AN IMPLANTABLE ELECTROCHEMICAL MULTI-ANALYTE SENSOR
ARRAY FOR METABOLITE MONITORING

A Thesis in
Chemical Engineering
by
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ABSTRACT

Currently, continuous monitoring of metabolites is under development to aid in the detection and management of diseases, such as diabetes, ischemia and sepsis. The methods in practice involve obtaining blood samples to monitor metabolites, which does not lend to patient compliance or the patient’s desire for frequent testing. In order to raise compliance with diabetic testing and aid in early detection of ischemia and sepsis an implantable electrochemical sensor array has been developed to monitor glucose, lactate, and pyruvate.

The glucose sensors were first fabricated on gold electrode arrays on flexible polyimide sheets by photo-polymerization of the biocompatible polymer poly(ethylene glycol) diacrylate (PEG-DA) to develop hydrogels and encapsulate the sensing elements. Using conventional silicon fabrication methods, arrays of five gold microdisk electrodes were fabricated using lift-off photolithography and sputtering techniques. A redox polymer was then electrostatically attached to the electrode and glucose oxidase (GOX) was entrapped inside the hydrogel on the array of electrodes by UV-initiated photo-polymerization of PEG-DA.

The glucose sensor components were then characterized electrochemically with cyclic voltammetry (CV) and square wave voltammetry (SWV) and optimized with CV, SWV and amperometry to improve the sensor’s performance over previous sensors. The developed glucose sensors were also tested in vivo to determine their ability to function in the harsh in vivo environment. The platform developed for the glucose sensor, with its ease of fabrication for multi-analyte sensors, was then implemented with the metabolites
lactate and pyruvate. All three sensors responded linearly to the desired analytes with linear ranges that spanned the biologically relevant concentrations.
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<td>AIBN</td>
<td>2,2’-azobis(2-methylpropionitrile)</td>
</tr>
<tr>
<td>bpy</td>
<td>2,2’-dipyridyl</td>
</tr>
<tr>
<td>CAM</td>
<td>ex ova chorioallantoic membrane</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry</td>
</tr>
<tr>
<td>DEA</td>
<td>Drug Enforcement Agency</td>
</tr>
<tr>
<td>DMA</td>
<td>dododecyl methacrylate</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>GOX</td>
<td>glucose oxidase</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>LOX</td>
<td>lactate oxidase</td>
</tr>
<tr>
<td>MPC</td>
<td>2-methacryloyloxyethyl phosphorylcholine</td>
</tr>
<tr>
<td>MTTF</td>
<td>Mean-Time-to-Failure</td>
</tr>
<tr>
<td>MUA</td>
<td>11-mercaptoundecanoic acid</td>
</tr>
<tr>
<td>NIDDK</td>
<td>National Institute of Diabetes and Digestive and Kidney Diseases</td>
</tr>
<tr>
<td>PAR</td>
<td>Princeton Applied Research</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline solution</td>
</tr>
<tr>
<td>PCP</td>
<td>phospholipid analogous vinyl polymer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEG-DA</td>
<td>poly (ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PEGDGE</td>
<td>poly(ethylene glycol) diglycidyl ether</td>
</tr>
<tr>
<td>PHEMA</td>
<td>poly(hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>POs-EA</td>
<td>poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
</tr>
<tr>
<td>PVI</td>
<td>poly (N-vinylimidazole)</td>
</tr>
<tr>
<td>PYX</td>
<td>pyruvate oxidase</td>
</tr>
<tr>
<td>SWV</td>
<td>square wave voltammetry</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLE</td>
<td>thin-layer electrochemistry</td>
</tr>
<tr>
<td>TPP</td>
<td>thiamine pyrophosphate</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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Chapter 1
Introduction and Specific Aims

The development of implantable sensors used for continuous metabolite monitoring has been under investigation for more than forty years.[1] Among those metabolites investigated have been glucose[2-9], lactate[10-14] and pyruvate[15-18] because they are important indicators of the serious diseases diabetes, ischemia and sepsis. Glucose, lactate and pyruvate are metabolic components of glycolysis, where glucose reacts to give pyruvate and then pyruvate is reduced by either aerobic respiration or anaerobic respiration (producing lactate in anaerobic). Therefore, the values of these three components are highly related and more information can be deduced from obtaining all of the values and ratios leading to the detection of illnesses sooner.

The most widely studied sensor desired for continuous monitoring is the glucose sensor for its use in diabetes management.[19-21] Currently, monitoring blood glucose levels for diabetic patients is invasive and painful, involving pricking the finger to obtain a blood sample to monitor glucose levels 3-4 times daily. The need for frequent tests and pain involved with testing leads to poor compliance. Monitoring of patients in a hospital setting for indication of ischemia or sepsis will be beneficial, since the current method of determination is the use of a handheld device (I-Stat [22]) that requires the collection of blood samples. The earlier these diseases are detected the chance of recovery will increase, but when blood samples are required the frequency of testing is decreased compared to a continuous monitoring system. Another situation where the continuous
monitoring of these analytes would be highly beneficial is the battlefield for soldiers. The three analytes under consideration are important metabolites for glycolysis that when monitored can provide useful information on the condition of the soldiers. For example, since lactic acid buildup is also an indicator of anaerobic respiration and fatigue, if the officers could monitor fatigue of troops then the troops can be replenished as needed.

In order to raise compliance for diabetic patients and to increase early detection of ischemia and sepsis, sensors in this dissertation are developed as implantable electrochemical sensor arrays that could continuously monitor the analytes, eliminating the need for painful repeated testing. The most widely studied sensor for in vivo testing is the glucose sensor; so the first step in development of the sensors is to optimize the glucose sensor then apply the techniques to lactate and pyruvate.

The sensor described measures the current related to the rate of reaction occurring between, for example, glucose and glucose oxidase (GOX) with a charge mediator (redox) polymer acting as the transport for the electron to the electrode. The sensor will be fabricated by photolithography to develop electrodes with sensing elements (redox polymer and enzyme) encapsulated in a poly(ethylene glycol) diacrylate (PEG-DA) hydrogel. Previous work has shown that the redox polymer works as a good electron transport [12, 18, 23-31], but in the previous sensors the sensors are not as reliable or biocompatible for implantation. One method of enhancing biocompatibility and ensuring mass transfer limitations to obtain linear responses would be the encapsulation of the sensor elements in PEG-DA hydrogels.[32, 33] To that end, the following specific aims were utilized for development of the sensor arrays:
Specific Aim #1: Develop and characterize components for fabrication of an electrochemical glucose sensor. Photolithographic methods are used to fabricate the base sensor array on a flexible substrate and are characterized optically and electrochemically to determine redundancy. The redox polymer is synthesized and characterized chemically and electrochemically to confirm synthesis.

Specific Aim #2: Fabricate redundant electrode arrays of glucose sensors utilizing components from Specific Aim #1, test the sensors and characterize the redox polymer when in the sensor to determine reversibility.

Specific Aim #3: Optimize the glucose sensor response, response time and sensitivity by varying the electrochemical method used for testing. The methods under consideration for testing are cyclic voltammetry (CV), amperometry and square wave voltammetry (SWV).

Specific Aim #4: Test and characterize sensors in vivo for accuracy once implanted in the subcutaneous tissue of a Sprague-Dawley rat.

Specific Aim #5: Apply the developed sensor techniques to fabricate lactate and pyruvate sensors and test electrochemically by SWV.
Chapter 2
Literature Review

Diabetes

The most widely studied sensor desired for continuous monitoring is the glucose sensor for its use in diabetes management.[19-21] Diabetes is a disease that affects 20.8 million Americans [34] and 171 million people worldwide, leading to 3.2 million deaths worldwide each year.[35] Diabetes is caused when the body has defects in insulin production, insulin action, or both. Insulin is a hormone that converts sugar, starches, and other food into energy needed for daily life and maintains glucose in the biological range. There are two extremes when glucose is out of the biological range, hypoglycemia and hyperglycemia, that both lead to major health complications. Hypoglycemia is when the glucose level is too low, between 0 and 4 mM, and can result in seizures or unconsciousness. Hyperglycemia is when the blood glucose level is too high, above 7 mM, if left untreated it can result in a diabetic coma and even death.[36]

Diabetes has been linked to numerous health complications, such as heart disease, blindness, kidney disease, high blood pressure, amputations, nervous system disease, and others.[34] The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) conducted a clinical study to determine the benefits of tighter blood glucose control on the patient’s health. The study found that when the glucose levels are monitored and kept as close to normal levels as possible the progression and onset of eye,
kidney, and nerve diseases was decreased greatly by 76%, 50% and 60%, respectively.[37]

The current method for monitoring is an invasive and painful method that involves pricking the finger to obtain a blood sample to monitor glucose levels. Due to the current methods compliance with checking 3-4 times daily can be easily forgotten or avoided. The blood data obtained gives no indication of direction or trend of blood sugar levels. Even the most motivated diabetic patient performing frequent tests may miss reoccurring highs or lows, particularly at night. To have better compliance of monitoring glucose levels a noninvasive, less painful and continuous monitoring system is desirable. The electrochemical sensors dominate the market and research focus, and are the focal point of research in this dissertation.[1, 38-40] The current research efforts are focused on developing an electrochemical, implantable, real-time, continuous monitoring sensor that can be attached to an insulin pump and can be stable without re-calibration for a minimum of three days, since the patients have to change their insulin pump every three days also. The developed sensor will improve on previously developed sensors through ease of fabrication, the use of biocompatible components, ability to develop redundant and multi-analyte sensors on one platform and improved performance through varying the electrochemical testing method. If this sensor is developed the diabetes patient will not have to monitor his or her glucose level but just maintain the insulin pump and sensor.
Previous and current sensor technology

Glucose sensors have been under investigation for decades for the use in diabetes management, the focus of the research has been to develop a continuous glucose monitor that is reliable and painless.[1, 21, 41] The sensors under investigation fall into three categories: non-invasive, minimally invasive, and invasive. The possibility of the non-invasive and minimally invasive has created excitement [19] and has promise but is still a ways off from being completely non-invasive and reliable [42]. There are continuous glucose monitors on the market (Glucowatch™ and Mini-Med Medtronic Continuous Glucose Monitoring System (CGMS®) System Gold™), but some erroneous data can occur so most suggest the trends of the glucose to be monitored and action taken appropriately [42] or blood glucose levels to be tested before any action is taken by the patient [19].

The non-invasive techniques are of great interest and have focused on interstitial fluid sampling [43-46] and transdermal spectroscopy [47-50]. Interstitial fluid sampling has primarily focused on reverse iontophoresis, which is the method of glucose sampling for the Glucowatch™. [4, 45, 46, 51, 52] The experimental setup for reverse iontophoresis involves two electrodes being placed in contact with the skin and an electric field is applied between them to promote anions in the skin to move toward the cathode and cations to move toward the anode. This movement of ions causes an electro-osmotic flow of the interstitial fluid toward the electrodes. The interstitial fluid is then collected in and diluted by the electrolyte that electrically couples the electrodes to the skin. The glucose extracted in the interstitial fluid can then be measured with a glucose
sensor that contacts the electrolyte. One advantage of this method is the collection of a physiologically relevant sample. Glucose concentrations in the interstitial fluid have been shown in a number of studies to have an identical to or closely correlated value to that in plasma, though the results vary significantly because of the different methods used for collection [9, 53-59]. The interstitial fluid collected is primarily transported through sweat ducts and hair follicles, leading to a low efficiency of the amount of skin exposed to the electric field compared to the amount actually used for transport. With the low transport efficiency the volume collected is small, then it is diluted with electrolyte causing very low glucose concentrations that can be difficult to detect properly. If the amount of fluid extracted can be increased, minimization of fluid dilution and a reliable sensor integrated, then the interstitial fluid sampling is a promising method for glucose monitoring. Other interstitial fluid sampling being tested with similar results is sonophoresis, where an acoustic field was utilized to extract the interstitial fluid. [60, 61]

Another non-invasive technique, transdermal spectroscopy, transmits visible, near-infrared or infrared light back-scattered from or through the tissue to determine glucose concentrations. [47-50] The transmitted or scattered light is then collected and the glucose concentration is determined from a change in the polarization of the light or a change in absorbance. The major advantage of these techniques is that they are really non-invasive and therefore painless. A major disadvantage of this method is the use of non-specific spectroscopic techniques because they attempt to measure glucose in a highly complex matrix of water, proteins, polysaccharides and lipids. As a result, the acquired signal is very complex and the glucose measurement must then be extracted from the obtained signal by chemometric methods, such as principal component
regression or partial least squares analysis. Since these techniques fail to provide a direct measure of blood or interstitial fluid glucose, there is considerable debate as to if glucose is actually being measured or rather a phenomenon correlated to glucose concentration under the experimental conditions.

Minimally invasive techniques involve a long-term implanted sensor and an external monitoring system [62], sometimes this technique is termed “smart tattoo” [63-66]. The “smart tattoo” utilizes fluorescence as the detection method and has the sensor implanted in the subcutaneous tissue while the fluorescent probe remains on the outside of the skin. This method of operation leads to higher specificity and sensitivity than seen in non-invasive methods and eliminates the need for a physical connection to the sensor through the skin.[66] This method holds promise but more research and testing is necessary before it can be used by diabetic patients.

Invasive, or implantable sensors have been under investigation the longest out of the techniques and have shown mixed but encouraging results. The implantable glucose sensors are developed for contact with undiluted physiological fluid via subcutaneous tissue (where the glucose levels are closely related to the blood glucose levels) [19, 21, 58, 67-72] or intravascular insertion [1, 73, 74]. One of the commercially available continuous glucose monitors, Mini-Med Medtronic CGMS® System Gold™, falls under the invasive category and has the glucose sensor implanted subcutaneously. This monitoring system wirelessly transmits calculated glucose concentrations from the implanted glucose sensor to the Mini-Med Paradigm® REAL-Time insulin pump where the insulin pump Bolus Wizard™ wizard calculates and suggests the insulin dosage. [19] The current continuous monitors on the market do not alleviate or minimize the
frequency of the blood collections for glucose monitoring, due to sometimes erroneous data collected before action is taken by the patient a blood glucose measurement should be obtained for verification. [19] This need for use of the “finger-prick” method with the current available monitors leads to more improvements and continued research in the area of glucose sensors.

Invasive sensors that are wired through the skin fall into two main categories: implanted sensor [74-79] and microdialysis [56, 80-82]. The difference in the two sensing schemes is the placement of the sensor, the implanted sensor has the sensor inside the body and the wires protruding out of the body, while the microdialysis system places a catheter in the body to extract fluid from the patient and places the sensor in an outside unit. The advantage in microdialysis is the separation of the sensors from the body, meaning the sensors do not have to withstand the harsh environment. There are commercially available microdialysis probes [83], but they are bulky (with the requirement of a pump) and call for an hour of fluid collection for before the analyte concentration is obtained. The bulkiness of the machine does not lend to portability and the readings are every hour not continuous as desired. The implanted sensors have many sources of error or failure due mostly to the harsh environment. Some examples of failure have been caused by membrane delamination or degradation, loss of enzyme activity or leaching, loss of mediator activity or leaching, corrosion and failure of electrodes or components and biofouling.[84] One key developmental issue is to increase biocompatibility so that the body does not exhibit a host response and attempt to dispel the implanted sensor. The error associated with these sensors makes the development of
reliable and accurate implantable sensors difficult but necessary for the patient to rely on the sensor with their life.

