaraldehyde. This denaturing can cause an enzyme to become unspecific, and bind to similar reactants besides the target reactant. One study compared PEGDE to other enzyme entrapment materials in enzyme specificity. The results are shown in Figure 13.

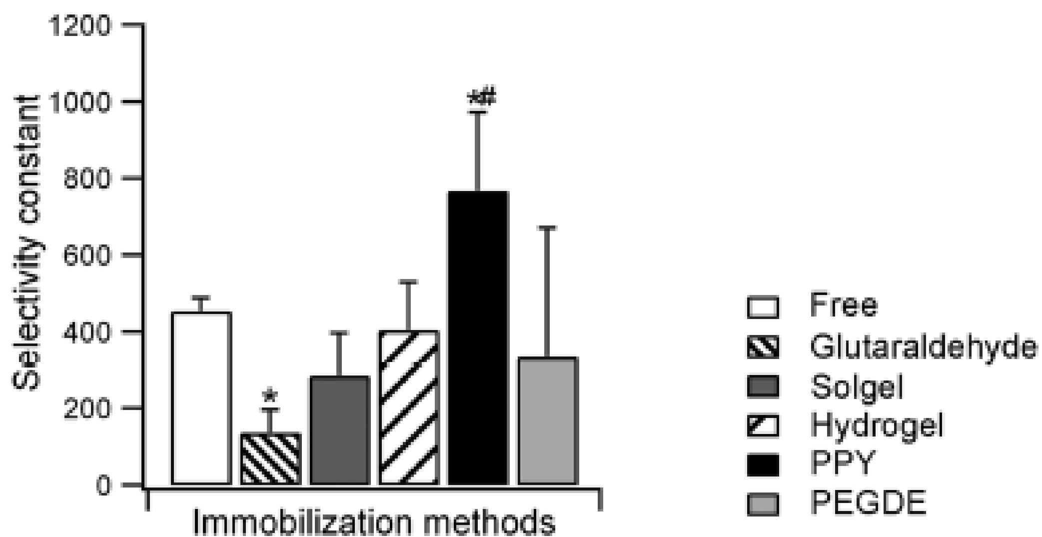


Figure 13: Enzyme Specificity Comparison (Vasylieva, 2011)

The higher the selectivity constant represents the impact of the immobilization technique on the selectivity of the enzyme. PEGDE has less impact on the selectivity of enzyme than glutaraldehyde.

These designs also utilize the properties of polypyrrole and chitosan. These design alternatives will all be tested to determine which film is optimal for the biosensor.

The selected device design shown in Figure 14 is a variation on the traditional layer-by-layer technique. The first layer is the polycation, polypyrrole, which is deposited onto the nanotube surface using electrophoresis (See Appendix A). Then the next layer, a polyanion lactate enzyme cross-linked with poly(ethylene glycol) diglycidyl ether (PEGDE) is deposited on top of the polypyrrole layer (Appendix A).

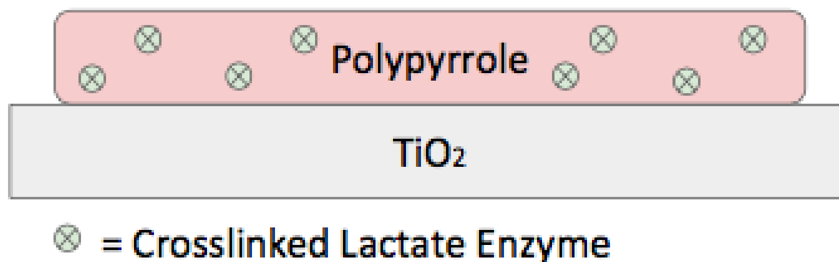


Figure 14: Single Polypyrrole Film and PEGDE Crosslink Design

The design in Figure 15 is an addition on the design of Figure 4. This design would have another layer of polypyrrole deposited on top of the PEGDE cross-linked lactate enzyme.

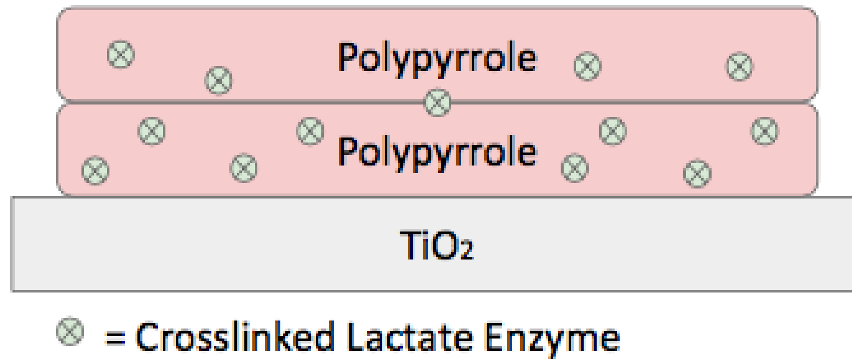


Figure 15: Double Polypyrrole Film and PEGDE Crosslink Design

The design in Figure 16 is a variation on the traditional layer-by-layer, similar to Figure 14 except chitosan is the polycation layer, in place of the polypyrrole. The chitosan is the first layer deposited on the surface of the TiO_2 (see Appendix A). Then the polyanion PEGDE cross-linked lactate enzyme will be deposited on top of the chitosan layer.

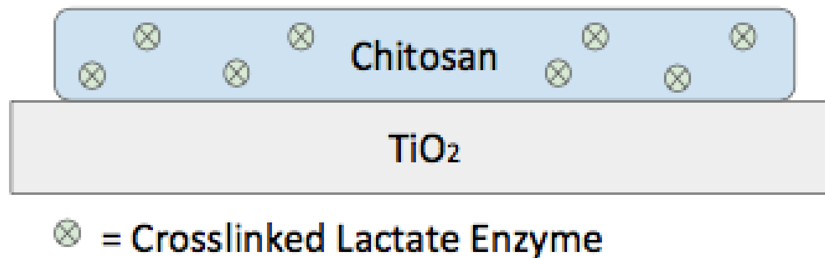


Figure 16: Single Chitosan and PEGDE Crosslink Design

The design in Figure 17 is an addition on the design of Figure 16. This design would have another layer of polypyrrole deposited on top of the PEGDE cross-linked lactate enzyme.

