Modified Electrodes for Amperometric Determination of Glucose and Glutamate Using Mediated Electron Transport

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The main goal of this research was to develop an easy to prepare and sensitive biosensor that would be able to detect glutamate in solution using ionic self-assembly methods. This was accomplished by preparing an ionically-self-assembled monolayer that included an electron transport mediator and an enzyme that would generate a current proportional to the concentration of analytes in solution. Biosensors were produced for the detection of glucose and glutamate.

Ferrocene poly(allylamine) (FePAA) was assembled on negatively charged self-assembled monolayer and shown to be electrostatically bound by cyclic voltammetry. Model films were made of FePAA and poly(styrenesulfonate) to determine if multilayer films could be assembled using electrostatic assembly. These experiments demonstrated that 7 bilayers is the maximum number of bilayers oxidizable by the heterogeneous reaction at the electrode surface.

ISAMs were then assembled on a 2 mm gold electrode and on a gold fiber microelectrode using FePAA and glucose oxidase. Using cyclic voltammetry, these ISAMs were shown to be able to oxidize glucose in solution. The LOD was determined to be lower for the microelectrode than for the 2 mm gold electrode, which was expected, while both compared well to the literature. The $K_m'$ were found to be smaller than other glucose
biosensors while the $I_{\text{cat}}$ increased with increasing number of bilayers. This demonstrated that the GluOx is making good electrical contact with the layer below. These glucose oxidase ISAMs, however, do not exhibit structural stability in flow-injection experiments.

As a solution to the ISAM modified electrodes degrading in the flowing system, a covalently modified surface was developed. Using cyclic voltammetry, these covalently modified surfaces were shown to be able to oxidize glucose in solution. The LOD of the covalently modified 2 mm gold electrode was calculated to be lower than the 2 mm ISAM modified gold electrode, due to the fast heterogeneous kinetics on the covalently modified electrode surface. The $K_m'$ and $I_{\text{cat}}$ for the covalently modified 2 mm gold electrode were found to be the similar to the 2 mm ISAM modified gold electrode indicating that the covalently modified electrodes will be a suitable replacement. The covalently modified surfaces exhibit excellent structural stability and detect much lower glucose amounts in flow-injection experiments.

ISAMs were subsequently assembled on gold fiber microelectrodes using FePAA and glutamate oxidase. Glutamate was able to be detected in solution at biologically significantly quantities using cyclic voltammetry. The $K_m'$ was shown to be comparable to literature values and $I_{\text{cat}}$ was shown to increase with increasing number of bilayers. These results demonstrate that an ISAM constructed using FePAA/GlutOx is a feasible way to detect glutamate in a system.
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Chapter 1: Introduction and Motivation

1.1 Description of an Electrochemical Biosensor

Since Clark’s original proposal in 1962 to implement enzymes in sensors for detection of biologically important analytes, the field of biosensors has made significant progress.\(^1\) This success is due to the sensitivity and excellent selectivity of the enzymes for the analytes of interest.\(^1\) The enzyme specificity comes from the active site in which only certain substrates can fit. Hence, the substrate must have a certain shape and contain certain functional groups to fit into the site and bind correctly. In general, a biosensor contains a molecular-recognition element directly interfaced to a signal transducer which together produce a response proportional to the concentration of analyte.\(^2\) In an electrochemical sensor, an electrode serves as the signal transducer, and the measurable response is an electrical current. The choice of molecular-recognition element depends on the analyte, and may range from redox proteins (cytochrome c) to enzymes (glucose oxidase) to DNA.

Electrochemical biosensors are quite attractive due to their many advantages over other detection methods. These advantages include low-cost, real-time operation and simplicity of starting materials. There are also disadvantages, the most concerning being the electron transfer efficiency between the molecular-recognition element and the electrode. This slow transfer efficiency is often due to both the location of the redox active site deep within the enzyme, and the inability of the enzyme to orient itself favorably with respect to the electrode surface for fast electron transfer.\(^3\) An example of
an enzyme that is often used in electrochemical biosensors, but is unable to be directly oxidized by an electrode is glucose oxidase.

### 1.2 Glucose Oxidase

Within biosensor development, the enzyme glucose oxidase (GluOx) has become a benchmark system. GluOx has favorable attributes that contribute to its common usage. These attributes include: high turnover rate, excellent selectivity, good thermal and pH stability and low cost. The enzyme is made of two identical subunits and one flavin adenine dinucleotide (FAD) coenzyme molecule. GluOx has a molecular weight of 186,000 g/mole and a size of 70 Å x 55 Å x 80 Å. A structure of GluOx can be seen in Figure 1.

![Figure 1. Glucose oxidase structure.](image)

The active site in GluOx contains the (FAD) coenzyme molecule, seen in Figure 1 as the red spacefill, which is tightly bound but not covalently attached to the enzyme. FAD works efficiently as a cofactor because of its reversible electrochemistry. FAD is the
fully oxidized state and to get to the fully reduced form of FADH₂ requires the addition of two hydrogen atoms, as shown in Figure 2.

Figure 2. FAD being reduced to FADH₂

To reoxidize FADH₂ back to FAD, molecular oxygen reacts “non-enzymatically” and hydrogen peroxide is produced as shown in Figure 3.¹⁹,²⁰

Figure 3. FADH₂ being oxidized to FAD

In the presence of glucose, FAD oxidizes glucose efficiently by shuttling electrons from glucose to oxygen dissolved in solution.¹⁹,²⁰ The overall reaction for glucose oxidation via GluOx (FAD) is shown below:

\[
FAD + (C₆H₁₂O₆) \rightarrow FADH₂ + (C₆H₁₀O₆) \quad (1)
\]

\[
FADH₂ + O₂ \rightarrow FAD + H₂O₂ \quad (2)
\]
FAD oxidizes glucose and FADH$_2$ and glucono-d-lactone are produced. The FADH$_2$ is then “non-enzymatically” regenerated by dissolved O$_2$ returning the enzyme to the FAD form and producing H$_2$O$_2$.