**Improving biocompatibility for implanted sensors**

Biocompatibility is related to the body’s response, in this case, to an implanted sensor. The ideal biocompatible surface would result in no foreign body response (see next chapter: Biofouling and foreign body response for detailed definitions and descriptions) and basically be invisible to the body. Significant research has been focused on improving biocompatibility with different materials and methods, many of which are described below. [84-86]

**Phospholipid-based biomimicry**

Utilizing phospholipids, components that make up the cellular membrane, some biosensors are constructed to mimic cells on the outside. [84, 87, 88] Phospholipid membranes are difficult to fabricate and fragile, leading to polymers modified with phospholipids being utilized to increase stability.[84] In Yasuzawa et al., a Langmuir-Blodgett technique was utilized to apply the glucose oxidase and phospholipid analogous vinyl polymer (PCP).[89] In Kudo et al., the phospholipid polymer 2-methacryloyloxyethyl phosphorylcholine (MPC) was utilized in a polymer form (poly(MPC-co-DMA) where DMA is dododecyl methacrylate) to entrap glucose oxidase in the sensor through solution casting.[90]
Polymer Membranes

Polymer membranes can be formed from naturally derived materials and synthetic materials to improve biocompatibility. Membranes made from naturally derived materials such as silk fibroin [91, 92], cellulose[93], and chitosan[94] have been used to increase biocompatibility. Eisele et al. showed that bacterial cellulose membranes increased the stability of glucose sensors in whole and diluted blood compared to Cuprophran (wood cellulose) membranes. [93] Chemical modification by acylation of the C-2 position of cellulose acetate increases biocompatibility of the membranes versus unmodified. [95] Cellulose acetate can be applied through pre-cast membranes, spin-coating or dip-coating procedures; the method of application is dependent on the size of the sensor. [86]

The synthetic polymer, Nafion is an anionic, inert polymer that has hydrophilic and hydrophobic properties. Nafion is composed of perfluorosulfonic acid and has shown to increase the lifetime of sensors and to decrease the inflammatory response in the short term and decrease interferents (believed to be due to the anionic property). Nafion can be applied as an outer coating to sensors through a dip-coating or spin-coating procedure to entrap the enzyme and improve biocompatibility of the sensor. [20, 84, 86, 96]

Surfactants

Surfactants are molecules made up of a polar hydrophilic head group and a hydrophobic hydrocarbon tail, which leads these molecules to favor being at the interface
between two phases. Membranes that were previously used, such as solvent cast cellulose acetate or dip-coated polyurethane membranes, can be modified by surfactants to decrease biofouling. Lindner et al. showed that membranes with lower surfactant plasticizer ratios exhibited lower anion interference and better biocompatibility.

### Hydrogels

Hydrogels consist of a cross-linked polymer system or a mesh-like structure that is flexible and can swell in the presence of water, producing a hydrophilic surface and masking the components below. The most widely used hydrogels consist of poly(hydroxyethyl methacrylate) (PHEMA), poly(ethylene glycol) (PEG), or poly(vinyl alcohol) (PVA) and tend to have reduced interaction with tissue. Hydrogels can be formed by applying UV light to, for the case in this dissertation, poly(ethylene glycol) diacrylate (PEG-DA) in the presence of a photoinitiator inducing a gelation process that forms the hydrogel. PEG-DA hydrogels are used to encapsulate sensing elements and allow the analyte to diffuse into the hydrogel for detection with a controllable mass transfer rate. The mass transfer into the PEG-DA hydrogels depends on the mesh size, which can be thought of as the size of the hole in the 3-D structure. The mesh size of the structure can be controlled by altering the molecular weight (length) or branching of the precursor PEG. The mesh size of hydrogels has been highly studied and described by Peppas, Merrill, Flory, and Rehner and allows scientists to calculate the mesh size and tailor their system according to their needs.
molecular weight of PEG is increased the mesh size and diffusivity will increase. [101-106]

Some problems that can occur with the fabrication of hydrogels are difficulty in adherence to the surface, mechanical stability problems when implanted, some precursors (monomer, solvent, cross-linking agent, UV light) can harm the enzyme or other sensor components, and some hydrogels can limit the diffusion of the desired analyte to the sensor. [84]

**Electrochemical Glucose Sensors**

Electrochemical sensors are commonly used for biosensing applications because they are sensitive and selective towards electroactive species, fast, accurate, compact, portable and inexpensive. An electrochemical sensor converts a chemical quantity into a measurable potential or current. [107] The inspiration for the electrochemical glucose sensors came from a dissolved oxygen sensor, termed Clark cell, developed by Clark in 1956. [39, 108] Updike and Hicks combined the Clark cell with a glucose oxidase membrane to develop a glucose sensor in 1967. [109] The development of this glucose sensor led to global interest in biosensors utilizing enzymes for specificity and electrochemical detectors as the interfacial signal. [39] The reaction produced in the enzymatic membrane used for glucose sensors is seen in Eq. 2.1,

\[
glucose + O_2 \xrightarrow{GOX} \text{gluconic acid} + H_2O_2
\]
where the increase in glucose will decrease the amount of oxygen present, decrease the pH (by the production of gluconic acid) and increase the amount of hydrogen peroxide. The GOX membrane lends itself to the three different detections, but the most commonly used one is the detection of hydrogen peroxide since the other two parameters are variable in the system. The reaction-produced hydrogen peroxide can be oxidized at a platinum working electrode with a potential of 700 mV vs. Ag/AgCl when operated by amperometry (more details about methods in Chapters 4 and 5). These sensors are dependent on excess oxygen being present to obtain accurate results. [39]

The first thought for making an implantable sensor would be to miniaturize the glucose sensors previously developed for use outside the body and implant it in the body. This system has many complicating factors. First, the sensors are not biocompatible, which would lead to the rejection of the sensor. Secondly, when the sensor is to be implanted in the body the oxygen levels cannot be controlled and therefore the previous sensors may be unreliable in vivo. Third, these sensors are operated at higher potentials, which leave them susceptible to interferences from electro-oxidizable species from the body, such as ascorbic acid, uric acid and acetaminophen. One method used for deducing the electroactive species signal is to use a second electrode to detect the background and subtract from the obtained signal, which still leaves the oxygen dependence. To alleviate the oxygen dependence and high operating potential drawbacks mediators have been implemented into the glucose sensors. Some initial mediators were diffusing mediators that can be toxic in the body. If diffusing mediators were implanted in the body, the mediator may diffuse into the body and result in interference with biological reactions and the sensor response would diminish. [26]
To eliminate the uncertainty of the oxygen concentration and danger in diffusing mediators, charge mediators have been utilized in the reaction to relay electrons to the electrode for detection. [12, 18, 23, 25-30] Gregg et al. used osmium redox polymers as the charge mediator, these complexes are stable in more than one oxidation state, so they accept and donate electrons freely. [24] These redox polymers are also larger and can be entrapped or “wired” in the sensor to deter any leaching of the mediator. [26] A highly studied redox polymer is POs-EA that has a decreased operating potential of approximately 0.4 V vs. Ag/AgCl when operated by amperometry (methods discussed more in Chapters 4 and 5) [24]. The reaction scheme for such redox polymers is seen below in Table 2-1, where “M” designates the redox polymer.

<table>
<thead>
<tr>
<th>Table 2-1: Mediator reaction scheme, where M indicates the redox polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose + GOX(FAD) → gluconic acid + GOX(FADH₂)</td>
</tr>
<tr>
<td>GOX(FADH₂) + 2(M)⁷⁺ → GOX(FAD)²⁺ + 2(M)⁶⁺ + 2H⁺</td>
</tr>
<tr>
<td>2(M)⁶⁺ + 2(M)⁷⁺ → 2(M)⁷⁺ + 2(M)⁶⁺ (e⁻ self-exchange)</td>
</tr>
<tr>
<td>2(M)⁶⁺ → 2(M)⁷⁺ + 2e⁻</td>
</tr>
</tbody>
</table>

Another step taken by Revzin et al. to increase the reliability of the sensor system was to introduce redundancy to the system. This method of redundant electrodes has shown to improve the reliability of the sensors and allows for detection of faulty readings. [18, 68, 110] The method used by Revzin et al. does not lend itself to multiple analytes being detected on the same array, because the fabrication process was dip-coating and the biocompatibility of the sensor system was not ideal with the enzyme and redox polymer being at the sensor/body interface. The arrays were fabricated using
Future Directions

The ultimate goal of the diabetes research is to develop a glucose sensor reliable enough to be able to communicate directly with an insulin pump and therefore a closed-loop insulin delivery system is desired. With the development of this system, patient compliance is expected to increase and the complications and deaths related to diabetes can be minimized and eventually eliminated. Glucose sensors, which use an enzyme (glucose oxidase) for its specificity, are currently not sensitive or stable enough to meet the demands of a closed-loop delivery system. The imprecision and inaccuracy of sensor performance are often caused by imprecision in sensor manufacturing; the immobilized enzymes are not deposited onto the electrode surface at the same density and with the same mass transfer limitations. The use of biomolecules leads to instability as the enzyme often can lose its activity over time or when exposed to detrimental species in the environment. The immobilization of the biomolecules also may not be stable and the enzyme can leach into the body. For implantable glucose sensors to be successful, the issues of reproducibility and instability must be addressed.

This thesis focuses on alleviating the difficulties with previous in vivo glucose sensors through the combination of many previously described improvements and other modifications. Techniques from the silicon micro-fabrication industry are borrowed to develop an array of electrodes that are fabricated on a flexible and biocompatible
polyimide substrate. The reliability of the sensor system is increased with the use of a redundant array, which lends itself to the ability of detecting multiple analytes with one array. The mediator POs-EA is used to decrease the operating potential of the sensor and to alleviate the oxygen dependence of the sensor. The photo-polymerization of PEG-DA is utilized to allow for biocompatibility, ease in fabrication and the ability to fabricate a multi-analyte sensing platform, which is an improvement over previously developed sensors. The components of the sensors are biocompatible, but the biocompatibility is not tested in this thesis due to the extensive time and resources needed for thorough testing of biocompatibility. The developed sensors are then tested through various electrochemical techniques to determine the optimum sensing and testing method. The developed sensor platform is then tested for its ability to work in vivo with glucose sensing and in vitro with additional analytes (lactate and pyruvate).
Chapter 3

Micro-Fabrication of Sensor Array for Metabolite Monitoring and the Statistics of its Reliability

Since diabetic patients rely on glucose sensors to monitor and control their glucose levels, the reliability of the glucose sensors is extremely important. There are many ways glucose sensors can fail when placed in an *in vivo* environment, which is summarized in Figure 3.1 and then described in more detail. One way to increase reliability of the glucose readings obtained is to use redundant sensors, which is the utilized method for the sensors described in this thesis. Micro-fabrication processes are mostly used for the processing of silicon, but sensor arrays for biosensing need to be developed on biocompatible platforms. [111] For this purpose polyimide was chosen as the substrate because of its flexibility and biocompatibility. Methods for fabrication of redundant sensor arrays on polyimide are described, and the technique used to increase biocompatibility is described. After the discussion of the fabrication and biocompatibility methods (biocompatibility testing is not completed in this thesis due to the extensive time and resources necessary to complete a thorough biocompatibility study), the statistics of increasing reliability through redundancy and the methods available for signal processing and fault detection are discussed.
Sources of Error and Failure of in vivo glucose sensors

Loss of Enzyme activity and leaching

In most electrochemical glucose sensors the enzyme glucose oxidase is used for its specificity to glucose. Enzymes can lose their activity because of protein denaturation and loss of active site. Glucose oxidase can also lose its activity when exposed to excessive amounts of hydrogen peroxide. Most enzymes utilized in the fabrication of biosensors for implantation are derived from non-mammalian proteins, which can lead to an allergic response if the enzymes leach (or leak out of the sensor) into the body.
Enzyme leaching can result when the attachment or entrapment of the enzyme is not robust. For example, if a method of entrapment via cross-linking polymers is utilized and the reaction does not go to completion during entrapment then the enzyme can leach out and cause the sensor loss of signal. [113]

**Loss of mediator activity and leaching**

Many *in vivo* electrochemical glucose sensors utilize a mediator to shuttle the electron to the electrode, and decrease the interference by electro-oxidizing species. [114] When small mediators are used the mediators tend to diffuse out of the membrane and into the body. If the mediator enters the body it may result in interference with biological reactions. [23, 26] The method of attachment for the mediator is very important, because if the mediator leaches out or loses activity the sensor may fail. The mediator may lose activity if it undergoes a detrimental reaction or denatures, causing the potential to shift or the electron mediator ability to be lost.

**Film delamination**

Most sensors have a film or membrane used as a sensing layer and/or a biocompatible layer. When this film is not attached properly or degrades, it can fail and delaminate from the electrode surface, causing the sensor to lose function through increased mass transfer resistance, diminished electron transfer, and/or its ability to deter the foreign body response. [84]
Corrosion and failure of electrodes, leads, and insulation

Some of the component-based failures of sensors that can occur include failure of the different parts of the electrode. If the metal used is prone to corrosion in the presence of water and is not properly protected the electrode can fail through metal corrosion. Other methods for failure also include the failure of the insulation or leads. The leads can fail if their connection is lost and the signal cannot be obtained, this may be the case if the leads are attached poorly or are also prone to corrosion. Another failure mode of the electrodes is electrode fouling, which occurs when small molecules come into contact with the surface of the electrode after penetration of the sensor. [84]

Biofouling and foreign body response

Biofouling is described when a sensor is implanted in the body and there is a buildup of cells, proteins, and other biological substances on the surface of the sensor. When the body detects a foreign object, a foreign body response is begun and acts to dispel the foreign body through the wound-healing stages. The stages for wound healing are homeostasis, inflammation, repair and scar formation and are affected by the material on the outer surface of the membrane. Homeostasis is the first step in the wound healing response and causes proteins from blood and platelets to adhere to the surface and causes membrane biofouling. Inflammation is the second step and occurs when proteins from the immune system and cells adhere to the surface, which causes more membrane biofouling and can cause enzyme degradation. Repair occurs next and is marked by the vascular tissue formation, which causes the diffusivity to change. Lastly, encapsulation
occurs when a fibrous capsule is formed around the sensor. With the fibrous capsule surrounding the implanted sensor, membrane biofouling has occurred and an even longer diffusive path is created for glucose to reach the sensor for detection, which leads to a smaller signal and eventually can lead to complete failure of the sensor. [84, 85]

**Fabrication of redundant sensor arrays**

**Materials and methods**

Glucose oxidase (GOX, EC 1.1.3.4, Type X-S, 128 units/mg solid from *Aspergillus niger*), ammonium hexachloroosmate(IV), 11-mercaptoundecanoic acid (MUA), poly(4-vinylpyridine), β-D-glucose, acetone, ammonium hexafluorophosphate, sodium dithionite, ether, 2-bromoethylamine hydrobromide, N,N-dimethylformamide (DMF), anion exchange beads, hydrochloric acid (HCl), poly(ethylene glycol) diacrylate (PEG-DA), and 2,2′-dipyridyl (bpy) were obtained from the Aldrich Chemical Company (Milwaukee, WI). Ethyl alcohol, ethylene glycol and acetonitrile were obtained from Fisher Scientific Company (Pittsburgh, PA). DAROCUR, the photo initiator, was obtained from Ciba. All reagents, unless otherwise stated, were used as received. Polyimide sheets (1/16” thick) were purchased from McMaster Carr (New Brunswick, NJ). Phosphate buffered saline (PBS) consisted of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate. Conductive silver epoxy was obtained from Ladd Research (Williston, VT). Ma-P 100, positive photoresist, and ma-D 330, photoresist developer, were purchased from MicroChem Corporation (Newton, MA).
Fabrication of base sensors

For fabrication of redundant sensor arrays on metal or flexible insulating substrates, processes used in the semiconductor industry can be utilized. [39, 115] Photolithography, wet etch, dry etch, sputtering, and evaporation are a few processes that have previously been used. [116, 117] This section describes the fabrication of the electrode arrays used throughout all experiments described, unless otherwise stated, and is summarized in Figure 3.2.