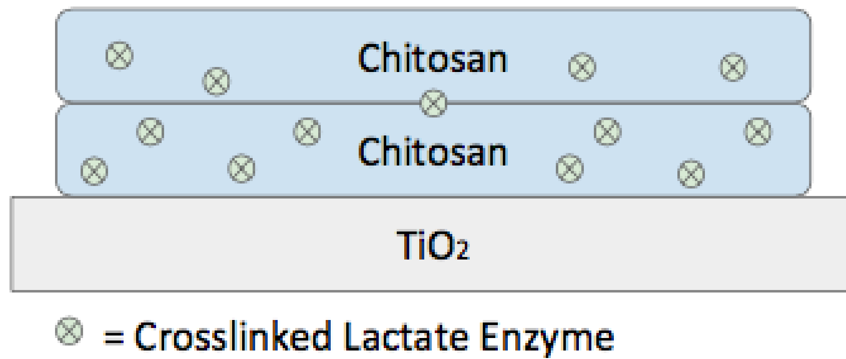


Figure 17: Double Chitosan and PEGDE Crosslink Design

Final Film Design

Our final film design was chosen to be lactate enzyme mixed within a chitosan film applied to the titanium dioxide electrode (Figure 18). Chitosan was a chosen material due to its ability to prevent interference from cells (for application beyond simple testing in substrate solution) and to prevent enzyme leakage (Senel, 2015). The data shown in Figure 13 also shows that polypyrrole significantly reduces the specificity of the enzyme, and chitosan has potential to perform better than this because it is a natural polysaccharide. This coating method is simple enough to synthesize many films to test in the amount of time we are allotted to complete this project, and materials needed are already mostly available in the lab, which allows us to reduce our spending. This kind of film will allow us to characterize chitosan as a material for this application, and evaluate its effectiveness with this kind of electrode. We also chose this design because it is easily adaptable to incorporating glutamate oxidase for detection of glutamate in future films and tests.

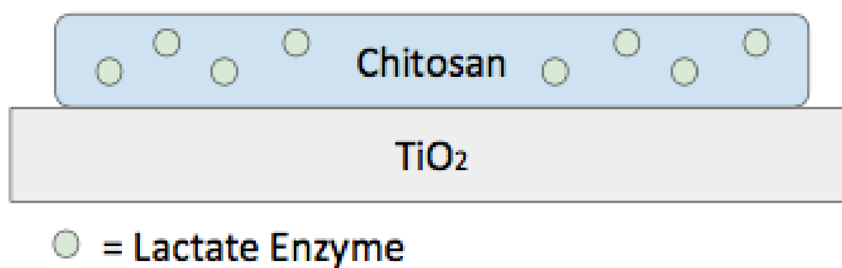


Figure 18: Single Chitosan Layer Design

Due to the fact that the titanium dioxide electrode was not available to coat in time to test, the films were applied to a glassy carbon electrode.

Chapter 5: Design Verification Results

Contact Angle

The chitosan mixture was tested by contact angle analysis. 30µL of the chitosan solution was deposited on a glass slide three times. It was important to perform the test in triplicate for statistical significance. The measured contact angles were compared to a chitosan film of a similar concentration in literature. The chitosan was also inspected for its consistency, to insure that it had the viscous behavior detailed in the literature. A photo (Figure 19) was taken of the water droplet within 20 seconds of its placement on the dried chitosan film. It sometimes took this long due to the difficulty of making the camera focus on the water droplet, which was very small.

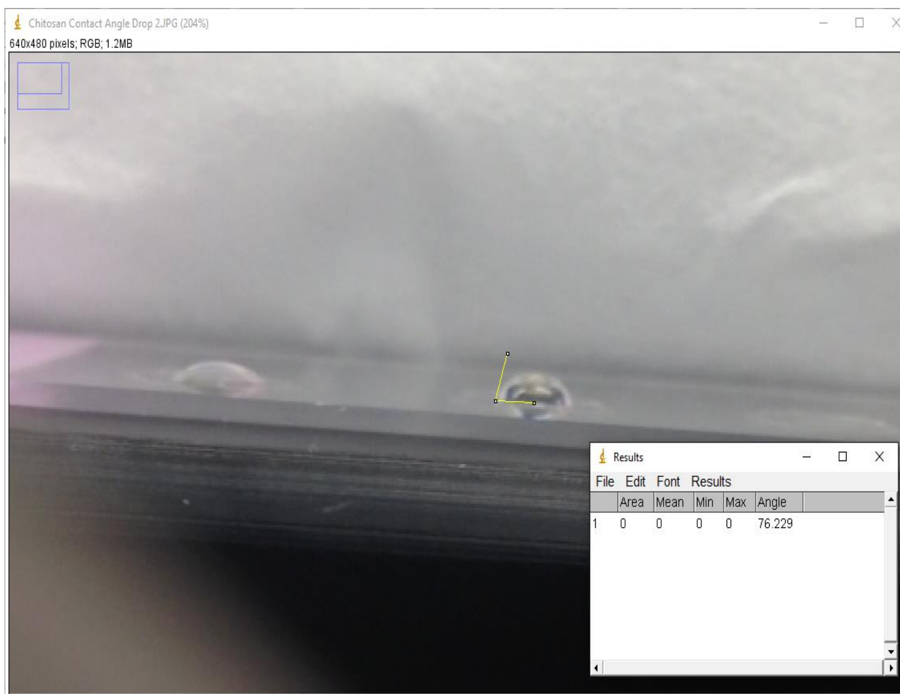


Figure 19: Chitosan Contact Angle

The three contact angle measurements calculated with Image J were 72˚, 76˚, and 55˚.

Cyclic Voltammetry

The cyclic voltammetry tests changed somewhat over the course of the project. It was important to make sure that the voltage span included the potential where the electrode is most

sensitive to detecting the enzymatic reaction. The final voltage range to be settled on for testing was the same the as the group developing the TiO_2 nanotube, -0.4V to 0.8V, however because the titanium dioxide electrode was not readily available for testing, all electrode testing was done on a glassy carbon electrode. The scan rate was 0.1 V/s. Film that contained lactate oxidase and film that contained lactate dehydrogenase were both tested. The results for the lactate oxidase are shown in Figure 20 and the results for the lactate dehydrogenase are shown in Figure 21.

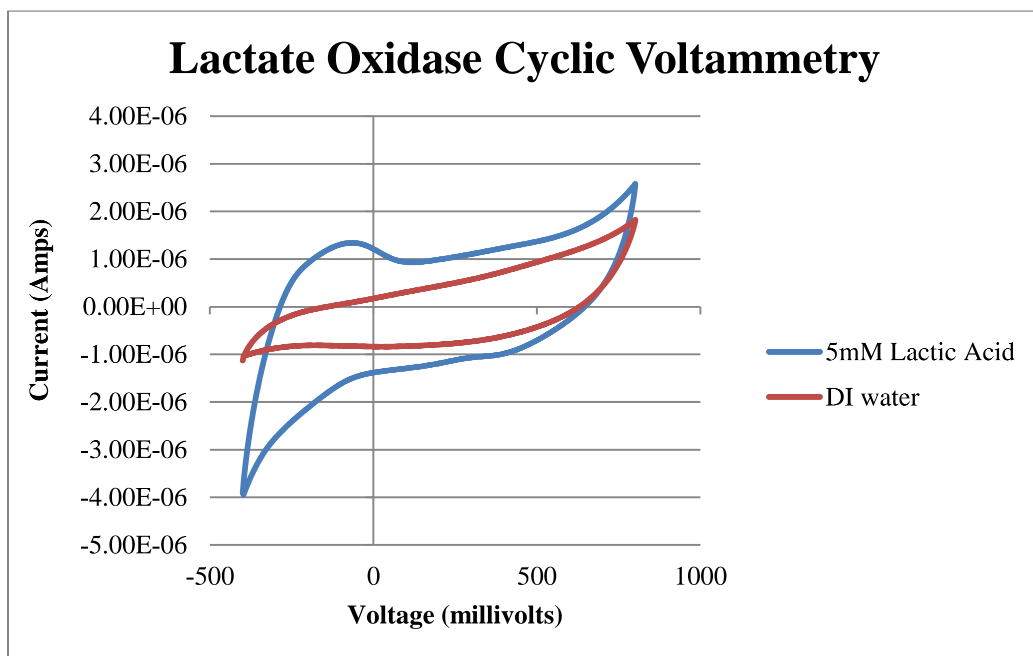


Figure 20: Lactate Oxidase Cyclic Voltammetry

At approximately the -0.2V mark there is a prominent peak in the current for the lactate oxidase coating, indicating a peroxide reaction. For the lactate dehydrogenase, there was a temporary plateau at the -0.2V mark, but no significant peak formed as in the lactate oxidase.