The crystal structure of GluOx has recently been published and shows that the FAD sits in a cleft, with an initial opening of 10 Å x 10 Å, closing to an opening only a few Å across.$^{18,21}$ This allows glucose to enter the active site and be oxidized due to the site specificity. Unfortunately, due to the orientation of the FAD in the cleft, the FAD oxidation by an electrode is inefficient; as the path length from the electrode to the FAD is too far for an electron to traverse. This makes the oxidation of the FAD kinetically unfavorable. Based on calculations done by Heller, electrons can be transported across distances as large as 20 Å, however the gap from FAD to the electrode surface is no closer than 25 Å.$^{3,22}$ Marcus et al. have shown that the oxidation of an enzyme at an electrode surface suffers from low efficiency based on the limited distance that an electron can tunnel as electron transfer decays exponentially with distance.$^{3,22}$ As the distance gets sufficiently large (much larger than 3 Å) the rate of electron transfer will suffer.$^{3,22}$ Heller states that GluOx cannot be oxidized at an electrode based on the hydrodynamic size of the enzyme and the fastest feasible electron transfer rate.$^{22}$ Under the right conditions, however, FAD oxidation can take place, as it is a thermodynamically favorable reaction.

Other FAD-containing enzymes will behave similarly to GluOx; however, the crystal structure for most of these has not yet been determined. For these oxidases the structure
surrounding the FAD center is not known. Cholesterol oxidase, however, has been crystallized and the FAD was found to be buried inside the enzyme as it is in GluOx.\textsuperscript{23} Since the FAD is also buried inside the center of the oxidase, it should exhibit similar inefficient electron transfer to an electrode surface.\textsuperscript{24} Other FAD enzymes that have not yet been crystallized include glutamate oxidase and lactose oxidase. The burying of the FAD redox site within the enzyme means that they will not be easily oxidizable at an electrode surface, and other methods will have to be used to efficiently oxidize these enzymes for use in an electrochemical sensor.

1.3 Methods to Make an Electrochemical Biosensor

There are three main ways to circumvent the electron transfer efficiency problem and use an enzyme in an electrochemical biosensor. One way is to affix the enzyme to an electrode surface and directly monitor the formation of hydrogen peroxide from the enzyme-substrate reaction in the presence of $O_2$. The hydrogen peroxide production is then monitored by oxidation of hydrogen peroxide at the electrode.\textsuperscript{25-27} While several glucose sensors based on this concept have been reported, this reaction is hard to monitor because hydrogen peroxide oxidizes at a relatively high positive overpotential.\textsuperscript{2, 17, 28} At this high positive overpotential, there is the possibility of interference during the detection.

A second method is to directly oxidize the enzyme at the electrode surface. There are very few enzymes, however, that have a small enough hydrodynamic radius to be efficiently oxidized at the surface of the electrode, as discussed previously. Most
enzymes, like glucose oxidase, have a large hydrodynamic radius (43 Å or greater) and a buried redox center.\textsuperscript{22, 29} The third, and most frequently employed method, is to use a redox mediator to shuttle the electrons from the FAD redox center of the enzyme to the electrode.

1.4 Mediated Electron Transport

Although the oxidation of FAD in the GluOx is thermodynamically spontaneous, the distance that the electron has to travel to the electrode surface is far enough that the oxidation of the FAD in the GluOx by the electrode is kinetically unfavorable. This makes the heterogeneous oxidation of the FAD in the GluOx an overall unfavorable process. Using a mediator to establish electrical communication between the oxidase and the electrode will help overcome the kinetic barrier to allow a type of heterogeneous oxidization of the FAD in the GluOx and is a simple solution to the problem.

An electron-transfer mediator is a molecule that has fast electron transfer kinetics and which has an oxidation potential positive of the thermodynamic oxidation potential of the kinetically hindered reaction. The determining factor for the thermodynamic process is the difference in the oxidation potentials between the mediator and the FAD. The larger the gradient, the more favorable the homogeneous reduction-oxidation reaction will be between the oxidized form of the mediator and the reduced form of the oxidase. Under these conditions, the mediator is oxidized by the electrode and then the oxidized form of the mediator is reduced by the FADH\textsubscript{2}. The net result is the FADH\textsubscript{2} is oxidized at a faster rate than is possible in the absence of the mediator.
In order to use a redox mediator to establish direct electrical communication with the electrode, the mediator must have several properties. A suitable mediator should have an appropriate oxidation potential - in the case of an oxidase, it should be at a potential more positive than the redox potential of the active site.\textsuperscript{19, 20} The oxidation potential of the FAD center in the GluOx active site is -0.447 V or -0.337 V vs. Ag/AgCl depending on the preparation.\textsuperscript{30, 31} Some examples of previously used mediators and their oxidation potentials are shown in Table 1.