Photolithography

Photolithography is defined as a process that uses light to develop a pattern. Photolithography uses photoresists to develop desired patterns and go through a multi-step process to obtain the final desired product. There are two main types of photoresists used for photolithography: positive and negative. A negative resist will result in the exposed regions remaining and the non-exposed regions being removed. The positive resist results in the regions that are exposed to UV light through a mask are removed and the non-exposed regions remain. When choosing a photoresist and patterning method a number of process issues need to be determined: the pattern of the mask, if a metal should be deposited first and removed through an etch step or a metal deposited after photolithography and excess removed through a lift-off procedure, the desired thickness of the photoresist, and the properties of the substrate and resist/developers to determine cross-reactivity.
When using photoresist, the first step is to obtain a clean substrate, which was achieved by washing the silicon or polyimide, which is flexible and biocompatible, with acetone and drying under nitrogen, and then depositing the photoresist, ma-P 100 (obtained from MicroChem Corp.), on the surface through spin coating (for an 8 micron thick layer the photoresist was spin coated onto the surface at a speed of 3000 rpm for 20 seconds). When there is an even layer of photoresist on the surface a hard-bake step is needed to remove the solvent, this is done by placing the substrate on a hot plate set to 100 °C for five minutes. Once the solvent is evaporated and the sample allowed to cool, the sample is brought into close contact with a mask with the desired pattern. When the mask and sample are exposed to UV light only the openings in the pattern allow the sample exposure to UV light; therefore, the exposed photoresist undergoes a chemical reaction leaving the unexposed regions unchanged.

A developer solution, ma-D 330 is utilized to remove the excess photoresist and obtain the desired pattern (the samples were soaked for 70 seconds in the developer and washed with deionized water). Once the pattern is obtained the next step is to deposit the metal for the electrode array. [39, 118]
**Metal Deposition**

Metal deposition can be accomplished by the sputtering or evaporation of a metal onto the surface. Sputtering is the preferred method in most situations because of the difficulties in the evaporation of alloys, it has a higher deposition rate, and results in better step coverage, increased adhesion, and higher purity of sputtered films, [39] and is therefore used to coat the samples for electrodes. Sputtering takes place when the target metal is in an argon plasma and bombarded by the positive ions of argon. The process then uses the momentum transfer from the bombardment of positive ions to sputter the metal away from the target and onto the desired substrate. A negative charge is produced on the target by its connection to a negative RF or DC power source, leading to the
positive ions being directed to the target. [39] The samples with photoresist were sputter-coated with 50 nm chrome (an adhesion layer) and 100 nm gold or to increase biocompatibility a 20 nm layer of titanium was used as the adhesion layer (completed at Pennsylvania State University and Lance Goddard Associates).

**Lift-Off**

The excess photoresist and metal is then removed by a lift-off technique, in which the sample is submersed in a solution that the photoresist is soluble in. In this case, the samples with the photoresist, ma-P 100, are submersed into acetone to obtain the desired pattern for the electrodes, which can be seen in Figures 3.3 and 3.4.

Figure 3.3: Photograph of fabricated electrode arrays on silicon (left) and polyimide (right), where in the picture of polyimide only the part to the left of the line would need to be implanted *in vivo*
Figure 3.4: Scanning electron micrograph (SEM) images of the electrode array with the diameter of the electrode equal to 500 µm

Deposition of Sensing Chemistries

After the sensor array is developed for increased reliability the next step in sensor fabrication is the deposition of the components used for sensing. Deposition of sensing chemistries can be accomplished by many means: covalent attachment, electrostatic attachment, entrapment or a combination of these methods. Covalent attachment is when the sensing elements are chemically bonded to the surface through chemical reactions creating covalent bonds. [87, 116] In Imamura et al., covalent attachment is the method utilized for sensor fabrication. After a self-assembled monolayer of aminoethane thiol is attached to the gold surface, ferrocene-modified glucose oxidase is covalently attached to the aminoethane thiol by glutaraldehyde. [119]
Electrostatic attachment can be accomplished when the elements used for sensing are charged oppositely to the surface or a layer attached to the surface. In Sirkar et al., the sensing elements are attached to the electrode via electrostatic attachment, utilizing the positive charge of the mediator and the negative charge of glucose oxidase. One of the benefits of electrostatic attachment is the ease of fabrication, when the charged surface is exposed to a liquid containing the oppositely charged element; the oppositely charged element then forms a layer on the surface. This layering approach can be repeated many times and the time needed per layer is less than one hour. [120]

Entrapment is another method to incorporate the sensing elements into the sensor and can be produced by entrapping the elements behind a membrane or inside a polymeric material. [86] Since glucose oxidase is much bigger than glucose the entrapment method can be utilized. [112] The glucose oxidase and mediator can be trapped in the sensor while the glucose is free to diffuse in and out of the sensor through the membrane leading to the reaction and detection of glucose. [86] In Csöregi et al., the two methods of entrapment are combined to develop the sensor. The sensing layer is fabricated by entrapping the mediator and glucose oxidase into a polymeric layer formed by cross-linking with poly(ethylene glycol) diglycidyl ether (PEGDGE). Another membrane was used for biocompatibility and trapped the rest of the sensor components behind a membrane developed by photo-cross-linking tetraacrylated poly(ethylene oxide) in solution with a photoinitiator via exposure to UV light. [68]

Membranes can also be used as a protective, diffusional, or biocompatible barrier. When implanting the sensor in the body, an outer membrane can be utilized as a size exclusion or protective barrier to deter larger molecules from interfering and also from
sensing elements leaking out. A diffusional barrier is utilized to alter the enzyme kinetics and extend the linear range of the sensor by increasing the diffusion-limited regime with respect to Michaelis-Menten kinetics. Also, as seen in Csöregi et al., a biocompatible membrane can be used to protect the rest of the sensor and deter a foreign body response (see previous section: Biofouling and foreign body response; also see Improving biocompatibility for implanted sensors section in the previous chapter for more examples). [68, 86]

The fabrication procedures for membranes vary, but many are not complicated and only involve casting or dip-coating. In the example above with poly(ethylene glycol) diglycidyl ether, a precursor solution is made up glucose oxidase, mediator, and PEGDGE. The solution is mixed then applied to the surface of the electrode and allowed to dry in order to crosslink the solution. [68] Other methods, such as photopolymerization or sol-gel formation, require specific instruments or more complicated procedures for fabrication, but have benefits and are discussed more below. [68, 88] For the biosensors described in this thesis all three methods of attachment are utilized and summarized in Figure 3.5.
Figure 3.5: Fabrication method for the PEG-DA hydrogel with sensing capabilities

The first step in the process is to attach a layer to gold because otherwise the other components will not adhere to gold. 11-mercaptoundecanoic acid (MUA) is utilized and self-assembles into monolayer on gold, with the thiol layer covalently attaching to the gold surface. The next step is to attach a mediator, POs-EA (which will be discussed in more detail in the next chapter), to the surface of the electrode, which is done through electrostatic attachment. The carboxylic acid end group of MUA is negatively charged and forms electrostatic attachments with the positively charged POs-EA. The final step in producing the electrochemical glucose sensors is to attach a membrane encapsulating the GOX enzyme. Photo-polymerizable PEG-DA hydrogels are used for this purpose because of their biocompatibility and ability to alter the mass transfer properties (which is discussed next in the section: Improving biocompatibility for fabricated sensor).
Improving Biocompatibility for Fabricated Sensor

Biocompatibility is related to the body’s response, in this case, to an implanted sensor. The ideal biocompatible surface would result in no foreign body response (see previous section: Biofouling and foreign body response for definitions and descriptions) and basically be invisible to the body.

Hydrogels consist of a cross-linked polymer system or a mesh-like structure that is flexible and can swell in the presence of water, producing a hydrophilic surface and masking the components below. The most widely used hydrogels consist of poly(hydroxyethyl methacrylate) (PHEMA), poly(ethylene glycol) (PEG), or poly(vinyl alcohol) (PVA) and tend to have reduced interaction with tissue. [32, 68, 84, 88, 99, 100] Hydrogels can be formed by applying UV light to, for this case, poly(ethylene glycol) diacrylate (PEG-DA) in the presence of a photoinitiator inducing a gelation process that forms the hydrogel. [32, 33] Biocompatible PEG-DA hydrogels are used to encapsulate sensing elements and allow the analyte to diffuse into the hydrogel for detection with a controllable mass transfer rate. [101-106]

Some problems that can occur with the fabrication of hydrogels are difficulty in adherence to the surface, mechanical stability problems when implanted, some precursors (monomer, solvent, cross-linking agent, UV light) can harm the enzyme or other sensor components, and some hydrogels can limit the diffusion of the desired analyte to the sensor. [84]
Statistics of reliability and redundancy

Microelectrode arrays have varying properties than that of a large single electrode that may lead to superior properties, such as enhanced signal-to-noise ratio. [121, 122]. Microelectrodes exhibit radial diffusion, because the diffusion layer thickness is larger than the dimensions of the electrode, which leads to higher analyte flux to the electrode than if linear diffusion were to dominate. When the limitation of the current in the system is caused by mass-transfer, then the microelectrode increase in mass transfer will result in higher current density. [28] An array of elements that can be addressed individually leads to the ability to increase the reliability of the system through the use of redundancy. [18, 110] Another advantage of the use of an array is the ability to vary the sensing elements on the array and therefore be able to detect multiple analytes with one array.

Reliability

Large numbers of redundant sensors allow signal averaging to improve accuracy and the use of fault detection algorithms to detect the failure of individual array elements. For example, the variance of a measurement based on the average of $N$ identical sensors is:

$$\sigma = \frac{\sigma_N}{\sqrt{N}}$$  \hspace{1cm} (3.1)
where $\sigma$ is the variance of the measurement, $\sigma_n$ is the variance of each individual sensor, and $N$ is the number of sensors. In addition, the reliability of the overall device will increase because of redundancy. In the most basic sense, reliability is defined as the probability of a component surviving for some period of time $t$. If $R_m(t)$ is the average sensor reliability among a group of $N$ sensors (i.e. the number of sensors functioning correctly at time $t$ divide by the total number of sensors), then the reliability of an array of these sensors operating in parallel is

$$R_s(t) = 1 - \left[1 - R_m(t)\right]^N$$  \hspace{1cm} \text{(3.2)}$$

As shown in Table 2-1 the reliability of the system where the average reliability of the individual components is 0.75 is greatly increased by increasing the number of elements in the array. Thus for an array of 4 sensors each with a reliability of 0.75, the reliability of the array is $1-(1-0.75)^4$ or 0.996, a large increase as compared to a single sensor. [18, 123, 124]

Table 3-1: Reliability of the system when varying number of elements in the array with $R_m(t)=0.75$

<table>
<thead>
<tr>
<th>Number in Array</th>
<th>Reliability of System ($R_s(t)$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>0.938</td>
</tr>
<tr>
<td>3</td>
<td>0.984</td>
</tr>
<tr>
<td>4</td>
<td>0.996</td>
</tr>
<tr>
<td>5</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Since the implanted glucose sensor readings have a direct impact on the patient, the reliability of the sensor’s reading is vital and can be influenced by developing a system that requires more functioning elements to produce a reading. The reliability of
the system where $K$-out-of-$N$ parallel elements must be functional for the system to be functional is described with respect to redundancy by Eq. 3.3:

$$
R_s(t) = \sum_{r=K}^{N} \binom{N}{r} [R(t)]^r [1 - R(t)]^{N-r}
$$

3.3

$R_s(t)$ is the reliability of the system, $R(t)$ is the reliability of an individual component, $N$ is the total number of components, $\binom{N}{r} = \frac{N!}{r!(N-r)!}$, [125] and $K$ is the minimum number of functioning components for a functioning array. [18, 126] As seen in Table 3-2, if the $K$-out-of-$N$ system of sensors is used, the reliability for each required number of functioning components is seen below for 5 components with $R(t)$ equal to 0.75.

<table>
<thead>
<tr>
<th>$K$</th>
<th>Reliability of System ($R_s(t)$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9990</td>
</tr>
<tr>
<td>2</td>
<td>0.9844</td>
</tr>
<tr>
<td>3</td>
<td>0.8965</td>
</tr>
<tr>
<td>4</td>
<td>0.6328</td>
</tr>
<tr>
<td>5</td>
<td>0.2373</td>
</tr>
</tbody>
</table>

As seen from the table, the reliability of the system when all five elements must be functional is very low, which means that in this system to increase reliability of the sensor functioning the number of functioning elements required needs to be examined and adjusted for optimal performance of the sensor.
Mean-Time-to-Failure

The most common metric for reliability is the mean-time-to-failure or MTTF where

$$MTTF = \int_{0}^{\infty} R(t)dt$$  \hspace{1cm} (3.4)

For many systems, component lifetimes are distributed exponentially, thus

$$R(t) = e^{-\frac{t}{\lambda}}$$  \hspace{1cm} (3.5)

where $\lambda$ is the component's mean lifetime. Thus for a single component

$$MTTF = \frac{1}{\lambda} \quad \text{and} \quad MTTF_s = MTTF\left(1 + \frac{1}{2} + ... + \frac{1}{N}\right)$$  \hspace{1cm} (3.6)

for a redundant array of identical sensors. As is apparent from Table 3-3, the MTTF of the array ($MTTF_s$) increases as the number of components increase. However, there is a diminishing return, i.e. each additional component contributes less to the MTTF. Thus an optimum number of components (or in our case sensors) exists to maximize reliability and minimize cost of the array.

Table 3-3: Varying the number of elements in an array and MTTF

<table>
<thead>
<tr>
<th>$N$</th>
<th>$MTTF_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$MTTF=5$</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>7.5</td>
</tr>
<tr>
<td>3</td>
<td>9.1667</td>
</tr>
<tr>
<td>4</td>
<td>10.4167</td>
</tr>
<tr>
<td>5</td>
<td>11.4167</td>
</tr>
</tbody>
</table>
Signal Processing and Fault Detection

A “one-point” *in vivo* calibration method is desired for implantable sensors, but the signal must be accurate enough for the patient’s life to be dependent on the sensor. The “one-point” calibration method relies on a zero point taken from the sensor in buffer with zero glucose present and then measuring one blood glucose concentration. [68]

In Csöregi et al., the “one-point” calibration method is tested on fabricated sensors and then a pair of redundant sensors is utilized to improve the reliability of the system. When using the pair of redundant sensors, the first step is to normalize the readings of the separate electrodes to each other by multiplying each reading of electrode A by the average output from electrode B divided by the average output from electrode A. The next step is to determine the standard deviation of the electrodes by obtaining 24 sets of readings of electrode B and the normalized electrode A and calculating the standard deviation. Then, the sets of readings that are outside of one standard deviation are deemed unreliable and not used. When using this method the sensors reliability to lie within the clinically correct zones of the Clarke error grid increased from 94% to 99%. [68]

Another method to process and detect faulty responses of the redundant sensor signal is called the likelihood test. The likelihood test that has previously been used is borrowed from nuclear reactor safety for pressure sensors deciding shutdown or no shutdown. Instead of shutdown or no shutdown for this case the sensor reading would be
rejected or accepted. For accuracy of the readings, the test chosen needs to reject readings that have even the smallest likelihood of being false and leading to the patient’s inappropriate response. [110]

When comparing the readings of the pair of implanted sensors a hypothesis must be tested. The null hypothesis is when the sensors should be equal, meaning the hypothesis is that there is no statistical difference between the two readings. To determine the validity of the null hypothesis, there can be two errors, type I and type II. Type I error is caused by rejecting the null hypothesis when it is correct, which is often termed a false positive. Type II error is accepting the null hypothesis when it is false. The likelihood test described takes into account the two types of errors above that can occur and skews the data in favor of type I errors versus type II to favor the safer of the two errors and decrease the number of incorrect treatments given by the patient. [110]

In Schmidtke et al., the likelihood test described above is applied to five pairs of implanted sensors and examined on the Clarke error grid for clinical relevance. The percentage of readings that fell into the clinically correct regions increased from 92.4% to 98.8% when utilizing the likelihood test. [110]

Conclusions

This chapter discussed the need for reliable implantable sensors and modes of failure the implanted electrochemical sensors can exhibit. The method used to increase the reliability of the sensors in this thesis is the use of redundancy. Fabrication techniques from the micro-electronic industry were applied to develop the sensors on a
flexible and biocompatible polyimide substrate. The statistics of using a redundant array of sensors were discussed in detail. Previous methods developed for signal processing and fault detection have shown to increase the reliability of sensors and can be utilized for the redundant array.
Chapter 4

Electrochemical Characterization of the Micro-fabricated Glucose Sensor Components

Introduction

Electrochemical Sensor Characteristics

Electrochemical sensors are commonly used for biosensing applications because they are sensitive and selective towards electroactive species, fast, accurate, compact, portable and inexpensive. An electrochemical sensor converts a chemical quantity into a measurable potential or current. [107] Electrochemical sensors are used in different ways by varying, measuring, and keeping constant different parameters in the sensor such as current and electrode potential. The main types of electrochemical testing for sensors are amperometry, potentiometry, cyclic voltammetry and pulsed voltammetry. Amperometry uses a constant potential and monitors the current, potentiometry uses a constant current and monitors the potential, cyclic voltammetry applies a cyclic potential and monitors the current and pulsed voltammetry applies a pulsed potential while monitoring the current. The methods under investigation for testing of the developed electrochemical sensors are cyclic voltammetry, square wave voltammetry, and amperometry.