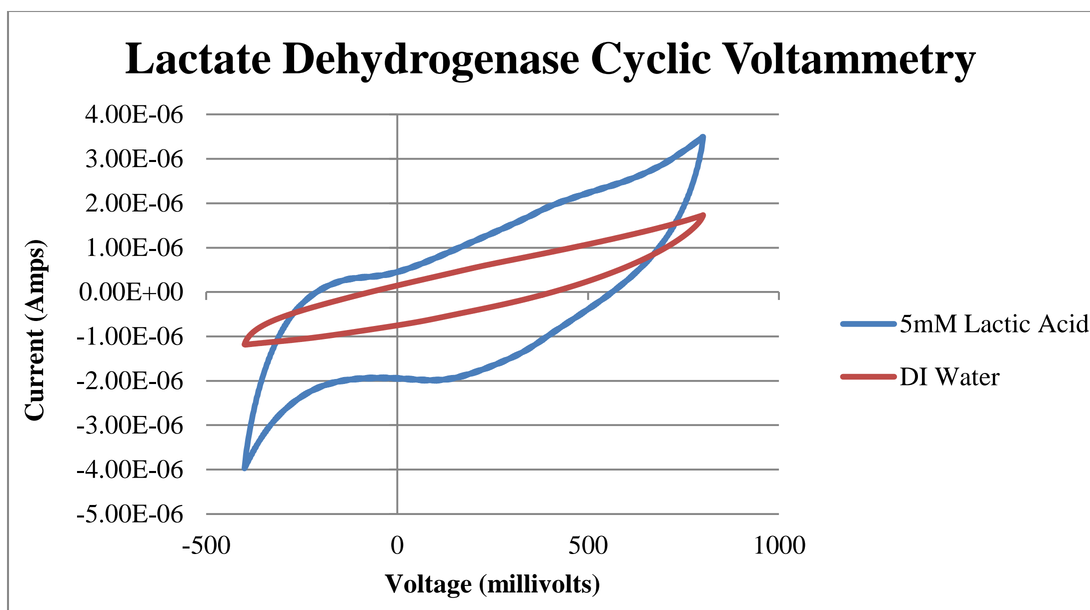


Figure 21: Lactate Dehydrogenase Cyclic Voltammetry

Amperometry

The next tests performed were amperometry at different concentrations of lactic acid in solution: 0, 100µM, 500µM, 5mM. The amperometry tests were conducted for 120 seconds on each concentration. The order of testing proceeded from the lowest concentration, the deionized water, to the highest concentration, a 5mM solution of lactic acid. The electrode was rinsed with deionized water between each test. Great care was taken with the rinsing to protect the coating from removal. These tests were also done with both lactate dehydrogenase (Figure 22) and lactate oxidase (Figure 23).

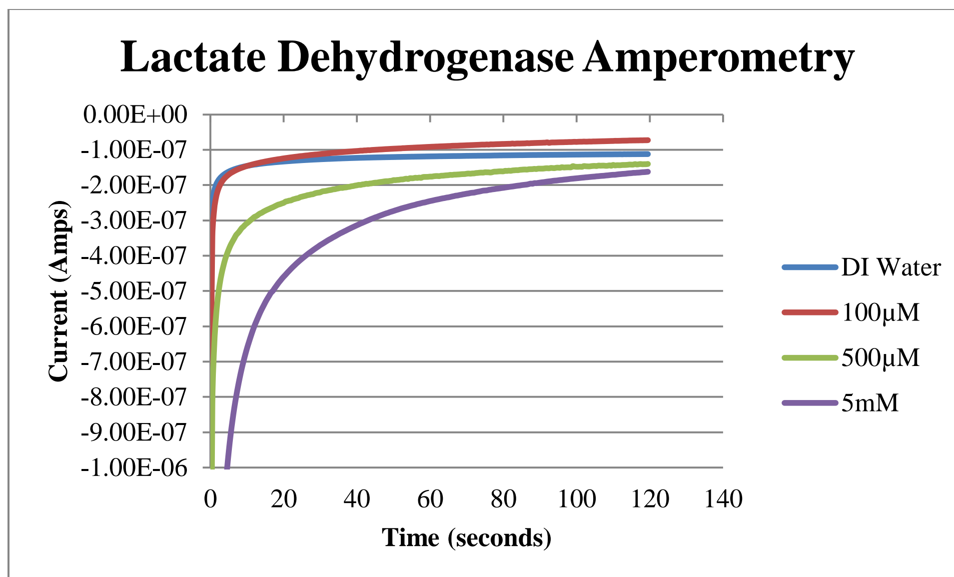


Figure 22: Lactate Dehydrogenase Amperometry

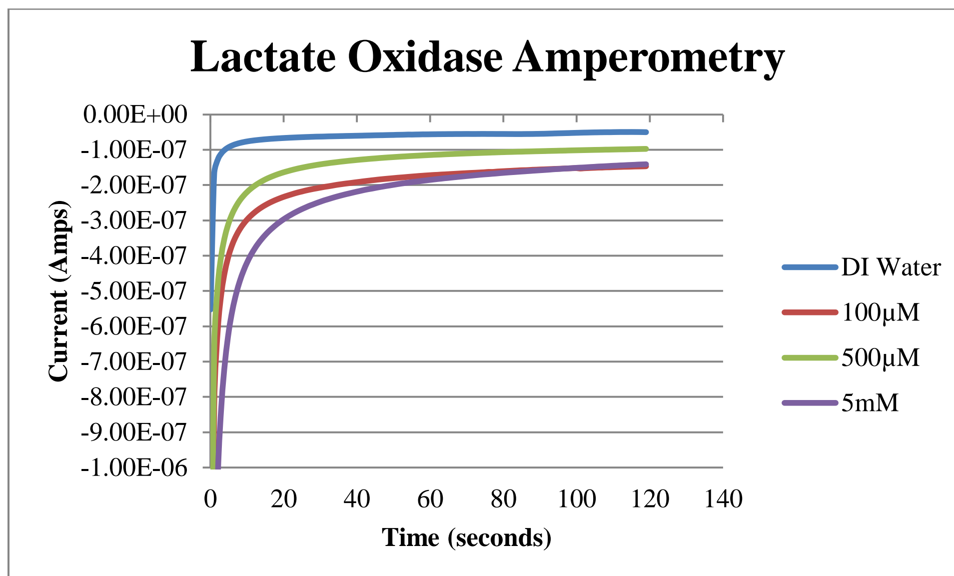


Figure 23: Lactate Oxidase Amperometry

The constant current was set to -0.2V based on the peak or plateau that formed at this potential in both enzymatic coating. As the concentration increases for both the lactate oxidase and dehydrogenase, the general trend of the absolute value of the current also decreases. However, in the lactate dehydrogenase tests, the 100µM concentration was an outlier, showing lower current than the deionized water. Additionally, in the lactate oxidase testing, the 100µM

concentration was also an outlier as it outputted a higher current than the 500µM concentration, and was only slightly lower than the 5mM concentration coating.