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</tr>
<tr>
<td>Indigo disulfonate\textsuperscript{32}</td>
<td>-0.188 V</td>
</tr>
<tr>
<td>Methylene blue\textsuperscript{33}</td>
<td>0.217 V</td>
</tr>
<tr>
<td>2,5-dihydroxybenzoquinone\textsuperscript{33}</td>
<td>0.137 V</td>
</tr>
<tr>
<td>Ferrocenecarboxaldehyde\textsuperscript{19, 20}</td>
<td>0.518 V</td>
</tr>
<tr>
<td>Ferrocenemethanol\textsuperscript{19, 20}</td>
<td>0.216 V</td>
</tr>
</tbody>
</table>

\textbf{Table 1. Mediators used with GluOx.}

The mediator must be able to cycled several times between its oxidized and reduced states in the process of shuttling charge between the oxidase and the surface of the electrode. This electron transfer from the enzyme to the mediator must occur rapidly to overcome the kinetic barrier effectively. In fact, the more efficient the electron transfer is between the mediator and the enzyme, the farther apart the mediator and enzyme can be.\textsuperscript{22} The heterogeneous electron transfer kinetics of the mediator can also limit the rate of the reoxidation of the enzyme, so this step also must be fast to keep the kinetic barrier
Finally, the mediator must be stable in both its oxidation states indefinitely for the mediation to continue.\textsuperscript{19, 20} Ferrocenes are one class of mediators frequently used since they have a wide range of redox potentials, are easy to derivatize, have fast electron transfer kinetics, and are stable in both the oxidized and reduced forms.\textsuperscript{19, 20} Several groups demonstrate that ferrocene–derivatives can mediate the oxidization of GluOx.\textsuperscript{17, 22, 28, 30}

When using a mediated electron transport scheme, only catalytic amounts of mediator and enzyme are needed as they are both regenerated during the catalytic cycle. Because of the electron shuffling, when glucose is added to the solution, the cyclic voltammogram results in a steady state response.\textsuperscript{34} Steady state behavior means that the flux of electrons to or from the electrode reaches a constant rate. This is due to the mediator constantly being regenerated within the system due to the oxidation of the glucose by the enzyme, and suggests that the rate of the reaction is limited by the rate of the glucose oxidation by glucose oxidase. A schematic of the mediation process is shown in Figure 4. In this example, glucose is being oxidized to glucono-d-lactone, as designated by the maroon arrow. While oxidizing the glucose, the GluOx(FAD) is reduced to GluOx(FADH\textsubscript{2}), as designated by the orange arrow. GluOx(FADH\textsubscript{2}) is subsequently oxidized to GluOx(FAD), shown by the maroon arrow, by the oxidized form of the mediator (Fe\textsuperscript{+3}) which in turn is reduced to Fe\textsuperscript{+2}. The final catalytic step is the Fe\textsuperscript{+2} being oxidized by back to Fe\textsuperscript{+3} the electrode surface, resulting in the current that is measured and beginning the cycle again. Since the Fe\textsuperscript{+3} is returned to Fe\textsuperscript{+2} by the reaction with GluOx(FADH\textsubscript{2}), the measured current is proportional to the turnover of the oxidase by glucose. This
mediation scheme can be, in theory, used to facilitate many different enzyme reactions, provided that they contain a FAD center in conjunction with a mediator.

Figure 4. Mediated electron transport for glucose oxidation in solution as iron at the electrode surface catalytically regenerates the oxidase.

1.5 Enzyme-Electrode Coupling Methods

The ferrocene-derivative mediators and the enzyme can also be incorporated into various types of matrices that can then be attached to an electrode surface. The advantage of having both the mediator and the enzyme bound at the surface is that a higher degree of organization can be achieved, and confining the system to a small dimension restricts the electron transfer distance between the mediator and the enzyme.\textsuperscript{29, 35} The utility of systems with the mediator and enzyme bound into thin films have been illustrated by applications in microdialysis and \textit{in vivo} measurements.\textsuperscript{36-38} The most common type of enzyme-containing matrix found in the literature are hydrogels. Hydrogels are cross-linked films that can contain enzymes within their pores and that swell in water.

Hydrogels are frequently formed by photopolymerization of the precursor molecules on the electrode surface. During the polymerization, the hydrogel forms around the enzyme
and the enzyme is encapsulated in the resulting pores. To photopolymerize a hydrogel film, a mixture of poly(ethylene glycol) diacrylate, vinylferrocene, 2,2′-dimethoxy-2-phenylacetophenone and the enzyme are combined on the surface of the electrode and illuminated with UV light (365 nm) for 2-10 sec. The progress of the reaction can be monitored by FTIR. These films have the advantage that they can be made into patterns using photolithography, and, in theory, used for bioarrays.

Another method to make enzyme containing hydrogels is illustrated by Calvo. Here the ferrocene poly(allylamine) backbone is cross-linked with epichlorohydrin in the presence of an enzyme at room temperature. Once the buffer used to dissolve the reactants has evaporated, the biosensor is ready to use. This produces a film that immobilizes the enzyme in the pores of the hydrogel and the mediator is bonded to the hydrogel matrix. The completion of the reaction was followed using electrochemical methods. This method of making hydrogels is marked by an increase in the long-term film stability, and homogeneous distribution of the redox and enzyme centers in the film.

Another common method of coupling the enzyme to the electrode is the method used by Adeloju et al., electropolymerization. This polymerization can take place either by free radical reaction or by a condensation reaction depending on the monomers involved. If the enzyme is present in the aqueous solution during the polymerization reaction, the enzyme can become encapsulated within the matrix at the electrode. Polymer films incorporating enzymes have been made using several different monomers, most
commonly polyaniline and polypyrrole. Polypyrrole has been cited 335 times in the literature, while polyaniline has been cited 174 times as matrices for biosensor preparation since 1996.\textsuperscript{5,43}

Glucose biosensors have been prepared by electropolymerization of pyrrole in the presence of GluOx by the application of a small positive potential. The magnitude and duration of the applied potential dictates the film thickness and the amount of encapsulated GluOx. To incorporate GluOx in a film of polypyrrole, both pyrrole and GluOx are added to the electrochemical cell in the presence of a platinum electrode.\textsuperscript{5} Current is passed through the cell for several minutes until electropolymerization is complete.\textsuperscript{44, 45} The films are commonly characterized using cyclic voltammetry. Electropolymerization has the ability to produce a film with more than one enzyme in a layer of polymer, provided that the polymer is conductive.\textsuperscript{20}