The basic experimental setup of an electrochemical cell consists of an electrolyte, a phase through which charge is transferred by movement of ions [39], three electrodes: reference electrode, counterelectrode and working electrode, a potentiostat used to
control the conditions of the experiment and a computer for easy user interface and data collection (see Figure 4.1). For the electrochemical sensors discussed herein, the potential is either kept constant (amperometry), cycled (cyclic voltammetry) or pulsed (square wave voltammetry) and the current is monitored.

---

**Figure 4.1:** Diagram of electrochemical cell used in experiments for electrochemical sensor testing, where the working electrode is the sensor being tested, the reference is usually silver/silver chloride (Ag/AgCl) and the counterelectrode is commonly a platinum wire. The electrodes are attached to a potentiostat that controls the conditions applied to the electrodes and is interfaced with a computer to collect the data and set the conditions.
Electrochemical Glucose Sensors

As discussed in Chapter 2, previously developed glucose sensors commonly contain glucose oxidase (GOX) for specificity and rely on the detection of either hydrogen peroxide or electrons through a mediated reaction. The first thought for making an implantable sensor would be to miniaturize the glucose meter used outside the body and implant it in the body. This system has many complicating factors. First, the sensors are not biocompatible, which would lead to the rejection of the sensor. Secondly, most sensors detect glucose by reacting the glucose with oxygen. In the environment there is an abundant source of oxygen for the sensor to work correctly but in the body there are varying amounts of oxygen, so this could lead to false signals when there is not enough oxygen present. Third, for the sensors outside of the body, diffusing mediators are sometimes used and can be toxic in the body. If diffusing mediators were implanted in the body, the mediator may diffuse into the body and result in interference with biological reactions and the sensor response would diminish. [26]

To eliminate the uncertainty of oxygen concentration and danger in diffusing mediators, charge mediators have been utilized in the reaction to relay electrons to the electrode for detection. [12, 18, 23, 25-30] Gregg et. al. used osmium redox polymers as the charge mediator, these complexes are stable in more than one oxidation state, so they accept and donate electrons freely. [26] A common redox polymer is POs-EA [24] (seen in Figure 4.2). This polymer is designated as “M” in the reaction scheme below:
Reaction Scheme:

\[
glucose + GOX(FAD) \rightarrow \text{gluconic acid} + GOX(FADH}_2\]

\[
GOX(FADH}_2) + 2(M)(\text{ox}) \rightarrow GOX(FAD) + 2(M)(r) + 2H^+2(M)(r) + 2(M)(\text{ox}) \rightarrow 2(M)(\text{ox}) + 2(M)(r) \text{ (e\textsuperscript{-} self-exchange)}
\]

\[
2(M)(r) \rightarrow 2(M)(\text{ox}) + 2e^-
\]

---

Figure 4.2: Schematic of POs-EA, the mediator polymer used to relay electrons to the electrode for detection

In this reaction, glucose will release an electron during its interaction with glucose oxidase, and the electron will be relayed to the electrode by POs-EA. This results in an increased electrochemical response with increasing glucose concentrations. [24, 28]

The sensor developed in this dissertation utilizes the mediator POs-EA to transport the electrons produced by the glucose reaction to the electrode for detection. Since the POs-EA in the glucose sensor is vital in transport it must undergo a reversible
reaction to be in the oxidized and reduced form. This chapter discusses the mediator in
the developed sensor and its reversibility when a part of the sensor as well as further
details on the electrochemical techniques cyclic voltammetry and square wave
voltammetry.

**Materials and Methods**

**Chemicals**

Glucose oxidase (GOX, EC 1.1.3.4, Type X-S, 128 units/mg solid from
*Aspergillus niger*), ammonium hexachloroosmate(IV), 11-mercaptoundecanoic acid
(MUA), poly(4-vinylpyridine), β-D-glucose, acetone, ammonium hexafluorophosphate,
sodium dithionite, ether, 2-bromoethylamine hydrobromide, N,N-dimethylformamide
(DMF), anion exchange beads, hydrochloric acid (HCl), poly(ethylene glycol) diacrylate
(PEG-DA) and 2,2′-dipyridyl (bpy) were obtained from the Sigma-Aldrich Chemical
Company (St. Louis, MO). Ethyl alcohol, ethylene glycol and acetonitrile were obtained
from Fisher Scientific Company (Pittsburgh, PA). DAROCUR, the photo initiator, was
obtained from Ciba. All reagents, unless otherwise stated, were used as received.
Polyimide sheets (1/16” thick) were purchased from McMaster Carr (New Brunswick,
NJ). Phosphate buffered saline (PBS) consisted of 1.1 mM potassium phosphate
monobasic, 3 mM sodium phosphate dibasic heptahydrate. Conductive silver epoxy was
obtained from Ladd Research (Williston, VT). Ma-P 100, positive photoresist, and ma-D
330, photoresist developer, were purchased from MicroChem Corporation (Newton,
MA). The polycationic redox polymer, poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine (POs-EA) was synthesized according to a procedure described previously. [24, 127]

**Synthesis of poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine (POs-EA)**

Synthesis of poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine (POs-EA), an osmium based polycationic redox polymer was done following modifications of established protocols. Osmium (bipyridine)$_2$ dichloride (Os(bpy)$_2$Cl$_2$) was synthesized according to a standard procedure with minor modifications. [128] In brief two equivalents of bipyridine (1440 mg) were mixed with one equivalent ammonium hexachloroosmate (IV) (2000 mg) in 100 mL ethylene glycol. The mixture was heated to reflux for 45 minutes and then precipitated with supersaturated sodium dithionite. The precipitate was repeatedly washed with water and finally with ether.

Os(bpy)$_2$Cl$_2$ (0.988 g, 1.728 mmol) and poly(4-vinylpyridine) (0.860 g, 8.18 mequiv) were heated under nitrogen at reflux in 36 mL of ethylene glycol for 2 hours. The solution was then cooled down to room temperature and 60 mL of DMF and 3.0 g of 2-bromoethylamine hydrobromide (14.6 mmol) were added and then stirred overnight at 45 °C. A crude polymer precipitate was formed by pouring the solution into rapidly stirred acetone. The hygroscopic precipitate was collected and dissolved in water. The solution was filtered and precipitated as the hexafluorophosphate (PF$_6$) salt by addition of a solution of ammonium hexafluorophosphate. The precipitate was dried in a vacuum at 40 °C. Dry PF$_6^-$ salt (0.98 g) was dissolved in 40 mL of acetonitrile and then diluted
with 100 mL of water and stirred over 10.4 g of anion exchange beads for 2 hours. The solution was filtered and evaporated under vacuum to ~20 mL. Concentrated HCl was added to the solution to adjust to pH 2. The solution was then dripped into rapidly stirred acetonitrile. A precipitate that formed was filtered and dried in a vacuum oven. The pure product was analyzed.

**Fabrication of electrode arrays on polyimide sheets**

Polyimide sheets are a flexible and insulating material that is an ideal platform for electrode manufacturing. Square pieces of polyimide were cut and washed with ethanol before coating with positive photoresist (ma-P 100). Modified literature procedure was used to fabricate the electrode arrays. [115] In brief positive photoresist (ma-P 100) was deposited on square polyimide sheets and spin coated at 4000 rpm for 30 seconds and then soft baked at 100 °C for 5 minutes. These polymer sheets with dry photoresist were brought in close contact with the photo-mask and exposed to 365 nm UV light. The polymer sheets were then placed in the developer solution (ma-D 330) for seventy seconds to remove the portions of photoresist that were exposed to UV light. The sheets were then rinsed with distilled water.

The photoresist patterns were then sputter coated with 10 nm of an adhesion layer of chrome followed by 150 nm layer of gold (performed at Pennsylvania State University using Lesker CMS-18 sputtering tool and by Lance Goddard Associates in Foster City, CA). The photoresist was removed using acetone to lift-off the excess chrome and gold from all non-patterned areas. The result was distinct patterns of gold with 500 µm
diameter electrodes with leads 10 µm and contact pads 2.5 mm x 2.5 mm. Wires were
attached to the contact pads by using conductive silver epoxy resin (Ladds Research,
Williston, VT).

**Glucose Sensors**

The electrode arrays were functionalized with a carboxylic end group by
immersing in 2 mM MUA in ethanol for 20 minutes and then washed with ethanol. The
electrodes were then dried under nitrogen. The thiol end group was chemisorbed to gold
to provide an anchor. Next, the redox polymer was immobilized on the electrodes by
depositing 2 µL of 10 mg/mL POs-EA onto the surface of the electrode and allowed to
cure overnight. Due to electrostatic interactions between the negatively charged MUA
and positive redox polymer a POs-EA layer will be formed. The excess POs-EA was
then removed by washing with deionized water and dried under nitrogen. To fabricate
the hydrogel on the glucose sensor array a 5 µL solution of 50 mg/mL GOX in 0.1 M
HEPES was combined with 5 µL of PEG-DA and 0.5 µL DAROCUR. The solution was
vortexed and then spread over the electrode array area and exposed to UV light for 0.2
seconds to form a hydrogel. The hydrogel was then allowed to swell and equilibrate for
at least one hour in a solution containing 0.1 M PBS before any testing was done. The
testing was done in a cell of PBS solution.
Electrochemical Testing

Electrochemical tests (CV and SWV) were performed using a Princeton Applied Research (PAR) 283 potentiostat on a three-electrode system with a platinum wire used as the counterelectrode and Ag/AgCl as the reference electrode. The array electrodes with POs-EA polymer and enzyme encapsulated with a PEG-DA hydrogel were used as working electrodes. The CV experiments were conducted at various scan rates (0.5 mV/s to 100 mV/s) over the potential range from 0 V to 0.6 V and returning to 0 V vs. Ag/AgCl. The SWV experiments were conducted over the potential range –0.2 V to 0.6 V vs. Ag/AgCl with varied frequencies (1 – 20 Hz), a step height of 1 mV and a step width of 20 mV.

Results and Discussion

To determine if the desired redox polymer was synthesized methods such as cyclic voltammetry, square wave voltammetry and FTIR can be used to identify the polymer. Fourier-transform infrared spectroscopy (FTIR) is an optical technique that involves an infrared beam probing a species to measure the response of the sample, this technique has proven useful in the chemical analysis of species. [129, 130] The species display peaks characteristic of defined bonds. The synthesized POs-EA exhibited peaks at 1410 cm\(^{-1}\), which is indicative of pyridine, and 3150 cm\(^{-1}\) which indicates a primary amine as seen in Figure 4.3. These peaks show that pyridine, which is a major component of the molecule, and a primary amine, the end group on the side chain of ethylamine, are present as expected for the polymer.
Electrochemical Analysis

Since POs-EA is an electroactive polymer it can be characterized by electrochemical techniques to compare the standard potential found in literature to the value obtained experimentally. CV and SWV techniques are used to determine the standard potential of the synthesized polymer. The POs-EA is also probed as when included in the glucose sensor that is being developed to determine its reversibility under the desired experimental conditions.
Cyclic Voltammetry

Cyclic voltammetry uses an electrochemical setup as seen in Figure 4.1 and cycles the potential between the reference and working electrodes while recording the current of the working electrode (see Figure 4.4 below for scanning parameters and the example graph of current versus time for POs-EA). The current measured during the test is graphed versus potential and the peaks in the graph are corresponding to the oxidation (Eq. 4.1 below) and the reduction (Eq. 4.2 below) potential of the electroactive species, in this case POs-EA.

\[
\begin{align*}
R \rightarrow O + ne^- & \quad (4.1) \\
O + ne^- & \rightarrow R & \quad (4.2)
\end{align*}
\]

where O is the oxidized species, \(e^-\) is the electron, \(n\) is the stoichiometric coefficient of the electron (1 for POs-EA), and R is the reduced species. As the potential is ramped up the oxidation of POs-EA occurs and the peak cathodic current is observed and as the potential is returned to the original potential the reduction of POs-EA occurs leading to the peak anodic current (as seen in Figure 4.5).
Figure 4.4: Experimental scans for cyclic voltammetry displaying the changes in potential versus time and the resulting current versus time
Figure 4.5: Diagram of a cyclic voltammogram of POs-EA in 0.1 M PBS with a scan rate of 30 mV/s where the background currents are labeled and $i_p$ is the peak current caused by cathodic (c) and anodic (a) reactions.

The cyclic voltammogram graph that is obtained for electrochemically reversible species can be divided into sections to understand the reactions occurring. When a potential is applied to the electrode the ions in solution will form a double layer of charges on the electrode surface, which leads to a capacitance of the electrode interface and can be seen as the lighter shade in Figure 4.5. The next part of the background seen under the sloped line in Figure 4.5 is due to the resistance of the solution, which is uncompensated. The uncompensated resistance has a sloped line because of the
relationship that the current is inversely proportional to the resistance \( i = E/R \) leading to the slope of the background being \( 1/R \) (which can be decreased by putting the reference electrode as close as possible to the working electrode and including an electrolyte).

The last part of the graph is the faradaic current obtained from the electroactive species. The concentrations of \( O \) and \( R \) are defined by the Nernst equation (Eq. 4.3) and at \( E^o \) (the standard potential) for electrochemically reversible systems the concentration of \( O \) and \( R \) are equal at the surface of the electrode.

\[
E = E^o + \frac{RT}{nF} \ln \left( \frac{[O]}{[R]} \right)
\]

where \( E \) is the potential applied between the working and reference electrodes, \([O]\) is the concentration of the oxidized species, \([R]\) is the concentration of the reduced species, \( E^o \) is the standard potential of the reaction, \( n \) is the number of electrons, \( F \) is Faraday constant (96485 Coulomb/mol), \( T \) is the temperature (in Kelvin) and \( R \) is the gas constant (at room temperature \( RT/F \) equals 0.059 V). The peaks seen in the CV are caused by the diffusion of the reacting species to the electrode, when the overpotential (the potential applied minus the standard potential) is increased more of the reacting species is reacted and less has had time to diffuse to the surface, leading to the peak in the current. The standard potential of the reaction is found by Eq. 4.4.

\[
E^o = \frac{E_{p,a} + E_{p,c}}{2}
\]

where \( E_{p,a} \) is the potential where the anodic current peaks and \( E_{p,c} \) is where the cathodic current peaks. The formal potential of the redox polymer, POs-EA, was approximately
0.3 V (Ag/AgCl), in agreement with previous results described in the literature.\textsuperscript{[28]} \(E^0\) is the standard potential of the reaction and related to \(\Delta G^0\) of the reaction as seen in Eq. 4.5:

\[
\Delta G^0 = -nFE^0
\]  

when the potential \(E\) is equal to the standard potential \(E^0\), in other words the overpotential is equal to zero, then delta \(\Delta G\) equals zero.