Real Time Testing

The real time testing of the electrode was performed using an amperometry test. This test used the same settings as the concentration testing amperometry. The test began with the electrode submersed in deionized water. At the 30 second mark 50µL of a 9.4M lactic acid solution were added to the deionized water. It was important to attempt to not touch the electrode setup with the micropipette, to prevent changes to the current flow not caused by increased lactic acid concentration. A second 50µL volume of 9.4M lactic acid solution was added to the testing solution at the 70 second mark. The lactate dehydrogenase (Figure 24) showed a small dip when the first amount of lactic acid was added. Following the second lactic acid addition, the current significantly increased and remained stable.

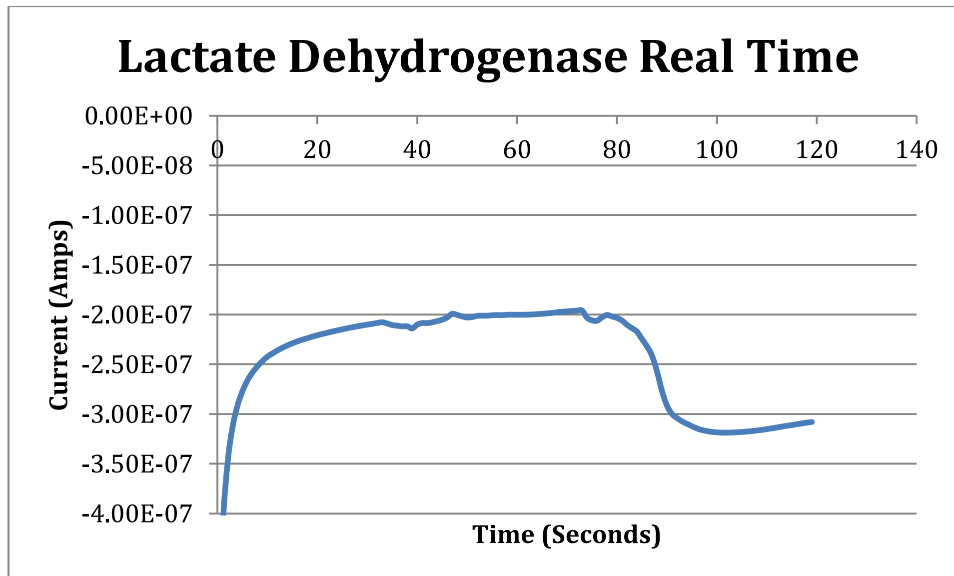


Figure 24: Lactate Dehydrogenase Real Time Test

The lactate oxidase (Figure 25) coating showed several spikes of increased current output after the first quantity of 9.4M lactic acid was added, along with a single current spike after the

second amount of lactic acid was added. However, unlike the lactate dehydrogenase coating, the current did not stay increased, but leveled out higher than the initial deionized water baseline.

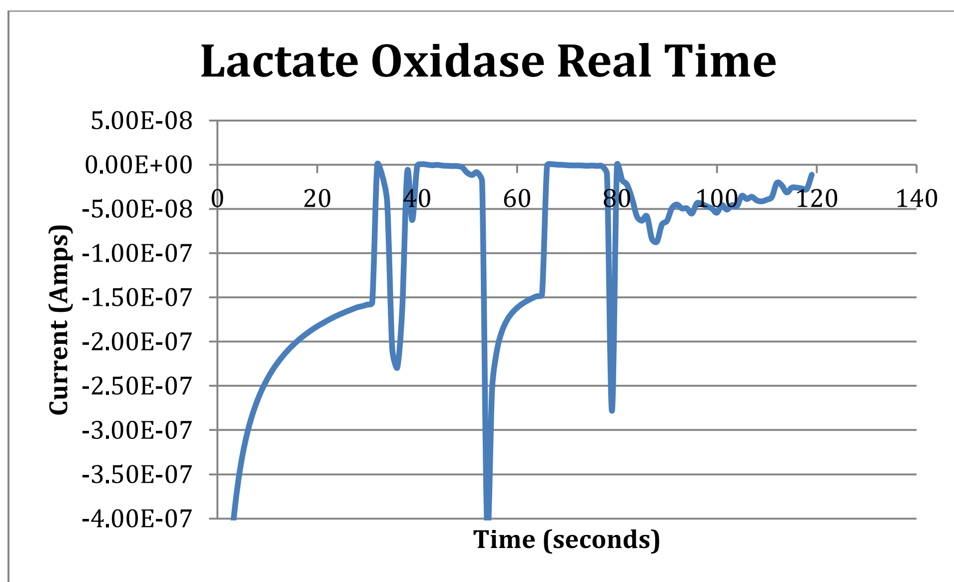


Figure 25: Lactate Oxidase Real Time Test

Colorimetric Lactate Assay Kit Testing of Chitosan Film

In order to test the viability of the enzyme in the chitosan film, an assay was developed to test for enzyme functionality using the lactate assay kit purchased through Abcam. While a detailed protocol for this assay can be found in Appendix B, chitosan film was made and put into wells in 20ul volumes. Three wells contained chitosan without enzyme, and three wells contained chitosan with enzyme. The films were allowed to dry in the fridge for a few days to preserve the enzyme, and then the films were blocked with BSA and incubated in lactate at a 6nmol/well concentration. The hope is that the BSA would block most of the potential lactate binding sites in the chitosan and the lactate could bind to the enzyme when incubating after the block. The assay was read on a plate reader at 450nm, and the absorbance values for the standards in this assay are shown in Figure 26. The standard bar graph shows a linear trend, which indicates the assay was run properly.