Finally, another common method to couple the mediator and enzyme reaction is to modify the enzyme itself with a mediator. This method attaches the mediator chain to the outer surface of the enzyme through an amide linkage.\textsuperscript{46} These modified enzymes can be used in solution or integrated into a polymer matrix that is deposited onto the electrode surface. In either case, the modified enzyme must closely approach electrode surface to regenerate the mediator. These systems, however, have demonstrated a low electron transfer rate to the surface.\textsuperscript{46}
All of these methods to attach the mediator and enzyme to the surface of the electrode are well developed and each has distinct advantages. Yet, only a few polymer systems can be used to produce these films. These enzyme-electrode attachment methods also rely on encapsulating the enzyme in the pores of the polymer matrix. The ability of the enzyme to be oxidized by the mediator-containing polymer is determined by the enzyme being within the electron transport distance of the mediator. In fact, it has been shown that the electron transfer in these kinds of biosensors can be slow due to low mediator loading in the matrix. To circumvent the complications of these methods, a simpler technique utilizing electrostatics to constructing an enzyme-mediator assembly composed of alternating layers of mediator containing polymer and enzyme can be used.

### 1.6 Electrostatic Assembly

The use of electrostatic assembly to form layers by through space interactions has become very popular in the past few years. This technique forms uniform thin films quickly and easily from solution. Decher first described these “fuzzy nanoassemblies” in 1997, and since then these films have been explored for a variety of uses, including for making thin films with biological activity.

The electrostatic assembly method requires alternately dipping a solid support with a charged surface into solutions containing oppositely charged polyions for a short time. The amount of adsorbed material is self-limiting since repulsion among the equally charged polyions will limit the amount of the polyion that will bind to the substrate. The quantity of polyion adsorbed will have more than the stoichiometric number of
charges relative to the substrate, meaning that the charge on the surface is reversed as a result of adsorption of the polyion.\textsuperscript{46, 48} Once the first layer has been deposited, the support is then dipped into another solution containing a polyion of opposite charge. This results in adsorption of the other polyion and a reversal in the sign of the surface charge. One deposition cycle is one polycation and one polyanion forming a complete bilayer. This is illustrated in Figure 5. Cycles of manual dipping can continue with alternating adsorption of polyanion and polycation and will result in stepwise growth of the film.

![Figure 5. Electrostatic Assembly of Multiple Layers.](image)

The greatest advantage of the ionically-self-assembly process is that in principle any polyion can be incorporated into the film.\textsuperscript{46, 47} The polycations and polyanions most often used are aqueous polymers, although some work has been done with organic soluble polymers.\textsuperscript{46} The most commonly studied polyions are the commercially available, synthetic polyions such as: poly(allylamine), poly(styrenesulfonate), poly(vinylsulfate) and poly(acryacid).\textsuperscript{46}
Enzymes can be incorporated into the films readily because enzymes are large polyions. Since enzymes have an overall charge, immobilization of the enzyme into a layer is simple and requires no chemical derivatization. In principle, electrostatic deposition of the enzyme will not result in loss of enzyme activity.\textsuperscript{46} In addition, the enzyme may reside in close proximity to many mediators in the underlying film, making oxidation of the enzyme by mediated electron transport possible.

1.7 Conceptual Basis of Dissertation

The overall goal for the research presented in this dissertation is two-fold. The principle objective is to develop a simple and reproducible procedure to prepare an amperometric biosensor. This amperometric biosensor should be able to detect biological analytes with good sensitivity and specificity. In addition, the sensor should be robust so that they can be used for detection of these analytes in a flowing system. The specific analytes targeted in this research are glucose and glutamate. Glucose sensing will be used as a benchmark system, while glutamate detection is the test case of an analyte of biological importance for which a good analytical method does not exist.

Glutamate is an excitatory amino acid that is released between synaptic terminals in the brain and has a role in memory, learning, neurological diseases and cell destruction.\textsuperscript{37, 49-52} Several studies have been devoted to determining glutamate concentrations in the brain as it is thought that glutamate released from the neurons can “contribute to ischemia, or severe brain injury.”\textsuperscript{49-53} Accurately determining glutamate concentrations \textit{in vivo} or in brain dialysate is challenging as the molecule is not easily detectable by
electrochemistry or UV-visible spectroscopy, which are the classical bioanalyte detection methods.

Figure 6. Structure of glutamate.

Two ways of determining glutamate concentrations using electrochemistry have been reported. The first is to monitor the hydrogen peroxide produced by the reaction of glutamate oxidase in the presence of glutamate. The oxidation of hydrogen peroxide, however, requires a large positive overpotential. At this large positive overpotential significant electrochemical interferences can occur.

A second way to measure glutamate concentrations is to trap the hydroxyl radicals produced by the presence of excess glutamate. Glutamate is always present in the synapses between the neurons, between at concentrations between 0.01 M and 0.001 M. There are times, however, when glutamate is found at concentrations in excess of this amount. The excess glutamate initiates a production of reactive oxygen species such as hydroxyl radicals. Salicylic acid can subsequently be reacted with the oxygen radicals to form 2,3-dihydroxybenzoic acid which can then be quantified by either electrochemistry or spectroscopy. Detection of 2,3-dihydroxybenzoic acid can provide a measure of the excess amount of glutamate present in the synapse. While this is an effective measurement system, it must be done by sampling off-line and is not a direct method for glutamate determination.
Using electrostatic assembly, the oxidation of glutamate oxidase through mediated electron transport will be a simple and effective method for glutamate detection. A method in which ferrocene poly(allylamine) and glutamate oxidase are electrostatically assembled on an electrode surface for the detection of glutamate has not been reported previously in the literature.