Since the purpose of the mediator is to easily donate and accept electrons it needs to be reversible, which can be determined by the CV graph. When a reaction is electrochemically reversible the peak separation has a defined value as seen in Eq. 4.6.

\[
\Delta E_p = \left|E_{pc} - E_{pa}\right| = \frac{RT}{nF} = \frac{59mV}{n}
\]  

For POs-EA, anodic and cathodic peaks were observed at 0.32 and 0.25 V (Ag/AgCl) at 20 mV/s with a peak separation of approximately 70 mV, indicating electrochemical reversibility (due to experimental limitations the normal range for reversibility is between 65-75 mV/n).\textsuperscript{[131]}

To determine chemical reversibility the relationship in Eq. 4.7 must be satisfied, which was the case for POs-EA.

\[
i_{pc} = -i_{pa}
\]  

Since the POs-EA was immobilized on the surface of an electrode and entrapped under a PEG-DA hydrogel, the nature of the polymer in this environment was determined. After the sensor arrays were fabricated, cyclic voltammograms of one element of the micro-array were performed at different scan rates (Figure 4.6). The CV scans do not appear consistent with thin-layer electrochemistry (TLE) (the POs-EA can
be thought of as a thin layer), because the expected CV shape for TLE appears as a Gaussian-shaped curve. This dependence suggests that the osmium layer is sufficiently thick and/or the electron transfer between osmium moieties is slow, leading to the system to mimic semi-infinite linear diffusion. Since the method of fabrication is a layering technique (MUA, P0s-EA, then the PEG-DA hydrogel), the film can be thought of as sufficiently thick and the electron transfer can also be hindered by the attachment of the PEG-DA hydrogel to the layers.

Figure 4.6: Varying scan rate from 0.5 to 100 mV/s on one electrode for the fabricated sensor in 0.1 M PBS to determine the behavior of the osmium polymer
Another diagnostic tool used to determine thin-layer electrochemistry is the dependence of the peak current versus scan rate. When the peak current is linearly dependent on the scan rate, as demonstrated by Eq. 4.8

\[ i_p = \frac{n^2 F^2 V C^o n V}{4RT} \]  

4.8

thin-layer electrochemistry is thought to occur. Semi-infinite linear diffusion occurs when the peak current versus the square root of the scan rate is a linear relationship defined by the Randall-Sevick equation (Eq. 4.9) for electrochemically reversible systems. [131]

\[ i_p = 2.69 \times 10^5 n^{3/2} AD^{1/2} c^* \nu^{1/2} \]  

4.9

Figure 4.7 shows a plot of peak current \( (i_p) \) versus scan rate \( (\nu) \) was linear at scan rates 0.5-20 mV/s (R-squared value of 0.9986). At high scan rates (above 20 mV/s) a deviation from linearity was observed, and the peak current was seen to depend linearly on the square root of the scan rate (not shown). The time scales used for the cyclic voltammetry experiments lay on the lower end of the scan rates so the electron transfer should be sufficiently fast and reversible for the experiments, since the lower end of the current versus scan rate curve yielded a linear relationship.
Square Wave Voltammetry

Square wave voltammetry (SWV) is an electrochemical technique suitable for both qualitative and quantitative analysis. One major advantage of this technique is that the charging current component, due to electrical charging of electrode double layer, is largely eliminated and the signal to noise ratio is enhanced. Another advantage of SWV is the decreased timescale necessary for the experiments. In SWV the potential is pulsed.
as seen in Figure 4.8 and the current signal is repeatedly sampled at two points (forward and reverse) relative to the time of application of the square wave voltage signal to the electrode. Various parameters can be altered with SWV to obtain the desired results: $\Delta E_p$ is the height of the pulse, $E_s$ is the step height, $t_p$ is the length of time the pulse is applied, $i_f$ is the forward current and $i_r$ is the reverse current. The range of $\Delta E_p$ and $E_s$ are 0 to 100 mV, with the normal practice being that the $E_s$ is much smaller than $\Delta E_p$ and these parameters determine the resolution of the scan. The $t_p$ is varied over 1 – 500 ms (giving a frequency of 1 – 500 Hz) and determines the timescale of the experiment. The SWV scan is quick as compared to typical CV scans and other pulse methods. [131]

The difference between the two current values ($i_f$ and $i_r$) can be plotted as a function of the applied potential. Forward, reverse and difference scans are obtained as seen in Figure 4.9. The resultant peaks correspond to the electroactivity of the species in the electrochemical cell. The major component of this difference current is the faradaic current, which flows due to an oxidation or reduction at the electrode surface.
Figure 4.8: Scan of potential versus time for Square Wave Voltammetry and the resulting current versus time plot
The SWV scans of POs-EA showed a reversible response for the Os$^{II}$/Os$^{III}$ redox couple, with forward and reverse peaks at similar potentials (Figure 4.9). The formal potential of this redox polymer entrapped in PEG-DA hydrogel was around 0.3 V versus Ag/AgCl, the same found when using CV.

Figure 4.10 shows square wave voltammograms of one element of the micro-array at different frequencies. The SWV peak difference current was proportional to frequency from 1 to 5 Hz (Figure 4.10) consistent with thin-layer electrochemical behavior. [132] At frequencies higher than 5 Hz, the dependency of peak current follows a linear relationship to the square root of the frequency, which is expected for reversible reactions with SWV. [133]
Figure 4.10: a) SWV scans at different frequencies of the sensor array in 0.1 M PBS, b) Current dependence on frequency for SWV
Conclusions

In this chapter the redox polymer, POs-EA, was synthesized and then characterized chemically and electrochemically. The POs-EA was found to have FTIR peaks indicative of the desired polymer and displayed the standard potential (as determined by SWV and CV) expected for the desired polymer. The polymer was also tested within the sensor system for its reversibility and reactivity, it was found to exhibit thin-layer electrochemistry behavior at the CV scan rates that will be utilized for further testing and within the confines of the sensor was seen to be chemically and electrochemically reversible as determined by the CV experiments. The POS-EA polymer was also probed with SWV to determine the characteristics of the polymer through the pulse method. With the POs-EA working as desired within the sensor structure the proposed glucose sensors can be further developed and characterized properly with the characteristics of POs-EA already determined.
Chapter 5

Electrochemical Optimization of Micro-fabricated Glucose Sensor Arrays

Introduction

In an electrochemical sensor, the measured current is proportional to the reaction rate. For a heterogeneous reaction, when the reaction occurs at the electrode-electrolyte interface, the current is described by Eq. 5.1.

\[ i = nF(A \text{Rate}) \]  \hspace{1cm} 5.1

where \( i \) is the measured current in amperes (A), \( n \) is the stoichiometric number of electrons involved in an electrode reaction, \( F \) is Faraday’s constant (96485.3 Coulombs), \( A \) is the electrode area in cm\(^2\), and the \( \text{Rate} \) is the reaction rate given in mol/s cm\(^2\). [131]

An alternative representation used to describe a heterogeneous reaction is current density, the current divided by the electrode area, which is defined by Eq. 5.2.

\[ j = nF(\text{Rate}) \]  \hspace{1cm} 5.2

where \( j \) is the measured current density in amperes/cm\(^2\). [131] The heterogeneous reaction rate depends on mass transfer to the electrode, in addition to the kinetic variables.

One consideration that must be taken into account when fabricating the electrochemical sensors is that the current is proportional to the reaction rate. For the
sensors described here in later sections, where there is an enzymatic reaction occurring in
the sensor, Michaelis-Menten kinetics have been used to describe the system. [134] If the
reaction rate proceeds infinitely fast by Michaelis-Menten kinetics the current would
saturate quickly, but if the system is mass transfer limited then a linear response can be
obtained (as can be seen in Figure 5.1). If Michaelis-Menten kinetics is applied to the
system then the reaction rate can be described by the following equation:

\[ \nu = \frac{V_{\text{max}} c_S}{c_S + K_m} \quad 5.3 \]

in which \( \nu \) is the reaction rate, \( V_{\text{max}} \) is the maximum reaction rate, \( c_S \) is the substrate
concentration, and \( K_m \) is the Michaelis-Menten constant. Since the reaction rate is
proportional to the current a similar expression can be written as follows:

\[ i = \frac{i_{\text{max}} c_S}{c_S + K_{\text{app}}} \quad 5.4 \]

in which \( i \) is the current measured, \( i_{\text{max}} \) is the maximum current, \( c_S \) is the substrate
concentration, and \( K_{\text{app}} \) is the apparent Michaelis-Menten constant. For fabricated
sensors to provide a linear response mass transfer limitations must be implemented
through the entrapment of the enzyme (as displayed in Figure 5.1).
Figure 5.1: Current versus concentration for Michaelis-Menten kinetics with and without mass transfer effects

The $K_m$ of the enzyme can be altered by encapsulation or adsorption onto a surface. [135, 136] In this chapter the use of the biocompatible polymer PEG-DA is used to entrap the enzyme glucose oxidase onto the surface of the previously described array of electrodes. Various electrochemical methods are utilized for the optimization of the sensing method to increase speed of response and sensitivity.

**Materials and Methods**

**Chemicals**

Glucose oxidase (GOX, EC 1.1.3.4, Type X-S, 128 units/mg solid from *Aspergillus niger*), ammonium hexachloroosmate(IV), 11-mercaptoundecanoic acid (MUA), poly(4-vinylpyridine), β-D-glucose, acetone, ammonium hexafluorophosphate,
sodium dithionite, ether, 2-bromoethylamine hydrobromide, N,N-dimethylformamide (DMF), anion exchange beads, hydrochloric acid (HCl), poly(ethylene glycol) diacrylate (PEG-DA) and 2,2′-dipyridyl (bpy) were obtained from the Sigma-Aldrich Chemical Company (St. Louis, MO). Ethyl alcohol, ethylene glycol and acetonitrile were obtained from Fisher Scientific Company (Pittsburgh, PA). DAROCUR, the photo initiator, was obtained from Ciba. All reagents, unless otherwise stated, were used as received. Polyimide sheets (1/16” thick) were purchased from McMaster Carr (New Brunswick, NJ). Phosphate buffered saline (PBS) consisted of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate. Conductive silver epoxy was obtained from Ladd Research (Williston, VT). Ma-P 100, positive photoresist, and ma-D 330, photore sist developer, were purchased from MicroChem Corporation (Newton, MA). The polycationic redox polymer, poly[4-vinylpyridine osmium (bipyridine)₂ chloride]-co-ethylamine (POs-EA) was synthesized according to a procedure described previously.[24, 127]

**Synthesis of poly[4-vinylpyridine osmium (bipyridine)₂ chloride]-co-ethylamine (POs-EA)**

Synthesis of poly[4-vinylpyridine osmium (bipyridine)₂ chloride]-co-ethylamine (POs-EA), an osmium based polycationic redox polymer was done following modifications of established protocols. Osmium (bipyridine)₂ dichloride (Os(bpy)₂Cl₂) was synthesized according to a standard procedure with minor modifications. [128] In brief two equivalents of bipyridine (1440 mg) were mixed with one equivalent ammonium hexachloroosmate (IV) (2000 mg) in 100 mL ethylene glycol. The mixture
was heated to reflux for 45 minutes and then precipitated with supersaturated sodium dithionite. The precipitate was repeatedly washed with water and finally with ether.

Os(bpy)$_2$Cl$_2$ (0.988 g, 1.728 mmol) and poly(4-vinylpyridine) (0.860 g, 8.18 mequiv) were heated under nitrogen at reflux in 36 mL of ethylene glycol for 2 hours. The solution was then cooled down to room temperature and 60 mL of DMF and 3.0 g of 2-bromoethylamine hydrobromide (14.6 mmol) were added and then stirred overnight at 45 °C. A crude polymer precipitate was formed by pouring the solution into rapidly stirred acetone. The hygroscopic precipitate was collected and dissolved in water. The solution was filtered and precipitated as the hexafluorophosphate (PF$_6^-$) salt by addition of a solution of ammonium hexafluorophosphate. The precipitate was dried in a vacuum at 40 °C. Dry PF$_6^-$ salt (0.98 g) was dissolved in 40 mL of acetonitrile and then diluted with 100 mL of water and stirred over 10.4 g of anion exchange beads for 2 hours. The solution was filtered and evaporated under vacuum to ~20 mL. Concentrated HCl was added to the solution to adjust to pH 2. The solution was then dripped into rapidly stirred acetonitrile. A precipitate that formed was filtered and dried in a vacuum oven. The pure product was analyzed.

**Fabrication of electrode arrays on polyimide sheets**

Polyimide sheets are a flexible and insulating material that is an ideal platform for electrode manufacturing. Square pieces of polyimide were cut and washed with ethanol before coating with positive photoresist (ma-P 100). Modified literature procedure was used to fabricate the electrode arrays. [115] In brief a positive photoresist (ma-P 100)
was deposited on square polyimide sheets and spin coated at 4000 rpm for 30 seconds and then soft baked at 100 °C for 5 minutes. These polymer sheets with dry photoresist were brought in close contact with the photo-mask and exposed to 365 nm UV light. The polymer sheets were then placed in the developer solution (ma-D 330) for seventy seconds to remove the portions of photoresist that were exposed to UV light. The sheets were then rinsed with distilled water.

The photoresist patterns were then sputter coated with 10 nm of an adhesion layer of chrome followed by 150 nm layer of gold (performed at Pennsylvania State University using Lesker CMS-18 sputtering tool and by Lance Goddard Associates in Foster City, CA). The photoresist was removed using acetone to lift-off the excess chrome and gold from all non-patterned areas. The result was distinct patterns of gold with 500 µm diameter electrodes with leads 10 µm and contact pads 2.5 mm x 2.5 mm. Wires were attached to the contact pads by using conductive silver epoxy resin (Ladds Research, Williston, VT).

**Glucose Sensors**

The electrode arrays were functionalized with a carboxylic end group by immersing in 2 mM MUA in ethanol for 20 minutes and then washed with ethanol. The electrodes were then dried under nitrogen. The thiol end group was chemisorbed to gold to provide an anchor. Next, the redox polymer was immobilized on the electrodes by depositing 2 µL of 10 mg/mL POs-EA onto the surface of the electrode and allowed to cure overnight. Due to electrostatic interactions between the negatively charged MUA
and positive redox polymer a POs-EA layer will be formed. The excess POs-EA was then removed by washing with deionized water and dried under nitrogen. To fabricate the hydrogel on the glucose sensor array a 5 µL solution of 50 mg/mL GOX in 0.1 M HEPES was combined with 5 µL of PEG-DA and 0.5 µL DAROCUR. The solution was vortexed and then spread over the electrode array area and exposed to UV light for 0.2 seconds to form a hydrogel. The hydrogel was then allowed to swell and equilibrate for at least one hour in a solution containing 0.1 M PBS before any testing was done. The testing was done in a cell of PBS solution.

**Electrochemical Testing**

Electrochemical tests (CV, amperometry and SWV) were performed using a Princeton Applied Research (PAR) 283 potentiostat on a three-electrode system with a platinum wire used as the counterelectrode and Ag/AgCl as the reference electrode. The array of electrodes with POs-EA polymer and enzyme encapsulated with a PEG-DA hydrogel were used as the working electrodes. All experiments were completed in 0.1 M PBS buffer with pH 7.2.

**Results and Discussion**

Amperometric biosensors based on redox polymer/enzyme complexes were shown to be miniaturizable and implantable. [110, 135, 137] Previously, enzymes entrapped in redox hydrogels using photopolymerization were shown to retain their
activity. [29, 32, 106, 138, 139] In this work redox polymers exchanged electrons with glucose oxidase entrapped in biocompatible poly(ethylene glycol) diacrylate (PEG-DA) hydrogels.

Reproducibility of Micro-fabrication

Each fabricated sensor array element was smooth and without any discontinuities (as seen previously in Figures 2.3 and 2.4). To determine whether the array elements had any connectivity problems, or whether they functioned independently, CV and SWV were done on each array member. Each electrode was 500 µm in diameter, individually addressable, and separated from the others by 1000 µm as to alleviate any cross-talk.