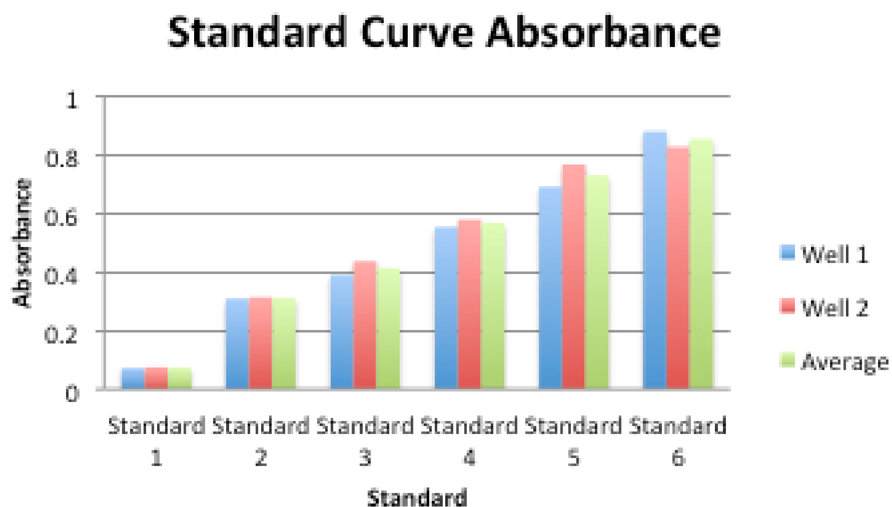


Figure 26: Standard Curve Absorbance

Figure 27 shows the absorbance values of both kinds of chitosan wells compared to other wells that aid in the understanding of the results. Standard 1 is a control with only buffer and reaction mix, showing that the chitosan films did at least contain some lactate above this level. The background control also provides a similar analysis. Standard 4 has lactate at the same concentration as what was put in the chitosan wells. This shows that the levels of lactate left in the wells after the incubation period was poor. Finally, there isn't a significant difference in this assay between the lactate levels in the chitosan without and with enzyme.

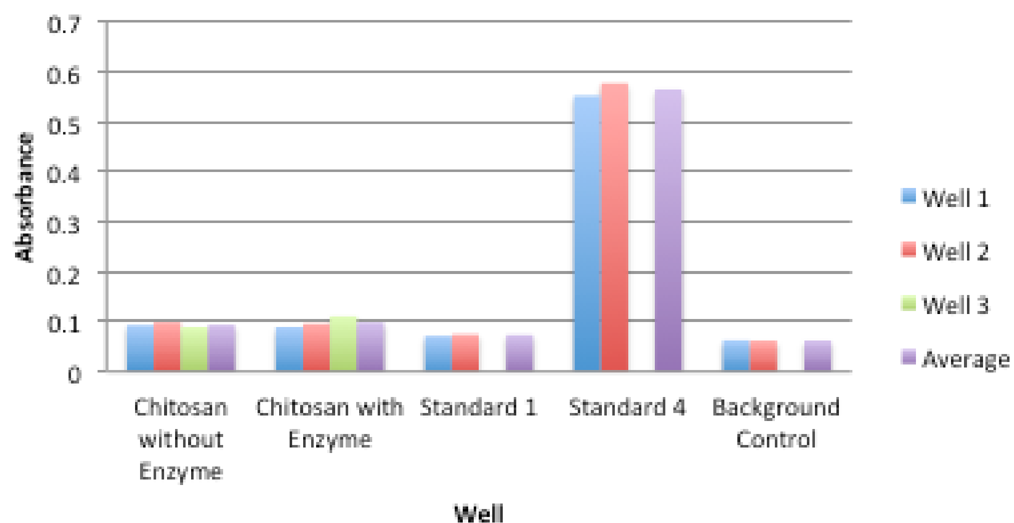


Figure 27: Chitosan Wells Compared to Relevant Wells

Chapter 6: Discussion

Validity Testing

There were multiple steps in coating development process that were tested for validity prior to testing with the Autolab machine. The initial step was to cross-reference the different possible biomaterial coatings with their enzyme and electrode compatibility, as detailed in Chapter 3. This was important because, as detailed in the budget section, the limited funds meant that there was little room for error by purchasing a material that would have absolutely no chance of working.

The first in lab validity testing was to test the contact angle of the chitosan solution. The results of the contact angle measurements showed that the dry chitosan was relatively hydrophobic. To improve the accuracy of this test, the literature that was used for the comparison also contained data on how the contact angle measurement of the the chitosan film changes over time after the water droplet (~10μL) was deposited. This was useful because there was difficulty in getting the camera to focus on an area as small as the each of the water drops. Therefore 10 to 20 seconds may have passed between deposition and capturing the image (Farris, 2011).

The second test was to perform a lactate assay, modifying the procedure to compare the mixture of 15:20 ratio of lactate dehydrogenase to chitosan solution with different standards. While the chitosan samples did have a stronger absorbance than the background wells, there was no significant difference between the chitosan wells with enzyme and those without. This indicates that the lactate may have been blocked from binding well with the chitosan, but did not bind well with the enzyme. The procedure for this assay was not the standard use of the kit, and variations or changes to the protocol could be made to address this problem. The same test was attempted with the lactate oxidase kit that was already in the lab from a previous MQP group. Unfortunately, the kit did not produce a measureable reaction, either with the oxidase and

chitosan mixture or the standards. This may have been caused by either age or other unknown factors.

The most important tests were performed using the Autolab machine in Professor Zhou's lab with GPES software. There were two main tests performed: cyclic voltammetry and amperometry. For both test the reference electrode made of AgCl and the counter electrode was a platinum coil, and the working electrode was the glassy carbon electrode. Both tests were conducted initially in deionized water, followed by increasing concentrations of lactic acid: 100µ, 500µM, and 5mM. For the sets of testing where both cyclic and amperometry readings were performed, the cyclic voltammetry was performed first followed immediately by the amperometry for each concentration. This was important so that the coating would not become saturate with higher lactic acid concentrations. If this happened, and then the electrode was tested in a lower concentration, it would be difficult to get an accurate reading due to the concentration gradient being flipped for the lactic acid solution. The solution the electrode is tested with should have higher levels of lactic acid than the coating.

The cyclic voltammetry testing was to determine if there was reaction occurring on the coated electrode in lactic acid when compared to immersion in deionized water. The voltage range of -0.4V to 0.8V was selected as the best testing range. This was decided so that the results on the glassy carbon electrode could be compared to the tests on the nanotubes. A peak in the graph around the -0.2V was what was looked for, as an indication of successful detection of the lactic acid substrate. The lactate oxidase, especially when tested in the 5mM solution of lactic acid, compared to the coated electrode in deionized water, showed this characteristic peak. However, the lactate dehydrogenase coating did not show a significant peak, though it did level off temporarily at. This may be due to the fact the dehydrogenase reaction does not produce

peroxide. The peroxide is mostly responsible for the spike at -0.2V, because this is the voltage where is it is the most reactive.

The amperometry tests were conducted for 120 seconds at a constant voltage of -0.2 V. There were mixed results for both the oxidase and dehydrogenase coatings. However, despite some outlier data, the general trend was that the absolute value of the current detected increased as the concentrations of lactic acid increased. This was expected, as the more lactic acid present, the greater the level of the reaction taking place, leading to current increasing. This is also consistent with the previous MQP's finding. However, due to ouliers being present in most rounds of testing, it is important to note that for *in vitro* and *in vivo* clinical use that the equipment is highly sensitive to small changes in current and is correctly calibrated. According to the testing, the current readouts for even 5mM of lactic acid are only in the μAmps range. Therefore, even small changes to testing procedures can cause less accurate data to accumulate.