The dissertation will present the results of these studies in the following manner.

Chapter 3 will describe the process of characterizing the electron transfer mediator material that will be used for the duration of this research, ferrocene poly(allylamine). To more accurately understand how ionically self-assembled monolayers (ISAMs) are prepared and how far electron transfer could occur through layers, bilayers of ferrocene poly(allylamine)-poly(styrenesulfonate) were constructed and characterized by cyclic voltammetry. This will allow a better understanding of how to assemble and electrochemically characterize the ISAM films.

Chapter 4 and 5 describe the assembly and characterization of ionically self-assembled GluOx biosensors. Films were assembled both on 2 mm gold electrodes and on microelectrodes and the advantages and disadvantages of these systems will be discussed. The GluOx biosensors assembled on microelectrodes represent an untapped methodology for GluOx biosensing. Electron-transfer through the films is discussed and the homogeneous kinetics of the GluOx in the film are examined. The GluOx biosensors
were subsequently tested in flow-injection experiments to determine long-term stability in sheer flow conditions.

Chapter 6 describes the design and characterization of a covalently modified ionically self-assembled GluOx biosensor. The characterization was carried out using XPS and RAIRS. This biosensor was designed specifically for use in the flow-injection experiments. Both the 2 mm gold electrode and the microelectrode biosensors were modified using this type of film. Electron-transfer through the films is discussed and the homogeneous kinetics of the GluOx in the film is examined. These films are compared and contrasted to the ISAM modified electrodes.

Chapter 7 describes the assembly and characterization of ionically self-assembled glutamate oxidase biosensors. Electron-transfer through the films is discussed and the homogeneous kinetics of the GluOx in the film is examined. These films were made on microelectrodes and represent novel research in biosensors.
Chapter 2: Experimental Approach

2.1 Introduction

This chapter recounts the experimental methods used to prepare and characterize the ionically self-assembled modified interfaces and furthermore will describe analytical methods including cyclic voltammetry, reductive desorption, x-ray photoelectron spectroscopy and reflection-absorption infrared spectroscopy. The experimental details will also be presented for each step of the biosensor preparation.

2.2 Cyclic Voltammetry

A sensitive, electroanalytical method for detection of analytes in solution or on the surface of an electrode is cyclic voltammetry. Cyclic voltammetry uses a triangular waveform to linearly ramp the potential through time and measuring the current as a function of potential. Figure 7 is an example of the triangle waveform used for cyclic voltammetry. The potential is varied linearly with time, and the rate at which the potential is varied is called the sweep rate. A cyclic voltammetry experiment starts by scanning from $t_1$ to $t_2$ in a linear fashion. The scan then reverses direction back to the original potential at $t_3$. 
Solution state cyclic voltammetry for a reversible system has several well-defined characteristics. At a standard 2 mm electrode, mass transport is dominated by linear diffusion. The oxidation and reduction peak in the cyclic voltammogram should be separated by $59/n \text{ mV}$ (where $n$ is the number of electrons transferred) and the peak separation is independent of scan rate. Additionally, the ratio of the peak currents (where the absolute value of the current reaches a maximum on the forward and the reverse scan) is one. Finally, for an electrochemically reversible system, the maximum current is dependent on the rate of diffusion of the analyte to the electrode surface; consequently, the peak currents are proportional to the square root of the scan rate.

### 2.2.1 Surface Confined Species

Surface confined species behave differently from solution state species. As the species is surface confined, the peak separation from the oxidative peak to the reductive peak will
be 0 mV for a perfectly reversible process. Also, the peak current is proportional to scan rate because there is no diffusion to the surface of the electrode.\textsuperscript{58}

For an electroactive species adsorbed to the surface of an electrode, cyclic voltammetry can be used to determine the surface density. This is done by integration of the area under the oxidative curve to quantify the total charge passed in the electrochemical process, equation 3:

$$\Gamma = \frac{Q}{nFA}$$  \hspace{1cm} (3)

Where $\Gamma$ is the surface coverage in mole/cm\textsuperscript{2}. $Q$ is the charge passed to oxidize/reduce the analyte, $n$ is the number of electrons in the electron-transfer process, $F$ is Faraday’s constant, and $A$ is the area of the electrode.

\subsection*{2.2.2 Enzyme Kinetics}

Enzyme kinetics are characterized by a number of parameters, the most ubiquitous being the Michaelis-Menten constant. The Michaelis-Menten constant ($K_\text{m}$) is a ratio of the rate constants in an enzyme-catalyzed reaction.\textsuperscript{59} For the reaction:

$$\text{GluOx(FAD)} + \text{Glucose} \xrightleftharpoons[k_2]{k_1} \text{GluOx(FAD)} - \text{Glucose} \xrightleftharpoons[k_4]{k_3} \text{GluOx(FADH}_2) + \text{Glucono-D-lactone}$$

$k_1$, $k_2$, $k_3$ and $k_4$ are rate constants for the enzyme reactions. At low glucose concentrations, the enzyme rapidly converts the glucose to the lactone. At high glucose concentrations, the enzyme becomes saturated and amount of lactone produced by the enzyme is determined by the turnover number of the enzyme, $k_3$ or as it is more commonly reported $k_{\text{cat}}$.\textsuperscript{59}
It is usually assumed that enzymatic reactions are irreversible so $k_4 = 0$, thus:

$$K_m = \frac{(k_2 + k_3)}{k_1} \quad (4)$$

Additionally, for many enzymes $k_3$ is very small relative to $k_2$, so that $K_m$ is approximately equal to $k_2/k_1$. $K_m$ therefore represents measure of the affinity of the enzyme for the substrate.