Enzyme-containing redox polymer films were formed through the UV-initiated free radical cross-linking of a redox polymer and PEG-DA in each electrode array element. Each sensor array element after micro-fabrication was smooth and without any discontinuities. To determine whether the array elements had any defects or whether they functioned independently cyclic voltammetry and square wave voltammetry were performed initially without the analyte (glucose) to evaluate the reproducibility of the micro-fabrication process and the stability of the film. The micro-fabrication process was quite reproducible as observed from almost overlaid cyclic voltammograms and square wave voltammograms for each element of the array (not shown). The entrapment of the redox polymer, glucose oxidase and PEG-DA was also uniform on all the electrodes, which is especially important when summing sensor signals of the arrays. The signal from all the array sensor elements mimics the behavior of one large sensor with electrode
area equal to the sum of all the electrode array elements. Figure 5.2 shows cyclic voltammograms of increasing number of array elements. Measurements done after combination of different array members show increasing peak currents with increasing number of array elements combined. Figure 5.3a shows square wave voltammograms of different combinations of increasing number of array elements. Measurements done after combination of different array members show increasing peak difference currents with increasing number of array elements combined (Figure 5.3b). The sensor arrays portray a situation where the area of the array elements is almost the same and the film assembly on each element being reproducible. With such a sensor array, multiple analytes can be measured simultaneously and through the introduction of redundancy, measurements can be derived from the average of the signal resulting from each array element. There is no evidence of cross-talk between the array members in these sensors.
Figure 5.2: Cyclic voltammograms of different combinations of the micro-array electrodes at 20 mV/s scan rate in 0.1 M PBS
Figure 5.3: (a) SWV of different combinations of array sensor elements in PBS at pH 7.2 (b) Change of SWV peak currents with different combinations of electrodes in the array sensor
Cyclic Voltammetric Analysis

Properly cross-linked GOX and POs-EA molecules are expected to retain the activity of glucose oxidase and to present signal stability. Signal stability would degrade if the enzyme were to leach from the hydrogel and was not seen in the fabricated sensors. The first step in determining the viability of the enzyme in the system was to determine the scan rate that would allow the reaction of glucose and glucose oxidase and the concurrent reactions to occur and the produced electrons be detected by the electrode. This was done by varying the scan rate to observe the behavior of the CV scans, when the electrocatalytic pathway is established for the reaction the reduction peak of the curve should decrease and the oxidation peak should be enhanced (seen Figure 5.4). The results for Figure 5.4 show that the optimum scan rate is 1 or 2 mV/s, and 2 mV/s is the scan rate used for other CV experiments unless otherwise indicated.
**Figure 5.4**: Glucose sensor cyclic voltammograms of one array element with varying scan rate to determine the optimum scan rate for detection of electrocatalysis (67 mM glucose in 0.1 M PBS)

**Figure 5.5** shows cyclic voltammograms of five combined elements of the micro-array in buffer containing 0, 5, 10, and 20 mM glucose solutions. In the presence of increasing glucose concentration, the current is found to increase. The rate of this catalytic reaction is proportional to the concentration of glucose present. Flavin adenine dinucleotide of glucose oxidase GOX(FAD) reacts with β-D-glucose to form a reduced form GOX(FADH₂) and gluconic acid inside the hydrogel. The reduced form of GOX(FADH₂) is in turn oxidized by the electrochemically generated Os³⁺ form of the
redox polymer, setting up a catalytic pathway which produces an enhanced oxidation peak.

The electrons are transferred from the enzyme to the redox polymer, shuttled between the redox sites in a self-exchange reaction until being transferred to the electrode surface. The catalytic current produced is proportional to the glucose concentration. Glucose oxidase is securely trapped in the hydrogel network and glucose diffuses through to access the glucose oxidase sites. Photo-polymerization of the acrylate end groups of the PEG-DA by the photoinitiator DAROCUR occurs and entraps the glucose oxidase enzyme on the redox polymer film.

Figure 5.5: Cyclic Voltammograms of the collective response of five sensor elements at different glucose concentrations at 2 mV/s scan rate in 0.1 M PBS
Figure 5.6 indicates that the sensor signal is linearly dependent on the concentration of glucose (0–20 mM) with an r-squared value of 0.9898. The calculated sensitivities for this sensor are $1.782 \, \mu\text{A}/(\text{cm}^2 \, \text{mM})$ for the whole array and $0.357 \, \mu\text{A}/(\text{cm}^2 \, \text{mM})$ for each electrode. Previously reported sensitivities for the same gold micro-electrodes without a biocompatible hydrogel were lower ($0.26 \, \mu\text{A}/(\text{cm}^2 \, \text{mM})$) than for this sensor. [115]
Cyclic voltammetry is a very helpful technique to prove the electrocatalysis of the enzyme in the system, but the major drawback using cyclic voltammetric analysis of the sensors is the time necessary for each cycle, for the scan rate of 2 mV/s the scan takes 10 minutes to complete. For a sensor that is desired to be real-time, a testing time of 10 minutes is not acceptable; therefore other scanning methods were investigated.

**Chronoamperometry Analysis**

Chronoamperometry is an electrochemical technique in which the potential is set at a constant potential equal to a high overpotential for the reaction in question and the current is monitored. For the reactions of POs-EA the potential is set at 0.4 V to allow for all of the reduced species to be oxidized and in turn when glucose is present the reaction can be detected when the oxidized species reacts with the GOX(FADH$_2$) form of the enzyme and the electron relayed to the electrode for detection. The current is monitored and increases proportionally to the reaction rate of the glucose reaction.

The glucose sensors were tested by amperometry and the stepwise curve in Figure 5.7 was obtained. Since the current is proportional to the reaction rate, which is proportional to the glucose concentration in solution, the increase in current with increasing glucose concentration is expected and observed.
Figure 5.7: Amperometry results of the glucose sensors with the concentration of glucose for each step labeled, where the potential was held at 0.4 V and the current versus time monitored.
Figure 5.8 contains the current density at each step from the amperometric scan versus the concentration of glucose in the test solution, which exhibited a linear response. The linear regression of the current density versus concentration has an R-squared value of 0.9857 and a sensitivity of 79.6 nA/(cm$^2$ mM), which is much lower than that of the cyclic voltammetry results and can still be improved on.
Square Wave Voltammetric Analysis

Square wave voltammetry (SWV) is an electrochemical technique discussed in Chapter 4 that has enhanced signal to noise ratio and scans that are that range from a few seconds to a couple minutes. Figure 5.9 shows square wave voltammograms of one element of the micro-array in buffer without glucose and in buffer containing 10 mM, 20 mM and 50 mM glucose solutions. In the presence of increasing glucose concentrations the peak difference current is found to increase. The catalytic current produced is proportional to the glucose concentration (Figure 5.10). As expected at high concentrations of glucose (above 30 mM) there is deviation of linearity due to saturation of glucose oxidase binding sites described by Michaelis-Menten kinetics.

Figure 5.9: Square wave voltammograms of one sensor element at different labeled glucose concentrations at 5 Hz
Figure 5.10: SWV sensor peak current response to increasing the glucose concentration when testing at 5 Hz

The frequency for the scans above were set to 5 Hz and gave a sensitivity of 0.250 nA/mM or 31.77 nA/(mM cm²), but since the amounts of enzyme and POs-EA are in excess the sensor should exhibit a linear response at higher frequencies also. The frequency was increased to 50 Hz (suggested range of frequency is between 1 and 500 Hz [131]) and the results of the peak difference current versus concentration can be seen in Figure 5.11. The sensor sensitivity increased to 0.150 µA/mM or 19.20 µA/(mM cm²), following a linear relationship with the r-squared value of 0.9852. If the amount entrapped in the sensor components is decreased so that they are not in excess the frequency will have to be lowered. Besides the increase in sensitivity and signal increasing the frequency decreases the amount of time needed per cycle from 120 to 12
seconds. Due to the marked improvements the frequency for the rest of the experiments in this thesis will be performed at 50 Hz, unless otherwise stated.

Figure 5.11: SWV response of glucose sensors with increasing glucose concentration when increasing the frequency of the testing to 50 Hz

Conclusions

Glucose sensor arrays have been fabricated on flexible polyimide sheets. Biocompatible enzyme-containing redox polymer films on the micro-arrays were formed
through the UV-initiated free radical cross-linking of PEG-DA. These micro-array sensors were individually addressable and were without discontinuities. There was no cross-talk between adjacent members. When sampled together the micro-array electrodes behaved as one large electrode with peak current equivalent to the sum of the individual elements of the array, especially important when diagnosing any array element failure.

The catalysis of glucose oxidation resulting from glucose oxidase enzyme exchanging electrons with redox polymer in PEG-DA hydrogel was observed through cyclic voltammetry. To improve response time amperometry was tested, but gave a lower sensitivity. Square wave voltammetry proved to be the optimum method of testing due to its high sensitivity and fast scanning time.
Chapter 6

In Vivo Studies of the developed Electrochemical Glucose Sensors

Introduction

Continuous metabolite monitoring is extremely desirable for use in earlier detection and control of serious diseases. One disease that has produced interest for decades is the increased glucose monitoring for diabetes. [1, 21] This interest has been firmly supported by the knowledge that increased and frequent monitoring of glucose levels in diabetic patients can reduce the risk of serious complications such as kidney, renal and heart disease by significant amounts. [37] The sensors under investigation fall into three categories: non-invasive, minimally invasive, and invasive. The possibility of the non-invasive and minimally invasive has created excitement [19] and has promise but is still a ways off from being completely non-invasive and reliable [42]. There are continuous glucose monitors on the market (Glucowatch, Mini-Med Medtronic Continuous Glucose Monitoring System (CGMS) and Therasense FreeStyle), but some erroneous data can occur so most suggest the trends of the glucose to be monitored and action taken appropriately [42] or blood glucose levels to be tested before any action is taken by the patient [19].

Many implantable glucose sensors show very promising results in vitro but then lose their sensitivity and even the ability to function when tested in vivo.[19] for this purpose the sensors previously described must be tested in vivo to determine their ability
to function in the complex environment. This thesis describes an implantable glucose sensor array that can increase reliability and biocompatibility through the use of an array and a PEG-DA hydrogel. Previous chapters have described the glucose sensor testing and optimization of testing techniques *in vitro* and this chapter describes the testing of the sensors *in vivo* to determine accuracy.

**Materials and Methods**

**Chemicals**

Ammonium hexachloroosmate(IV), 11-mercaptoundecanoic acid (MUA), poly(4-vinylpyridine), acetone, ammonium hexafluorophosphate, sodium dithionite, ether, 2-bromoethylamine hydrobromide, N,N-dimethylformamide (DMF), anion exchange beads, hydrochloric acid (HCl), poly(ethylene glycol) diacrylate (PEG-DA), and 2,2′-dipyridyl (bpy), glucose oxidase (GOX), Inactin (DEA approved), dextrose and human insulin were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Ethyl alcohol, ethylene glycol and acetonitrile were obtained from Fisher Scientific Company (Pittsburgh, PA). DAROCUR, the photo initiator, was obtained from Ciba. All reagents, unless otherwise stated, were used as received. Polyimide sheets (1/16” thick) were purchased from McMaster Carr (New Brunswick, NJ). Phosphate buffered saline (PBS) consisted of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate. Ma-P100, positive photoresist, and ma-D 330, photoresist developer, were purchased from MicroChem Corporation (Newton, MA).
Ag/AgCl electrodes were obtained from C&R Medical (Camarillo, CA) and wire glue was obtained from Anders Products (Melrose, MA). All chemicals were use as received unless otherwise stated. Inactin solutions were prepared using sterile isotonic saline.

**Synthesis of poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine (POs-EA)**

Synthesis of poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine (POs-EA), an osmium based polycationic redox polymer was done following modifications of established protocols. Osmium (bipyridine)$_2$ dichloride (Os(bpy)$_2$Cl$_2$) was synthesized according to a standard procedure with minor modifications. [128] In brief two equivalents of bipyridine (1440 mg) were mixed with one equivalent ammonium hexachloroosmate (IV) (2000 mg) in 100 mL ethylene glycol. The mixture was heated to reflux for 45 minutes and then precipitated with supersaturated sodium dithionite. The precipitate was repeatedly washed with water and finally with ether.

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at 40 °C. Dry PF$_6^-$ salt (0.98 g) was dissolved in 40 mL of acetonitrile and then diluted with 100 mL of water and stirred over 10.4 g of anion exchange beads for 2 hours. The solution was filtered and evaporated under vacuum to ~20 mL. Concentrated HCl was added to the solution to adjust to pH 2. The solution was then dripped into rapidly stirred acetonitrile. A precipitate that formed was filtered and dried in a vacuum oven. The pure product was analyzed.

**Fabrication of electrode arrays on polyimide sheets**

Polyimide sheets are a flexible and insulating material that is an ideal platform for electrode manufacturing. Square pieces of polyimide were cut and washed with ethanol before coating with positive photoresist (ma-P 100). Modified literature procedure was used to fabricate the electrode arrays. [115] In brief positive photoresist (ma-P 100) was deposited on square polyimide sheets and spin coated at 4000 rpm for 30 seconds and then soft baked at 100 °C for 5 minutes. These polymer sheets with dry photoresist were brought in close contact with the photo-mask and exposed to 365 nm UV light. The polymer sheets were then placed in the developer solution (ma-D 330) for seventy seconds to remove the portions of photoresist that were exposed to UV light. The sheets were then rinsed with distilled water.

The photoresist patterns were then sputter coated with 10 nm of an adhesion layer of chrome followed by 150 nm layer of gold (performed at Pennsylvania State University using Lesker CMS-18 sputtering tool and by Lance Goddard Associates in Foster City, CA). The photoresist was removed using acetone to lift-off the excess chrome and gold
from all non-patterned areas. The result was distinct patterns of gold with 500 µm
diameter electrodes with leads 10 µm and contact pads 2.5 mm x 2.5 mm. Wires were
attached to the contact pads by using wire glue.

**Glucose Sensors**

The electrode arrays were functionalized with a carboxylic end group by
immersing in 2 mM MUA in ethanol for 20 minutes and then washed with ethanol. The
electrodes were then dried under nitrogen. The thiol end group was chemisorbed to gold
to provide an anchor. Next, the redox polymer was immobilized on the electrodes by
depositing 2 µL of 10 mg/mL POs-EA onto the surface of the electrode and allowed to
cure overnight. Due to electrostatic interactions between the negatively charged MUA
and positive redox polymer a POs-EA layer will be formed. The excess POs-EA was
then removed by washing with deionized water and dried under nitrogen. To fabricate
the hydrogel on the glucose sensor array a 10 µL solution of 25 mg/mL GOX in 0.1 M
HEPES was combined with 5 µL of PEG-DA and 0.5 µL DAROCUR. The solution was
vortexed and then spread over the electrode array area and exposed to UV light for 0.2
seconds to form a hydrogel. The hydrogel was then allowed to swell and equilibrate for
at least one hour in a solution containing 0.1 M PBS before any testing was done. The *in
vitro* testing was completed in a cell of PBS solution.
Electrochemical Testing

*In vitro* electrochemical tests (SWV) were performed using a Princeton Applied Research (PAR) 283 potentiostat on a three-electrode system with a platinum wire used as the counterelectrode and Ag/AgCl as the reference electrode. The array electrodes with POs-EA polymer and enzyme encapsulated with a PEG-DA hydrogel were used as working electrodes. SWV was done in 0.1 M PBS buffer pH 7.2.

**In vivo studies**

Male Sprague Dawley rats were obtained from Harlan ranging from 350 to 400 grams. The rats were anesthetized using Inactin with a dosage of 80-100 mg/kg weight of rat and were kept in the proper anesthetic window throughout the experiments and euthanized with CO$_2$ after completion of the experiments. (IACUC approved protocol #23204) When the rat was anesthetized fully the hair was removed and the skin scrubbed in the area where the sensor was to be inserted. The rat was then placed and remained on a heating water jacket to keep the rat from hypothermic conditions. A hole was cut into the side of the rat and blunt dissection was used to make a pocket in the subcutaneous tissue where the sensor end was inserted. The reference electrode was applied to the surface of the shaved skin and the electrodes were held in place by pressure applied to gauze pads on the outside of the animal.

The reference electrode applied acted as the reference and counter electrode and each array member in the sensor array was tested as the working electrode by applying a square wave from 0 to 0.6 V vs. Ag/AgCl using the Model 283 Princeton Applied
Research potentiostat. Blood samples were obtained every ten minutes and the blood glucose values determined with the FreeStyle Freedom meter (Therasense, Inc., Alameda, CA).