A limitation that was detected during the cyclic voltammetry and amperometry testing was the relationship between chitosan and acidic environments. As stated in Chapter 2, chitosan shows a greater to propensity to dissolve in acidic solutions. However, the time was relatively short that the chitosan film spent immersed in each acidic solution, on the order of several hours. It appears that the combination of the immersion within an acidic solution and the rinsing in between each test caused the coating to slowly come off. However, this would be classified as a testing limitation as opposed to the chitosan being a failure for the coating. If the coating was used to detect in vitro with blood, or was implanted *in vivo*, the acidic pH issues would no longer be present due to blood having a pH of 7.4.

The real time testing was inconclusive. Each time the lactic acid was added to the testing solution, there should have been a sharp spike in the current, followed by the amperometry

current readout leveling off at a larger current value. For the lactate oxidase the current spiked after the administration of the lactic acid, but did not remain higher than the deionized water baseline. The current also did not level off as expected. The lactate dehydrogenase did not show any of the characteristic spikes. However, after the second quantity of lactic acid was administered, the current rapidly increased and remained at that level for the duration of the test. It is likely that when the lactic acid was applied, it did not instantaneously diffuse to equilibrium, accounting for some of the unusual current outputs detected from both coatings. In addition, there was the possibility during the application of the lactic acid the electrode setup was slightly jarred, imperceptible to the applicator. Any change orientation of the electrodes towards one another during the cyclic voltammetry and amperometry tests can cause a change in the current output for that specific test.

Duration

One of the major limiting factors to this project was the length of time allotted for its completion. The full details of this project were received in early September 2015. The full report was due for submission on April 28, 2015. Therefore, all of the background research, planning, acquisition of necessary materials, testing, and writing were required to be completed in less than eight months. The time span especially affected the project, in that if would not have been possible for a lactate oxidase kit to be delivered by the time it would have been necessary. This necessitated the ordering of the similar enzyme lactate dehydrogenase. This was an unexpected turn that required more research that had not been planned on. Fortunately, there was lactate oxidase enzyme left over from the 2013-2014 biosensor MQP that was stored properly at -20˚C. By testing the lactate oxidase in the chitosan coating, it was determined that the enzyme was still active.

Budget

The budget was another limiting factor of this project. The lactate dehydrogenase kit was purchased for $500, surpassing the initial budget of $450. It was very fortunate for this project that Professor Jain was willing to purchase the lactate dehydrogenase kit for her lab and allowed us to use what we needed. Professor Jain's lab also already had chitosan, lactate acid, and lactate oxidase. Additionally, Professor Zhou's lab provided two glassy carbon electrodes, the Autolab machine, alumina powder, and the materials for the nanotubes and nanoparticles.

As can be seen in the budget, most of these materials are fairly expensive, at least in the context of startup cost. For example, only minimal amounts of the already depleted chitosan powder were used to make the chitosan solution. In fact, after the correct molar ratios were determined, the 25mL of chitosan solution made lasted the entire for the entire remainder of the project. Therefore, if this coating was made for a commercial purpose, it would likely be economically viable for customers to purchase. This is because for this project the only small amounts of the bulk required for purchase were utilized, something that would not be an issue with mass production.

Application

The purpose of utilizing chitosan as a biosensor coating was to compare it to the polypyrrole coating developed by the 2013-2014 MQP. The main improvement was the ease of application for the chitosan coating compared with the polypyrrole. In addition to the process of polishing the glassy carbon electrode, the polypyrrole coating required a half hour of the electrode immersed in deionized water containing polypyrrole flakes, subject to a constant voltage with the Autolab machine and GPES software. The full details of this procedure can be found in the 2013-2014 MQP report. The chitosan application by drop analysis is much simpler, by just dropping the chitosan/enzyme mixture on the surface of the electrode and allowed to dry.

This requires less of overview time, meaning that the person applying the coating has to be physically present less time.

However, a drawback to the chitosan drop method is the volume of the coating used varies with the surface area size of the electrode. Therefore when first depositing the coating on a new electrode is a process of trial and error. However, to avoid using up to much of the enzyme, the volume required for deposition can be first tested using deionized water and the chitosan solution without any enzyme. In addition, the dehydrogenase coating appeared to spread evenly across the electrode surface after deposition. Contrastingly, the oxidase coating was resistant to spreading, producing a smaller area of coating coverage, despite attempts at dropping the mixture over the entire surface. As both coatings were deposited on the carbon electrode in 10μL quantities, it is a reasonable assumption that the oxidase coating was thicker than the dehydrogenase coating.

Chapter 7: Final Design and Validation

Standards

Due to the nature of the biosensor produced as a result of this project, there are several material properties that it must possess to be considered safe for widespread patient use. These properties are especially important for any future *in vivo* implantations. The major standard that this product must meet to be manufactured and marketed in industry is biocompatibility, outlined in ISO 10993. Some of the product properties that must be tested for under ISO 10993 are cytotoxicity, hemocompatibility, and carcinogenicity (Food and Drug Administration, 1995). Actual testing of the biosensor with respect to these properties was not completed as part of this project due to the focus on providing an *in vitro* proof of concept. However, the materials selected (chitosan, titanium dioxide, etc.) were specifically picked due to being detailed multiple literary sources as having good biocompatibility, low immune response, and no have no evidence of carcinogenicity.

An example of an *in vitro* test to determine cytotoxicity and overall biocompatibility is direct contact test. In this test, biosensor is placed in separate cells cultures consisting of cell types that would be present at the site of implantation, such as blood, bone, muscle, and nerve cells. After several days, the experimental cells, containing the biosensor, would be compared to control cell culture samples of each type to determine if any abnormal behavior is occurring, including cell death. If the *in vitro* testing were successful, then the next step would be to conduct *in vivo* testing to determine if there are differences upon implantation into a mammalian body. Animal trials should be done starting with lower orders of mammals, such as rats. Upon successful completion in small mammal testing, the device should then be implanted within mammal even closer to human physiology, such and pigs and primates. The final set of testing prior to FDA approval would be human clinical trials.

Economics

It is very expensive to monitor cancer growth and development, especially in the brain, with current technology. The current gold standard, an MRI scan, is estimated to cost $18,167 over a 25-year span for the average patient (Moore, 2009). This high cost is especially significant when taking into account that most patients only get an MRI scan every six months, as well as any other hospital expenses incurred. By using this type of sensor either *in vitro* or *in vivo,* there would be an improvement over the current gold standard of MRI scans. This is because the *in vitro* testing could be performed at a doctor's office, which is less expensive than hospital visits. Eventually, this biosensor technology might be capable of being used by the patient in a similar method to a glucose meter that diabetics use. Additionally, a biosensor implanted in *in vivo* would provide real time data signals, and only require doctor appointments at the end of the biosensors lifespan or if abnormal metabolite levels are detected.