### 2.2.3 Kinetics of Mediated Electrode Reactions

The kinetics of the enzymatic reaction on a modified electrode can be determined using cyclic voltammetry. The kinetic breakdown of the mediated electron transfer is shown in Figure 8. There are two distinct parts to the reaction, the heterogeneous reaction and the homogeneous reactions. The heterogeneous reaction occurs only at the electrode-electrolyte interface and, for mediated electron transport, is considered to be a fast reaction. For biosensors described in these experiments, the heterogeneous reaction is the oxidation of the $\text{Fe}^{+2}$ to $\text{Fe}^{+3}$ at the electrode. The homogeneous reactions are the reduction of the $\text{Fe}^{+3}$ by GluOx and the oxidation of glucose by GluOx. The first homogeneous reaction is described by the rate constant $k$. The second homogeneous reaction is the same as would be in solution and can be described by Michaelis-Menten kinetics.

![Reaction Diagram](image-url)

Figure 8. Breakdown of mediated electron steps.
There are several ways to determine $K_m$. The most common method is the Lineweaver-Burke method, which plots the inverse of current versus the inverse of substrate concentration. This method though has the lowest precision.$^{59}$ The better method is the Hanes-Woolf graphical analysis method. In this method concentration divided by current is plotted against substrate concentration. As the Hanes-Woolf method uses a single reciprocal plot, this method has much less error associated with it, and is the method used in these investigations to determine $K_m$. From the Hanes-Woolf plot, the $K_m$ and the steady state current, $I_{cat}$ can be determined. The steady state current is a measure of the maximum catalytic current of the enzyme-substrate reaction.$^{13, 41, 60}$

### 2.2.4 Limit of Detection Determination

The limit of detection (LOD) is determined using limiting current versus concentration plots. LOD values were calculated by first determining the instrumental noise for the system. This was determined by applying a potential of 0.5 V to a bare gold electrode in 0.1 M KCl for 100 seconds. The standard deviation was taken of the resulting current and this was used to calculate the LOD for the calibration curves. LOD values are quantified by multiplying three times the standard deviation of the instrumental noise and dividing that quantity by the average of the slope of the mean calibration curve.
2.2.5 Limit of Linearity Determination

The limit of linearity is the point at which the calibration curve deviates from linearity. The non-linearity was determined using the regression statistics package in Excel. After plotting the most linear portion of the calibration curve, the “Residuals Output” was examined to determine if the points chosen were indeed linear. When a standard residual is greater than 2, the point can be discarded. This method is called “n-fit.” When “n” is greater than 2, then the deviation is greater than normal, and the data point can be discarded. The limit of linearity is used to illustrate over what range the biosensor can be used for quantitative analysis.

2.2.6 Cyclic Voltammetry Conditions

All cyclic voltammetry experiments were conducted with a CH Instruments model 600A potentiostat (Austin, TX) and a static electrochemical cell shown in Figure 9.

Figure 9. Electrochemical cell used in cyclic voltammetry experiments.
All solutions were degassed with argon for 10 minutes prior to measurements and the applied potential was reported relative to a Ag/AgCl reference electrode.

An acetate buffer (0.05M) with 0.01 M KCl was prepared as the electrolyte for the glucose experiments. The acetate buffer was prepared from 0.05 M sodium acetate and 0.05 M acetic acid in deionized water making a solution of approximately pH 4.8. The sodium acetate, acetic acid, sodium chloride and hydrochloric acid were used as received from Aldrich. This solution was subsequently adjusted to pH 5.0 using dilute HCl while monitoring with a pH meter.

A phosphate buffer (0.05M) with 0.01 M KCl was prepared as the electrolyte for the glutamate experiments. The phosphate buffer was prepared from 0.05 M sodium phosphate monobasic and 0.05 M sodium phosphate dibasic in deionized water making a solution of approximately pH 6.8. The sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride and sodium hydroxide were used as received from Aldrich. This solution was subsequently adjusted to pH 6.6 using dilute NaOH while monitoring with a pH meter.

### 2.3 Thiol Deposition

Monolayers of 3-mercapto-1-propane-sulfonate and 11-mercaptoundecanoic acid were formed by exposing clean gold electrodes to 0.001 M solutions of freshly prepared 0.01 M H$_2$SO$_4$ and ethanol, respectively. 3-mercapto-1-propane-sulfonate, H$_2$SO$_4$ and ethanol were all used as received from Aldrich. 11-mercaptoundecanoic acid was previously
prepared by Baltzersen. The gold substrates are cleaned prior to adsorption by placing them in a fresh piranha solution (1:3 H₂O₂/98% H₂SO₄) for several minutes. **Caution, piranha solution is highly corrosive and reacts violently with organic materials. Use precaution when working with this solution and following usage, dispose of the solution properly.** The substrate was a 1”x1” glass slide coated with 1000 Å vapor deposited Au on top of 50 Å of Cr as an adhesive layer (Evaporated Metal Films, Ithaca, NY).

Gold electrodes, purchased from Bioanalytical Systems Inc.(West Lafayette, IN), were cleaned by cycling in 0.01 M H₂SO₄ between -0.5 and 1.5 V at 1.0 V/s for 10 min, or until no further surface contamination was observed. The electrodes have an exposed working surface of 2 mm. The gold electrodes were allowed to sit undisturbed for 24-48 hours to allow for maximum monolayer packing. The electrodes were removed, thoroughly rinsed with deionized water and immediately used. Gold microelectrodes were prepared for modification in the same manner as the 2 mm gold electrodes and were used immediately. Thiol monolayers were characterized by reductive desorption and reflection-absorption infrared spectroscopy.