Many different experimental setups were tested to determine the optimum in vivo setup. For the initial tests the sensors were inserted into the subcutaneous tissue in the stomach facing outward toward the skin and held in place with VetBond. The array was inserted as one piece with alligator clips attaching separate wires to the sensor array. In the optimum experimental setup, the exposed wires below the insertion of the sensor array are protected with a non-conductive gel, while the inserted part of the sensor has the PEG-DA hydrogel covering the sensor. To make the array and wires into one unit, conductive wire glue was used to adhere the ends of wires that are all combined into one. As seen in Figure 6.1, the other ends of the wires are stripped and are made easily accessible for fast switching between working electrodes. The sensor array unit was then inserted into subcutaneous tissue on the side of the rat facing down toward the tissue and held in place by applying pressure to gauze pads that were placed over the sensor area (Figure 6.2).
Figure 6.1: Fabricated sensor array for *in vivo* experimentation as a single unit, where the end of the sensor array is implanted into the rat and the potentiostat wire is connected to the other end.
Figure 6.2: Experimental setup for *in vivo* testing where the fabricated sensor array is inserted array facing down into a subcutaneous incision in the anesthetized rat’s side made by blunt dissection and the reference electrode is applied to the surface of the skin and works as the reference and counter electrode.
Results and Discussion

For *in vivo* experimentation, all electrodes were first hydrated in PBS buffer with no glucose added and tested *in vitro* to determine the viability of the sensor. Once the rat was determined to be in the correct anesthetic window, blunt dissection was performed in the subcutaneous region of the side and isotonic saline injected to decrease the possibility of air pockets that could cause higher resistance and interfere with the sensor. The sensor is then inserted and testing begun. As seen in Figure 6.3 the *in vivo* measurements are demonstrate an increase in peak potential and decrease in peak current than that *in vitro*, this can be caused by an increase in uncompensated resistance [140]. This behavior may be attributed to numerous factors: the reference electrode being placed on the surface of the skin forces the current to flow through the skin, the subcutaneous tissue consists of densely packed cells that may increase resistance, the sensor is facing away from the reference electrode which can increase the resistance, and other factors. When the electrodes were explanted from the rat and re-tested *in vitro* the peak current returned to the initial potential (data not shown). The peak current was lower after sensor implantation but was increasing as a function of time and may return to its previous peak current if allowed time. This problem with uncompensated resistance can be reduced by altering the sensor design to include reference and counter electrodes that would be in solution with the working electrodes. Another method for reduction of the uncompensated resistance is to decrease the frequency.
As seen in Figure 6.3, an increased background current appears on the right side of the curve. This excess current can be caused by the oxidation of the polymer coating on the sensor wires. The coating was thought to be a true insulator, but at higher potentials when exposed to the electrolyte solution it displays oxidation. This oxidation occurred in vitro when the polymer gel was in contact with the electrolyte. When the gel was not in the solution no excess signal was observed, but in vivo the gel was coated on the electrodes high enough to be exposed to interstitial fluid. Future experiments would need to have truly non-conductive gels utilized, such as poly(methyl methacrylate) or poly(dimethylsiloxane). Since the excess background was seen past the peak potential of
the Os polymer *in vivo* the observed peak potential at 0.4 V was the position in each concurrent SWV scan that the peak current was assumed.

In Figure 6.4, the SWV scans of one sensor can be seen, and the highest peaks occur during the time the sensor is still equilibrating. When the sensor has reached an equilibration state (when the gel has been allowed to swell properly, approximately 30 minutes—since it had already been allowed to swell *in vitro* prior to insertion), the sensor response is monitored with respect to changing the rat’s glucose concentrations with injections of dextrose and insulin.

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Figure 6.4: SWV scans of one sensor while implanted *in vivo* in a subcutaneous incision in a rat, where the highest scan is still during the equilibration time
Figure 6.5 shows the trend of the glucose sensor current and blood glucose measurements. The arrows indicate the addition of glucose (up arrows) and insulin (down arrows) via an intraperitoneal (IP) injection. The glucose sensor showed an increase and decrease in sensor response when compared to the altering of glucose concentrations. The sensor value peaks and valleys were shifted compared to the blood glucose level obtained by the Freestyle Freedom meter, which has been seen previously with other sensors implanted subcutaneous. [19, 141, 142] These shifts can be decreased by slower infusion rates and smaller amounts injected per infusion as seen in Armour et al. [141]
Figure 6.5: Example of one sensor’s peak current response in vivo compared to injections and the measured blood glucose, the up arrow indicates IP glucose injections to increase the glucose concentration and the down arrow indicates an IP insulin injection to decrease the glucose concentration.

For sensor calibration a two-point calibration was utilized on the descending section of the glucose curve. Figure 6.6 displays one sensor calibrated with the two-point calibration and the corresponding blood glucose levels. The glucose curve is still shifted and the calibration method needs to be improved, the decrease in resistance with a sensor array including working, counter and reference electrode should help with the calibration.
To determine the relevance of glucose sensors tested in vivo, the Clarke error grid analysis was utilized. [5, 143] As seen in Figure 6.7, the grid is split into five sections: A is clinically accurate, B is clinically relevant, C, D and E are clinically irrelevant. Three sensors were analyzed with the method and gave 7% in the D region, 5% fall in the B region and 88% fall within the A section. If the resistance was decreased and more
testing completed (IACUC approval necessary) then the percentage in the clinically relevant section should increase.

Figure 6.7: Clarke Error Grid for Glucose sensors tested *in vivo*. Three sensors (n=42) where A is clinically accurate, B is clinically relevant, and C, D and E are clinically inaccurate. The x-axis displays the blood glucose levels as measured by the Freestyle Freedom glucose meter and the y-axis is reading obtained by the calibrated implanted glucose sensor.
**Conclusions**

The previously developed glucose sensors were tested *in vivo* in anesthetized rats for their viability in subcutaneous tissue. The experimental parameters for the animal studies were optimized and sensor response obtained. The sensors display the same trend as would be expected for the injections of insulin and glucose. When analyzed by the Clarke error grid analysis 88% of the readings fell within the clinically accurate region, which means the sensor gives promising results and the results can be improved by decreasing the resistance and using a truly non-conductive polymer for insulation.
Chapter 7
Utilization of Micro-fabricated Electrode Sensor Arrays for the Detection of Lactate and Pyruvate

Introduction

The development of implantable sensors used for continuous metabolite monitoring has been under investigation for more than forty years. [1] Among those metabolites investigated have been glucose [2-4], lactate [10, 11] and pyruvate[15-17] because they are important indicators of the serious diseases: diabetes (described in previous chapters), ischemia and sepsis. Glucose, lactate and pyruvate are metabolic components of glycolysis, where glucose reacts to give pyruvate and then pyruvate is reduced by either aerobic respiration or anaerobic respiration (producing lactate in anaerobic). Therefore, the values of these three components are highly related and more information can be deduced from obtaining all of the values and ratios leading to the detection of illnesses sooner.

Ischemia is defined as the lack of blood supply (and in turn oxygen) to tissue or an organ [144] and can occur in organs such as the heart, brain, liver, intestine and limbs. Ischemia can be caused by g-forces in military flying where the body forces the blood supply to the extremities of the body, it can also be caused by the restriction of blood flow caused by atherosclerosis, a tumor, traumatic head injury, stroke, or other factors. Lactate is an important indicator of ischemia, because high lactate levels indicate anaerobic respiration occurring (or no oxygen). High lactic acid levels can also be an
indicator of fatigue and an early indicator of symptoms such as cramping and weakness, caused by too much exercise.

Sepsis is defined as a “poisoned state” which is caused by pathogenic microorganisms absorbed into the bloodstream. Sepsis can occur in anyone, but those that are more susceptible have a weakened immune system, which could be caused by chemotherapy, an injury (e.g., bullet or car crash), are very young or old, or have genetic factors that increase the risk of sepsis. There are three degrees of sepsis: uncomplicated sepsis, severe sepsis and septic shock. Millions of people each year contract uncomplicated sepsis, which produces flu-like symptoms and does not normally require hospitalization. Severe sepsis occurs when sepsis is in conjunction with problems with a vital organ (heart, lungs, liver or kidney) and the patients are likely to be very sick and possibly die (30-35% of cases). Septic shock occurs when the sepsis is coupled with low blood pressure that does not respond to treatment and problems with one or more of the vital organs occur. The patients with septic shock need immediate admission to the intensive care unit (ICU), but even with treatments in the ICU the death rate is 50% of patients. One metabolic indicator used for diagnosis of sepsis is lactate values above 2 mM.

Monitoring of patients in a hospital setting for indication of these diseases will be beneficial, since the current method of determination is the use of a handheld device (I-Stat) that requires the collection of blood samples. The earlier these diseases are detected the chance of recovery will increase, but when blood samples are required the frequency of testing is decreased compared to a continuous monitoring system. Another situation where the continuous monitoring of these analytes would be highly beneficial is
the battlefield for soldiers. The three analytes under consideration are important metabolites for glycolysis that when monitored can provide useful information on the condition of the soldiers. For example, since lactic acid buildup is also an indicator of anaerobic respiration and fatigue, if the officers could monitor fatigue of troops then the troops can be replenished as needed.

One method used for the continuous monitoring of analytes is microdialysis, in which a catheter is inserted into the area of interest and a buffer is pumped through the pump and into the interstitial fluid then fluid from the body is pumped back through the probe for detection. There are commercially available microdialysis probes [83], but they are bulky and require an hour of fluid collection for each analyte concentration to be obtained. The bulkiness of the machine does not lend to portability and the readings are every hour not continuous as desired.

The described micro-fabricated array lends itself to the development of multi-analyte sensors. The use of photo-polymerization of the biocompatible PEG-DA polymer allows for variation in precursor solutions that are deposited onto the array member for multiple analytes being able to be detected. The previously described method of a micro-array for glucose sensor fabrication is utilized to fabricate lactate and pyruvate sensors to detect the analytes in the biological range. The reaction scheme for the newly developed sensors can be seen in Figure 7.1, where the analyte, enzyme, and product are outlined in Table 7-1.
Figure 7.1: Reactions that take place in the electrochemical sensor. The gray box symbolizes the PEG-DA hydrogel that entraps the enzyme and the components for each sensor are described in the table below.

Table 7-1: The different components of the lactate and pyruvate sensors as related to the figure above.

<table>
<thead>
<tr>
<th></th>
<th>Lactate Sensor</th>
<th>Pyruvate Sensor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analyte</strong></td>
<td>lactate</td>
<td>pyruvate + phosphate</td>
</tr>
<tr>
<td><strong>Enzyme</strong></td>
<td>lactate oxidase</td>
<td>pyruvate oxidase</td>
</tr>
<tr>
<td>(LOX)</td>
<td>(PYX)</td>
<td></td>
</tr>
<tr>
<td><strong>Product</strong></td>
<td>pyruvate</td>
<td>acetylphosphate</td>
</tr>
</tbody>
</table>
Materials and methods

Chemicals

Ammonium hexachloroosmate(IV), 11-mercaptoundecanoic acid (MUA), poly(4-vinylpyridine), acetone, ammonium hexafluorophosphate, sodium dithionite, ether, 2-bromoethylamine hydrobromide, N,N-dimethylformamide (DMF), anion exchange beads, hydrochloric acid (HCl), poly(ethylene glycol) diacrylate (PEG-DA), and 2,2′-dipyridyl (bpy), N-vinylimidazole, 2,2′-azobis(2-methylpropionitrile) (AIBN), ethanol, tetrahydrofuran (THF), hexane, lactate oxidase (LOX, from Pediococcus sp. 20 units/mg), pyruvate oxidase bacterial (PYX, 1.5 units/mg), thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), lactic acid, and sodium pyruvate were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Ethyl alcohol, ethylene glycol and acetonitrile were obtained from Fisher Scientific Company (Pittsburgh, PA). DAROCUR, the photo initiator, was obtained from Ciba. All reagents, unless otherwise stated, were used as received. Polyimide sheets (1/16” thick) were purchased from McMaster Carr (New Brunswick, NJ). Phosphate buffered saline (PBS) consisted of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate. Ma-P 100, positive photoresist, and ma-D 330, photoresist developer, were purchased from MicroChem Corporation (Newton, MA).
Synthesis of poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine (POs-EA)

Synthesis of poly[4-vinylpyridine osmium (bipyridine)$_2$ chloride]-co-ethylamine (POs-EA), an osmium based polycationic redox polymer was done following modifications of established protocols. Osmium (bipyridine)$_2$ dichloride (Os(bpy)$_2$Cl$_2$) was synthesized according to a standard procedure with minor modifications. [128] In brief two equivalents of bipyridine (1440 mg) were mixed with one equivalent ammonium hexachloroosmate (IV) (2000 mg) in 100 mL ethylene glycol. The mixture was heated to reflux for 45 minutes and then precipitated with supersaturated sodium dithionite. The precipitate was repeatedly washed with water and finally with ether.

Os(bpy)$_2$Cl$_2$ (0.988 g, 1.728 mmol) and poly(4-vinylpyridine) (0.860 g, 8.18 mequiv) were heated under nitrogen at reflux in 36 mL of ethylene glycol for 2 hours. The solution was then cooled down to room temperature and 60 mL of DMF and 3.0 g of 2-bromoethylamine hydrobromide (14.6 mmol) were added and then stirred overnight at 45 °C. A crude polymer precipitate was formed by pouring the solution into rapidly stirred acetone. The hygroscopic precipitate was collected and dissolved in water. The solution was filtered and precipitated as the hexafluorophosphate (PF$_6$-) salt by addition of a solution of ammonium hexafluorophosphate. The precipitate was dried in a vacuum at 40 °C. Dry PF$_6$- salt (0.98 g) was dissolved in 40 mL of acetonitrile and then diluted with 100 mL of water and stirred over 10.4 g of anion exchange beads for 2 hours. The solution was filtered and evaporated under vacuum to ~20 mL. Concentrated HCl was added to the solution to adjust to pH 2. The solution was then dripped into rapidly stirred
acetonitrile. A precipitate that formed was filtered and dried in a vacuum oven. The pure product was analyzed.

Fabrication of electrode arrays on polyimide sheets

Polyimide sheets are a flexible and insulating material that is an ideal platform for electrode manufacturing. Square pieces of polyimide were cut and washed with ethanol before coating with positive photoresist (ma-P 100). Modified literature procedure was used to fabricate the electrode arrays. [115] In brief positive photoresist (ma-P 100) was deposited on square polyimide sheets and spin coated at 4000 rpm for 30 seconds and then soft baked at 100 °C for 5 minutes. These polymer sheets with dry photoresist were brought in close contact with the photo-mask and exposed to 365 nm UV light. The polymer sheets were then placed in the developer solution (ma-D 330) for seventy seconds to remove the portions of photoresist that were exposed to UV light. The sheets were then rinsed with distilled water.

The photoresist patterns were then sputter coated with 10 nm of an adhesion layer of chrome followed by 150 nm layer of gold (performed at Pennsylvania State University using Lesker CMS-18 sputtering tool and by Lance Goddard Associates in Foster City, CA). The photoresist was removed using acetone to lift-off the excess chrome and gold from all non-patterned areas. The result was distinct patterns of gold with 500 µm diameter electrodes with leads 10 µm and contact pads 2.5 mm x 2.5 mm.
**Lactate Sensors**

For fabrication of the lactate sensors, the enzyme lactate oxidase has been seen to lose its activity when in aqueous solutions for extended periods of time. [147, 148] Lactate oxidase was therefore complexed electrostatically with the polymer poly(N-vinylimidazole), which has shown marked improvement in enzyme stability.[147]

Poly (N-vinylimidazole) (PVI) is synthesized by modification of the procedure from Li et al. [149] In short 20 mL N-vinylimidazole and 0.04 g of AIBN were dissolved in 50 mL of absolute ethanol under nitrogen and stirred at 70 °C for 40 hours. The reaction mixture was allowed to cool, then the poly(N-vinylimidazole) was precipitated in an excess of THF/hexane (3:8, v/v). The precipitate was washed repeatedly with the non-solvent mixture and dried overnight in a vacuum oven at 80 °C.