Environmental Impact

The enzymatic biosensor will contain biological material, especially when utilized for blood testing. Therefore, after its use the sensor will need to be placed in a biological or medical waste container for proper disposal. Assuming that the medical waste is handled according to the law, the TiO_2 nanotubes and enzymatic coating should not pose any threat or negative effect to the environment. Even if the biosensor was incorrectly disposed of, the chitosan and enzyme will eventually degrade as they are of biological origin. The TiO_2 material is also known to be biologically inert in virtually all circumstance, meaning that even incorrectly disposed biosensors should not have a significant impact on life in the natural environment.

Societal Influence

We could market our product in many ways. Two main methods of marketing the biosensor would be to sell to healthcare professionals and institutions or directly to

pharmaceutical to be purchased by patients. If the product cost was reduced to make the biosensor affordable for people with average incomes, it could be possible for many brain tumor patients to monitor their own lactate and glutamate levels. Lactate is a very important metabolite in the bloodstream of ordinary people with brain tumors, athletes and newborn babies. The impact on the ordinary people can be huge with the developed biosensor. People will be able to monitor their lactate levels with one simple test from home.

Political Ramifications

The high upfront cost of imaging machines such as MRI and CT, as well as the cost to use and maintain, make these machines difficult to rely upon for monitoring brain tumors in impoverished countries. If this biosensor technology could be mass-produced, the price of the sensor has the potential to decrease to feasible levels for these countries to use. However, this still leaves the issue of being able to afford medical treatment for the brain cancer. In spite of this, technology such as the biosensor developed during this project is the first step towards removing cost as limiting factor in detecting cancer.

Ethical Concerns

When real time monitoring is used with a biosensor there needs to be device receiving the information that the sensor is picking up. This may be a computer that a doctor or medical professional can access for diagnostic purposes. If the chain of information in this kind of remote monitoring system is compromised then a patient’s right to confidentiality and privacy could be violated.

Health and Safety Issues

In the case of developing this biosensor to have an *in vivo* application, the safety of the patient needs to be the greatest priority. Because the biosensor is detecting levels of lactate in the patient’s blood, safety issues such as risk of infection should be considered. While there are other

implantable devices on the market such as an insulin pump for someone with diabetes, the risk of infection should be outweighed by the benefits of accurate and reliable real time early tumor detection to detect tumors at a smaller size and increase the patient's chance for a longer life.

Manufacturability

The ease of reproducing the biosensor manufactured during the course of this project is likely to be high. The biosensor is not very complicated to construct. This claim is based on the fact that three undergraduate students were capable of researching, designing, and constructing this biosensor within a time span of 28 weeks of active work. Therefore, laboratories and companies with far more experience should be able reproduce the biosensor that we created. The biosensor was difficult to develop, build, and test on the budget received for this project. However, this cost is relatively affordable within the allocated budgets for most companies.

Chapter 8: Conclusions and Recommendations

Further Testing

Cell Media and Blood Testing

The eventual purpose for the technology developed during this project is aimed at either an *in vitro* sensor for lactate, similar to a glucose meter used by diabetics, or an implanted sensor *in vivo* for real time monitoring. Therefore, testing the ability of the biosensor to detect lactic acid in media containing glioblastoma cells and later blood from cancer patients would be highly beneficial. The main advantage that testing with these substances is that they contain other substances besides the lactic acid and deionized water solution used for the tests detailed in this report. Therefore, this would test the sensor's ability to detect the lactic acid, even with the presence of other interfering substances. An additional benefit is that the cell media and blood have relatively neutral pHs. This would remove the weakness of the chitosan in the coating starting to dissolve, which would be bad in the case of an eventual implantation.

Glutamate Biosensor

With an increased budget, a glutamate biosensor could be engineered in addition to the lactate biosensor. A glutamate biosensor would provide the ability to test glutamate levels, which are also beneficial in early brain tumor detection. Glutamate dehydrogenase and glutamate oxidase enzymes both exist, as detailed in the background section. Therefore, in theory, by following the same procedure as for the lactate biosensor, either glutamate enzyme can be immobilized to produce the same redox reaction. Each biosensor created requires funds for materials and testing, thus an increased budget would allow for this capability.

Carbon v. Platinum

The current biosensor model utilized a glassy carbon material for the electrode, however with increased time a platinum based biosensor could be engineered as the electrode material.

Theoretically, a platinum electrode should produce more accurate and reproducible results due to increased enzymatic stability. Platinum electrodes require more time to engineer and prepare, however if additional time was granted, this would be a worthwhile endeavor.

Titanium Dioxide Nanotubes

The initial goal of this project was to test the chitosan coating on titanium dioxide nanotubes being developed concurrently by a chemical engineering MQP group. However, time constraints prevent the successful testing of both together. The nanotubes are infused with metallic nanoparticles, such as platinum or nickel that react with hydrogen peroxide, and generate a current. For this purpose, the lactate oxidase enzyme would be the ideal pick. This is opposed to lactate dehydrogenase, which does not cause peroxide to form as a byproduct of catalyzing a reaction with lactate.

Coatings

Fresh Coatings

An increased budget would allow us to but a fresh coating on the biosensor for each test that is run. Both lactate coatings utilized on the electrode have a limited number of uses with the same coating until results are no longer accurate or detectable. An increased budget would allow for coating materials, and thus those coating to be put on the electrode for each test. This would almost guarantee accurate test results each time, without the risk of skewed test results.

Alternatives

There are other types of biomaterials that have been successfully used in enzymatic coatings for biosensors. One of these materials is polypyrrole. As detailed in the alternative designs section of Chapter 4, polypyrrole can be used alone, or in combination with chitosan to form a biosensor coating. It is recommended that a future team look into a chitosan and polypyrrole mixture for an

enzyme immobilization coating, as polypyrrole coating has been tested by a previous MQP team with promising implications.

Future Medical and Commercial Utilization

The technology present in this biosensor can be utilized in other areas of society besides cancer detection.

Athlete Performance Testing

As lactate is the waste product of consumed glucose, it can be an important metabolite to monitor for athlete performance testing. Lactate levels will indicate how hard muscles are working (ie. how much glucose they are using up). In the most strenuous and extreme of activities, 25uM of lactate can be detected in the bloodstream. Thus, exertion and maximum performance testing may be conducted using a lactate biosensor.

Fetal Health Monitoring

Immediately before birth, lactate levels from the scalp blood of the fetus can be drawn for health purposes. The lactate levels in the fetal blood will serve as an indication of the heart health of the fetus, and tell doctors whether or not the baby will likely survive the birthing process (since oxygen levels will decrease upon birth). Elevated lactate levels are an indication that the fetus is not receiving enough oxygen and their heart health is not strong enough to survive the birthing process without assistance.