### 2.4 Reductive Desorption

Reductive desorption is an electrochemical technique that can be used to determine surface coverage. Using a gold surface with self-assembled monolayers as the working electrode, the reductive-desorption measurements give information about the monolayer formed on the surface. The area, shape and position of the reductive peak can give
information about the monolayer coverage, and lateral interactions within the layer. For example, the longer the chain length, the more negative the potential needs to be to desorb the molecules. This reflects the stronger van der Waals forces between the alkyl chains. Several different kinds of interactions can be imagined between the chains such as: hydrogen bonding, \( \pi-\pi \) interactions as well as interactions between the head groups (repulsive or attractive), and all which will have different influences on the reduction potentials.

For alkanethiols adsorbed on gold, the Au-S bond can be broken at a reasonable potential, usually between -0.8 to -1.2 V vs. Ag/AgCl causing the molecule to desorb from the surface.\(^6^3\)

\[
\text{Au—SR} + e^- \rightarrow \text{Au} + \text{SR}^-
\]  

(5)

Walczak et al. showed that the charged required to desorb the monolayer from the substrate can be used to determine the surface coverage of monolayer.\(^6^4\) This surface coverage can be calculated using:

\[
\Gamma = \frac{Q}{nFA}
\]  

(6)

Where \( Q \) is the charge passed to break the Au-S bond, \( n \) is the number of electrons in the electron-transfer process (in this case \( n = 1 \)), \( F \) is Faraday’s constant, and \( A \) is the area of the electrode.\(^6^4\) The value of \( Q \) is determined by integration of the area under the oxidative curve.
2.4.1 Reductive Desorption Conditions

The surface coverage of the monolayer-covered electrodes was determined using reductive desorption measurements. Reductive desorption experiments were conducted with a CH Instruments model 600A potentiostat (Austin, TX) and a drop electrochemical cell similar to that described by Chidsey. The contact area that a drop of electrolyte solution makes with the modified substrate, 0.66 cm² for these measurements as determined by chronocoulometry, defines the surface area of the working electrode. A potassium hydroxide solution (Electronic grade, Aldrich) (0.05 M) was used as the electrolyte. A blank gold slide is first used to determine the solution resistance which then can be used for positive feedback instrumental resistance compensation. The potential was swept between 0 and −1.1 V versus a saturated Ag/AgCl reference electrode at a rate of 50 mV/s. All solutions were degassed with N₂ for 10 minutes prior to measurement.

2.5 Reflection Absorption Infrared Spectroscopy (RAIRS) Conditions

RAIRS spectra were obtained using a Nicolet model 710 infrared spectrometer (Madison WI) using a liquid nitrogen cooled MCT (HgCdTe) detector. The spectrometer was fitted with a Spectra-Tech model FT-80 fixed grazing angle specular reflectance sample apparatus at an incident angle of 80° and a beam diameter of 2 cm. The spectra were referenced to a clean, gold substrate prepared in the same manner so that there is no material on the surface. The infrared source light was p-polarized using a Zn-Se wire grid polarizing filter (Cambridge Physical Sciences, IGP 228). The sample chamber is purged (Balston Filter Products 75-60) with dry air. Reflection spectra of the monolayers
were collected with 2 cm⁻¹ resolution and 1000 interferometer scans. Spectral acquisition was done using Nicolet’s Omnic (version 3.0) software.

2.6 Synthesis of Ferrocene Poly(allylamine)-FePAA

Ferrocene carboxaldehyde (10 mg) was dissolved in 5 mL of anhydrous methanol and added dropwise within 60 minutes to 30 mL of anhydrous methanol that contained 40 mg of poly(allylamine) and 0.26 mL of triethylamine. All were used as received from Aldrich. The methanol was dried using molecular sieves. The poly(allylamine) had previously been purified by dialyzing against water for 3 days to eliminate low molecular weight oligomers and subsequently rotovapped to remove the water. This methanol solution was stirred for one additional hour at room temperature under a N₂ atmosphere. The solution was then cooled back to 0°C while 10 mg of sodium borohydride (used as received from Aldrich) was added slowly and stirred for another 90 minutes, still under a N₂ atmosphere. The final product was dried in a vacuum oven at 35°C and the residue was extracted with deionized water. This aqueous solution was then dialyzed 3 times to purify the final product. The FePAA was characterized by X-ray photoelectron spectroscopy.

2.7 X-ray Photoelectron Spectroscopy Conditions

X-ray photoelectron spectroscopy spectra were obtained using a Perkin-Elmer 5400 model instrument (nitrogen purged) with a Mg/Al radiation source and hemispherical analyzer. Measurements were taken at a pressure of approximately ~10⁻⁷ mbar and a take off angle of 45 degrees with respect to the surface normal. The cold-finger was used for
degassing of the samples. Survey spectra were collected for between 5-10 minutes using pass energy of 44.750 eV and a 300 W electron beam power. The high-resolution spectra were collected for between 5-20 minutes. The gold surfaces used for these experiment has a Ti underlayer (Evaporated Metal Films (Ithaca, New York)) and the samples were dried at 70°C for 30 minutes prior to placing them in the XPS to remove all excess water.