The electrode arrays were functionalized with a carboxylic end group by immersing in 2 mM MUA in ethanol for 20 minutes and then washed with ethanol. The electrodes were then dried under nitrogen. The thiol end group was chemisorbed to gold to provide an anchor. Next, the redox polymer was immobilized on the electrodes by depositing 2 µL of 10 mg/mL POs-EA onto the surface of the electrode and allowed to cure overnight. Due to electrostatic interactions between the negatively charged MUA and positive redox polymer a POs-EA layer will be formed. The excess POs-EA was then removed by washing with deionized water and dried under nitrogen. The lactate oxidase enzyme was obtained from Sigma then aliquoted and lyophilized for storage. To fabricate the hydrogel on the sensor array a solution of 25 mg/mL lyophilized LOX and 10 mg/mL PVI in 10 µL 0.1 M PBS was combined with 2 µL PEG-DA, and 0.2 µL
DAROCUR. The solution was vortexed and then spread over the electrode array area and exposed to UV light for 0.2 seconds to form a hydrogel. The hydrogel was then allowed to swell and equilibrate for at least an hour in PBS before any testing was done. The testing was done in a cell of 25 mL of 0.1 M PBS with varying amounts of lactic acid (0 to 50 mM).

**Pyruvate Sensors**

The electrode arrays were functionalized with a carboxylic end group by immersing in 2 mM MUA in ethanol for 20 minutes and then washed with ethanol. The electrodes were then dried under nitrogen. The thiol end group was chemisorbed to gold to provide an anchor. Next, the redox polymer was immobilized on the electrodes by depositing 2 µL of 10 mg/mL POs-EA onto the surface of the electrode and allowed to cure overnight. Due to electrostatic interactions between the negatively charged MUA and positive redox polymer a POs-EA layer will be formed. The excess POs-EA was then removed by washing with deionized water and dried under nitrogen. The pyruvate oxidase enzyme was obtained from Sigma, then aliquoted and lyophilized for storage. To fabricate the hydrogel on the sensor array a 5 µL solution of 25 mg/mL PYX, 10 µM FAD and 0.1 M TPP in 0.1 M PBS was combined with 2 µL of PEG-DA and 0.2 µL DAROCUR. The solution was vortexed and then spread over the electrode array area and exposed to UV light for 0.2 seconds to form a hydrogel. The hydrogel was then allowed to swell and equilibrate for at least an hour in a solution containing 10 µM FAD.
and 0.1 M TPP in 0.1 M PBS before any testing was done. The testing was done in a cell containing the PBS/FAD/TPP solution with varying amounts of pyruvate (0 to 13.2 mM).

**Electrochemical Testing**

Electrochemical tests (SWV) were performed using a Princeton Applied Research (PAR) 283 potentiostat on a three-electrode system with a platinum wire used as the counterelectrode and Ag/AgCl as the reference electrode. The array electrodes with POs-EA polymer and enzyme encapsulated with a PEG-DA hydrogel were used as working electrodes.

**Results and Discussion**

Previously, amperometric biosensors based on redox polymer/enzyme complexes were shown to be miniaturizable and implantable. [110, 135, 137] Also, enzymes entrapped in redox hydrogels using photopolymerization were shown to retain their activity. [29, 32, 106, 138, 139] In this chapter, redox polymers exchanged electrons with an enzyme (LOX or PYX) entrapped in a biocompatible poly (ethylene glycol) diacrylate (PEG-DA) hydrogel. The fabricated lactate and pyruvate sensors were evaluated *in vitro* using SWV to determine their sensitivity and linear range.

Biocompatible PEG-DA was used to cross-link the lactate oxidase enzyme with the electroactive redox polymer, POs-EA. Properly cross-linked molecules are expected to retain the activity of lactate oxidase enzyme and to present signal stability. If the
enzyme were lost through leaching from the electrode surface then the signal stability would suffer, which was not observed. Figure 7.2 shows an increase in the peak of the current difference in the scans with increasing lactate concentration, from 0 to 50 mM. The oxidized form of the lactate oxidase enzyme reacts with lactate to form a reduced form of LOX and pyruvate. The reduced form of LOX is in turn oxidized by the electrochemically generated Os$^{3+}$ form of the redox polymer, setting up a catalytic pathway that produces an enhanced oxidation peak. The electrons are transferred from the enzyme to the redox polymer, shuttled between the redox sites in a self-exchange reaction until being transferred to the electrode surface for detection [31].

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**Figure 7.2:** SWV scans of lactate sensors of one sensor array element at varying lactate concentrations from 0 to 50 mM lactate
Figure 7.3: Peak current of the difference SWV scans when increasing the lactate concentration, where the linear part of the graph (0-15 mM) has an r-squared value of 0.9674

In Figure 7.3, the peak current of the SWV scans is graphed versus the concentration of lactate in the test solution. Lactate oxidase is securely trapped in the hydrogel network and lactate is able to diffuse inside the hydrogel to access the lactate oxidase sites. Photopolymerization of the acrylate end groups of the PEG-DA by the photoinitiator DAROCUR occurs and entraps the lactate oxidase enzyme on the redox polymer film. This reaction does not diminish the activity of the glucose oxidase enzyme as seen by the retained activity and sensitivity of the sensor to lactate. Figure 7.3 shows
that the lactate sensors exhibit a linear response from 0 to 15 mM with an r-squared value of 0.9674. The sensitivity for the lactate sensor in the linear range is 1.0726 \( \mu \text{A/mM} \) (which is 136.6 \( \mu \text{A/(mM cm}^2 \)). When the lactate concentration was increased above 15 mM deviation from linearity was observed, which can be attributed to the mechanism of the enzyme. The enzyme follows Michaelis-Menten kinetics and leads to a diffusion-limited region and a reaction-limited section, which can be seen in Figure 7.3 by the two very defined regions. In the diffusion-limited region the sensor displays a linear response and while in the reaction-limited region the sensor exhibits a plateau of peak current due to the saturated binding sites of lactate oxidase. The obtained linear region is ample for lactate, since normal values of a healthy resting person are between 1 and 2 mM [11] and levels above 7-8 mM are sometimes associated with fatal outcomes [150], although for extreme amounts of exercise the lactate levels can reach 20 mM [151] but this can be termed as extremely high and action should be required.

After the lactate sensors displayed the expected and desired results the same method of fabrication was used to develop pyruvate sensors. The pyruvate sensors were tested with the electrode array via encapsulated pyruvate oxidase and the result of the peak difference current from SWV scans versus the pyruvate concentration is seen in Figure 7.4.
Figure 7.4: Peak current difference from SWV scans when varying pyruvate concentrations from 0 to 13 mM

The pyruvate sensors displayed a linear range from 0 – 0.87 mM (r-squared value is 0.9542), which spans the biological range of 40 – 120 µM. The linear range of the sensor is expected to decrease because of the enzyme’s lower $K_m$ value equal to 0.34 mM [18]. The sensitivity of the sensor was 0.19 µA/mM or 24.2 µA/(mM cm$^2$). The $K_m$ value of the enzyme can be thought of as a measure for the diffusion and reaction limited regions, when the $K_m$ is higher the diffusion-limited region is extended, but when the $K_m$ has a lower value the diffusion-limited region is shorter. Since, as discussed in Chapter 5, the mode of the sensor relies on the diffusion-limited region to give the linear
response, the lower $K_m$ value of pyruvate oxidase leads to a lower maximum in the linear range.

Since the electrode arrays and enzymes in this study were from different batches, the signals from the electrodes were not the same. Some variations in fabrication methods, such as different solutions of MUA and POs-EA being used, slight faults in micro-fabrication process or differences in times of deposition, can lead to deviations in obtained responses. These types of variations in sensor fabrication occur frequently and lead to the commercially available sensors to have a calibration standard used. The calibration standard is either a standard test strip included with the purchased test strips (as seen in the Precision QID meters by Medisense) or a number on the test strip package indicating the calibration that is input into the glucose meter to use an already programmed calibration standard (as seen in the FreeStyle Freedom meter by Therasense). To make the fabricated sensors on the same scale a calibration can be achieved by subtracting the current amount at zero analyte concentration and dividing by the plateau value of the curve. Figure 7.5 shows the results from three pyruvate oxidase sensors on the normalized scale with the scale bars indicating the standard deviation of the mean values. If fabrication techniques lead to non-uniform sensors a normalization process can be utilized to calibrate all of the fabricated sensors in vitro before use by patients to eliminate any variations.
Conclusions

In this chapter the method of fabricating electrochemical glucose sensors was utilized to develop lactate and pyruvate sensors. The linear range of these sensors ranged from 0 – 15 mM for lactate and 0 – 0.87 mM for pyruvate, both encompassing the biological range for the desired analytes. The fabrication process of these sensors lends itself to allow for the combination of sensors in this chapter and the previously described
glucose sensor on a single array to detect all three analytes at once. The curves for pyruvate were also normalized to correlate different sensors in one graph, and this method can be utilized for calibration of sensors to minimize any fabrication faults.
Chapter 8

Conclusions and Future Work

Conclusions

This thesis investigates the development of an electrochemical multi-analyte sensor array using a biocompatible PEG-DA hydrogel. The sensor array can increase the reliability of the previous sensor by use of redundancy. The array also lends itself to the fabrication of multiple analytes in the same array. Also, the array can incorporate a sensor utilized to measure the background current \textit{in vivo} and aid in the one-point calibration method [152].

Glucose sensor arrays have been fabricated on flexible polyimide sheets. Biocompatible enzyme-containing redox polymer films on the micro-arrays were formed through the UV-initiated free radical cross-linking of PEG-DA. These micro-array sensors were individually addressable and were without discontinuities. There was no cross-talk between adjacent members. When sampled together the micro-array electrodes behaved as one large electrode with peak current equivalent to the sum of the individual elements of the array, especially important when diagnosing any array element failure. The catalysis of glucose oxidation resulting from glucose oxidase enzyme exchanging electrons with redox polymer in PEG-DA hydrogel was observed through cyclic voltammetry. To improve response time amperometry was tested, but gave lower
sensitivities. Square wave voltammetry proved to be the optimum method of testing due to its high sensitivity and fast scanning time.

The fabricated glucose sensors were then utilized in vivo to determine their viability in subcutaneous tissue. The optimum experimental settings for the animal experiments were determined and data from the glucose sensors displayed the trend of the signal to increase and decrease when expected for varying glucose concentrations. Further in vivo testing is desired, but IACUC approval is necessary.

To implement the multi-analyte array the sensor design established for the glucose sensors was applied to the analytes lactate and pyruvate. The sensors were tested with square wave voltammetry and exhibited the expected curve with the linear range of the sensor dependent on the enzyme and substrate utilized. The linear ranges obtained for these sensors were in the desired range for the analyte concentration in vivo as seen in Table 8-1 below.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity $\mu$A/(mM cm$^2$)</th>
<th>Linear Range (mM)</th>
<th>Biological Range (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose</strong></td>
<td>19.20</td>
<td>0 – 20</td>
<td>0 – 20</td>
</tr>
<tr>
<td><strong>Lactate</strong></td>
<td>136.6</td>
<td>0 – 15</td>
<td>0 – 8</td>
</tr>
<tr>
<td><strong>Pyruvate</strong></td>
<td>24.2</td>
<td>0 – 0.87</td>
<td>0.04 – 0.12</td>
</tr>
</tbody>
</table>

Table 8-1: Summary of the developed sensors by their sensitivity and linear range
Sensor Design Improvements

The current sensor and *in vivo* setup has the reference Ag/AgCl electrode attached to the surface of the skin and acting as the counterelectrode also. This current setup can lead to polarization of the reference electrode and altered signals, possible increase in the peak potential. One method designed to alleviate this problem is to design an electrode array that incorporates the reference and counter electrodes. The sensor design in Figure 8.1 has been developed, and can be fabricated through multiple photolithographic steps. Discussions have been conducted for these sensors to be fabricated by a local company, State of the Art (State College, PA), with extensive experience in microfabrication techniques.

The sensor in Figure 8.1 has electrode diameters equal to 500 µm, and the width of the array is designed to fit into an 18 gauge breakaway needle, alleviating the necessity of blunt dissection. The sensor utilizes both sides of the sensor to increase the number of array elements per area of inserted sensor. The design of the sensor can still be altered to incorporate more electrodes and smaller electrodes.

Another improvement desired for experimentation is an upgrade in the potentiostat used. The current potentiostat used for experimentation is only capable of testing one electrode at a time, but with a multiplexer attachment numerous electrodes can be tested simultaneously. With the ability to test all of the electrodes in the array simultaneously the amount of data can be greatly increased and in turn the reliability of the sensor system will be increased. As seen in Chapter 3 in the section Signal processing and fault detection with an increase in the sensor redundancy to as little as two
sensors the reliability of the sensors can be increased greatly and faulty readings can be detected. [68, 110]

Figure 8.1: Design of an improved sensor array that includes the reference and counter electrodes in the implanted portion of the array, where the titanium/gold is the working electrodes, platinum acts as the counterelectrode and silver/silver chloride acts as the reference electrode.

**Improvement of Eliminating Interferences**

The current method to subtract the contribution from the interfering substances is to have a zero sensor (which can also be used for the one-point calibration method *in vivo*) [68, 152] and subtract the background. Another method to deter interfering substances is to utilize sensors that operate at lower potentials that the interferences do not oxidize at. Heller et al has developed Osmium complexes that have a standard potential at –160 mV, where the interferences would not have an effect. [153] This
polymer is more difficult and more expensive to synthesize, but could lead to better sensor capabilities.

Another method developed by Heller and co-workers was to make a layered sensor that utilized oxygen in the glucose reaction in the outer layer and the inner layer utilized a soybean peroxidase enzyme to detect the hydrogen peroxide produced. The layered sensor had a working potential of approximately 0 V, but requires oxygen for the reactions to occur. [154, 155] This sensor could be coupled with the glucose sensor to increase reliability by varying the methods of detection within on sensor array.

**Biocompatibility Studies**

The biocompatibility of these sensors has not been determined *in vivo*, so a next step would be to determine the biocompatibility of the PEG-DA hydrogel sensor. This can be investigated by postmortem tissue explanation around the sensor and tissue not exposed to the sensor, and then a histological evaluation should be completed to determine the activity of the tissue in the vicinity of the implanted sensor. IACUC approval would be required before these experiments could be performed.

If the histological examination is determined inconclusive, then other models for biocompatibility can be utilized. One model uses a cage implant system that inserts the sensor to be tested in a mesh cage made of stainless steel, then the cage is implanted into an animal. This method allows for extraction of fluid samples that have collected in the chamber to monitor inflammatory response, while continuing to test the sensor *in vivo* and not sacrificing the animal until all experiments are completed. [19] Another method
that has been developed uses the ex ova chorioallantoic membrane (CAM) to model tissue reactions that are similar to mammalian cells, including fibrosis and inflammation. [156] The CAM model would not require IACUC approval, but for the mesh cage method it would be required.

If the biological response still needs improvement, nitric oxide (NO) has been shown to decrease the wound healing response when released from a sensor. Studies have shown that sensors prepared to release nitric oxide, mimicking the behavior of cells, have decreased the foreign body response. [1, 157] A scheme could be devised to encapsulate NO in nanocapsules that could be entrapped in the PEG-DA hydrogels and degrade with time to release NO to the surrounding tissue and decrease the foreign body response.

**Utilization of Array Capabilities to Detect More Analytes**

Since an array is utilized, other desirable analyte sensors can be fabricated on the array to monitor continuously. Other analytes include: pH, pO$_2$ and pCO$_2$ (blood gas measurements), electrolytes such as Na$^+$, K$^+$, and Ca$^{2+}$, and oxygen saturation (SaO$_2$). [1] One benefit of the PEG-DA hydrogel fabrication is that different precursor solutions containing varying sensing elements can be deposited onto the surface of the array without causing problems with the other sensors.
Bibliography


83. CMA, M.I., Micrdialysis for Clinical Use. 2007.


VITA

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Becky L. Clark was born and raised in Palatka, Florida. She graduated from Palatka High School and St. Johns River Community College in June 2000. Miss Clark then relocated to Tallahassee, FL where she received her Bachelor’s of Science in Chemical Engineering from the Florida State University in 2003. She then moved to the Pennsylvania State University to pursue her Doctor’s of Philosophy in Chemical Engineering under the tutelage of Dr. Michael V. Pishko in the area of implantable electrochemical biosensors. After completion of her doctorate degree, she will be working in the micro-fabrication field.