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Appendices

Appendix A: Design Protocols

Applying Polypyrrole to Electrode

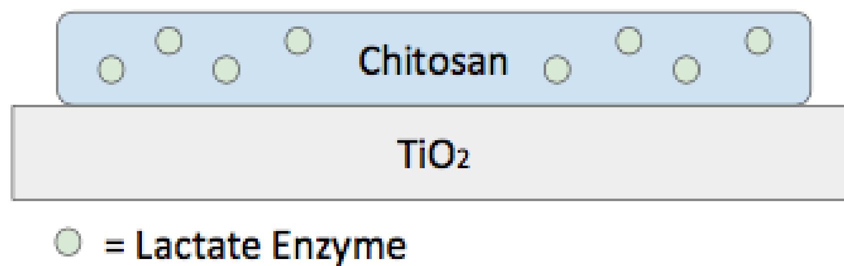
Before preparing the polypyrrole for application to the electrode, the electrode must first be prepared. This includes polishing the electrode, then ultrasonicating it in a solution such as methanol for 1-2 minutes. After the electrode is polished and has completely dried, the solutions may be applied. Before applying the polypyrrole, many research teams suggest creating a 'polypyrrole prelayer'. This is meant to help the enzymes adhere in a smooth layer and allow for the electrode to obtain an accurate reading. In order to apply this prelayer, the electrode is soaked in a 0.1M solution of LiCl04 and 0.05M pyrrole solution. This solution should be applied just long enough to coat the surface of the electrode and allowed to dry at room temperature. The pH of the polypyrrole layer should be around 6.2 (Razola et al., 2002). After this prelayer has been added to the electrode, the actual polypyrrole layer can be added. The polypyrrole is prepared by adding 6ul of polypyrrole to 1mL of phosphate buffered saline (PBS) with a pH of 7.4. Once this solution is thoroughly mixed, immerse the electrode in the solution and allow to completely dry. Once the electrode has dried, rinse the electrode with deionized water and let dry (Ammam, 2009).

Applying Chitosan to Electrode

The process of applying chitosan to the electrode of the lactate biosensor involves preparing the material into a usable form in order to achieve its full functional potential. The simplest and most efficient way to prepare the chitosan to make a film for the biosensor is to begin with chitosan chips or flakes, purchased from a chemical company such as Sigma-Aldrich or Fisher Scientific (Zhang et al., 2006). By examining several protocols from various research

teams who have applied chitosan to their biosensor electrode with other materials, we have formulated the following protocol:

Beginning with chitosan flakes purchased from a chemical manufacturer, we will prepare a 0.10 wt.% chitosan solution. This seems to be the most effective concentration of chitosan for creating a film, especially if it is to be layered with other materials. To prepare this solution, a measured value of (depending upon the desired chitosan solution necessary) will be massed and dissolved in heated 0.1M hydrochloric acid (HCl). The HCl should be heated to around 80°C in order for the chitosan to properly dissolve into solution. Adjust this solution to a pH level of 4.5 using either HCl to lower the pH or sodium hydroxide to raise the pH (if at all necessary). Use either a pH test strip or an automated pH bench top meter to check the solution for necessary adjustments. Filter the solution through a 0.45-micron or lower filter in order to ensure sterility and the absence of foreign particulates. This solution can be stored in a 4°C refrigerator until it is ready for use. Once this chitosan solution has been prepared, it can be added to the electrode, typically in a 15-20ul quantity, however, for our electrode we only applied a 10ul volume in order to reduce the film thickness. This would allow the substrate to access the enzyme throughout the film during testing. At this point, the solution should become somewhat gelatinous and another solution layer may be added to the electrode (Zhang et al., 2006). The figure below from Chapter 4 (Figure 18) illustrates how the chitosan will be applied atop the TiO2 electrode.



Crosslinking Oxidase with PEGDE

In the protocol found for crosslinking with PEGDE, the authors used glutamate oxidase, where our biosensor detects lactate. The following protocol would be modified to account for this. The glutamate oxidase was immobilized on the surface by crosslinking with PEGDE. Once the polypyrrole or chitosan was successfully polymerized on the electrode, the oxidase enzyme was immobilized in a solution with 57mg/ml of glutamate oxidase, 81mg/ml BSA, 10mg/ml PEGDE and 1% glycerol in PBS (0.01M). This solution was applied to the electrode and the electrode was heated to 55 degrees Celsius for 2 hours to complete the crosslinking of the enzyme (Vasylieva, 2011).

Appendix B: Lactate Assay Protocol for Chitosan Film Testing

Materials/Solutions:

- Make a 2% BSA solution in PBS

Part 1: Block Chitosan Wells

1. Add 50-100ul of 2% BSA in PBS to each chitosan well
2. Let it stand for 1-2 minutes
3. Pipette liquid out carefully
4. Repeat this 2X (3 times total)
5. Add 50-100ul PBS to each chitosan well
6. Let it stand for 1-2 minutes

7. Pipette liquid out carefully
8. Repeat (2 times total)

Part 2: Binding in Chitosan

1. Mix 10ul of standard with 990ul of buffer
2. Add lactate standard of 6nmol/well standard to each chitosan well. Let it stand for 1 hour (standard 4 is 18ul standard, 126ul buffer)
3. While lactate is incubating/binding, make standard curve:
    a. Follow the chart and make 150ul of each standard in a tube, then take 50ul for each well and make the standard in duplicate
    b. Make standards
        i. Standard 1: 120ul buffer
        ii. Standard 2: 6ul standard, 144ul buffer
        iii. Standard 3: 12ul standard, 138ul buffer
        iv. Standard 4: 18ul standard, 132ul buffer
        v. Standard 5: 24ul standard, 126ul buffer
        vi. Standard 6: 30ul standard, 120ul buffer
    c. Take 50ul per standard well, standards in duplicate.
4. After 1 hour: Add 50-100ul of PBS into each chitosan well
5. Let it stand for 1-2 minutes.
6. Repeat (2 times total).

Part 3: Reaction Mix and Incubation

1. Make enough reaction mix for every standard well and sample well. The kit suggests the equation: Xul component * (number of reactions + 1) where Xul components are:

a. 46ul lactate assay buffer, 2ul lactate substrate mix, and 2ul lactate enzyme mix (50ul reaction mix per well).

2. Mix each well by pipetting up and down gently.
3. Background Reaction Mix
   a. 48ul lactate assay buffer and 2ul lactate substrate mix (50ul/well) using the same formula above.
4. Incubate at room temperature for 30 minutes.

Part 4: Plate Reader

1. Read at 450nm.

Appendix C: Material Cost

The materials used in our film design include chitosan, L-lactic acid (substrate), a L-lactate assay kit (contained lactate dehydrogenase enzyme used in the film), as well as lactate oxidase enzyme from a lactate assay kit. The Chemical Engineering group used materials not included in our MQP budget to synthesize the titanium dioxide nanotubes with deposited nanoparticles.

Material Cost Analysis:

Material	Description	Price	Availability
Chitosan	Sigma Aldrich (10G), in powder/flake form	$33.10	In lab
L-Lactic Acid	85-90% pure L-lactate, approximate molarity of 9.4M	Unknown	In lab
L-Lactate Assay Kit (Colorimetric)	Abcam, enzyme used is lactate dehydrogenase	$549.00	Ordered from Abcam website
Lactate Assay Kit	Sigma Aldrich, enzyme used is lactate oxidase	$458.00	In lab from previous MQP