2.8 Preparation of Microelectrode

Microelectrodes are prepared by attaching 2 cm of 50 μm gold fiber (used as received from Alfa Aesar) to 5 cm of Ni wire (standard wire wrap wire) using silver conducting paint (Silver Print Paint, GC Electronics, Rockford, IL). After the paint has dried (approximately 30 minutes), the bonded fibers are inserted into open-ended melting point tubes. These tubes are then placed inside a capillary puller, and the glass capillary is melted around the end of the gold wire, leaving a few millimeters of the wire exposed. The tips of the electrodes are then backfilled with epoxy to make the final seal around the gold wire. The electrodes are allowed to cure overnight at room temperature then for an additional two hours at 100°C. The final microelectrode has an exposed cylindrical tip a few millimeters in length.

2.9 FePAA – poly(styrenesulfonate) ISAM Electrode

The FePAA – poly(styrenesulfonate) ionically-self-assembled monolayer (ISAM) modified electrode was formed by taking either a 3-mercapto-1-propane-sulfonate and 11-mercaptoundecanoic acid modified gold electrodes (as described in Thiol Deposition) and placing it in a solution of 0.001 M (by repeat unit) FePAA in 0.01 M, pH 5 acetate
buffer for 20 minutes. At pH 5, approximately 30% of the amines are protonated, as determined by IR while all of the sulfonate groups in poly(styrenesulfonate) are going to be deprotonated. After adsorption, the electrode was rinsed in 0.01 M, pH 5 acetate buffer. The substrates were then placed in 0.001 M (by repeat unit) poly(styrene sulfonate) in 0.01 M, pH 5 acetate buffer for 20 minutes. After adsorption, the electrode was rinsed in 0.01 M pH 5 acetate buffer. This completed one bilayer. This procedure continued if subsequent bilayers were needed.

2.10 Glucose Oxidase and Glutamate Oxidase ISAM Electrodes

The glucose oxidase ISAM modified electrode was formed by taking a 3-mercapto-1-propane-sulfonate modified gold electrode and placing it in a solution of aqueous FePAA for 20 minutes. After adsorption, the electrodes were allowed to dry in air. The excess polymer was removed by cycling between 0 and 0.6 V at 0.005 V/s until 3 consecutive cycles were consistent. The electrode was then immediately placed into the glucose oxidase solution. Glucose oxidase was adsorbed onto the FePAA modified electrode from a solution containing $7 \times 10^{-6}$ M glucose oxidase in 0.01 M, pH 5 acetate buffer. The glucose oxidase (EC 1.1.3.4 type X-S from *Aspergillus niger*, 186 kDa, 179 units/g solid) was used as received from Sigma. The adsorption continued for 45 minutes, after which the electrode was thoroughly rinsed with distilled water and used immediately. All glucose detection was done under an argon atmosphere.

If a glutamate oxidase (GlutOx) ISAM was to be prepared, the FePAA coated electrode would be placed into a solution containing 0.25 mg glutamate oxidase in $20 \times 10^{-5}$ L, 0.01
M, pH 6.6 phosphate buffer. The glutamate oxidase (EC 1.4.3.11 from *Streptomyces sp.* 10.1 units/mg solid) was used as received from Sigma. The adsorption continued for 45 minutes, after which the electrode was thoroughly rinsed with distilled water and used immediately. All glucose detection was done under an argon atmosphere.

If multiple bilayers were being deposited, each GluOx or GlutOx layer would be checked for enzymatic activity.

### 2.11 Covalently Modified ISAM Glucose Biosensor

The covalently modified ISAM glucose biosensor was prepared by forming a covalent bond between 11-mercaptoundecanoic acid and FePAA. This forms a covalent amide bond between the carboxylic acid on the thiol and the amine on the FePAA. Four mg of FePAA is put into 20 mL of 0.01 M pH 5 acetate buffer containing 150 mg KCl. This solution is sonicated to ensure the complete dissolution of the polymer. Once the solution has returned to room temperature, 150 mg of 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride (used as received from Aldrich) was added and degassed for 20 minutes. The 11-mercaptoundecanoic acid modified substrate was then placed in this solution for 60 minutes. The substrate was removed from the solution, and thoroughly rinsed with deionized water. Glucose oxidase was adsorbed onto the covalently modified electrode from a solution containing 7 x 10^{-6} M glucose oxidase in 0.01 M, pH 5 acetate buffer. The glucose oxidase was used as received from Sigma. The adsorption continued for 45 minutes, after which the electrode was thoroughly rinsed with distilled water and used immediately.
2.12 Flow-Injection Experiments

Flow injection experiments are a good way to determine the stability of the surface modification over the long term. Many biosensors are tested using flow-injection experiments to determine the limit of detection of the electrode under turbulent conditions. The flow injection setup is performed at a constant potential under a constant flow. The analyte is injected directly into the tubing and the current is constantly measured. When the analyte reaches the electrode, the analyte is oxidized and the current increases. The flow set up for the 2 mm electrode is shown in Figure 10 and the flow set up for the microelectrode is shown in Figure 11.

![Flow apparatus for the 2 mm electrode.](image.png)

Figure 10. Flow apparatus for the 2 mm electrode.
2.12.1 Flow Injection Experiments Conditions

Flowing experiments were performed using a Waters 501 HPLC Pump (Milford, MA) delivering solvent at 0.5 mL/min flow rate. Samples were introduced by injections using a syringe and an on-capillary 6-port sample injection valve with a 20 µL sample loop. The mobile phase was 0.01 M acetate buffer, pH 5 and degassed for all experiments. For GluOx ISAM modified flow-injection experiments the KCl concentration was 0.05 M, while for covalently modified ISAMs glucose biosensors, the KCl concentration was 0.14 M. The current was constantly monitored at 0.500 V vs. Ag/AgCl using a potentiostat (Princeton Applied Research, model 273) controlled by a locally written data collection program. For 2 mm gold electrode experiments, a standard flow cell was attached to the pump using 1/16” diameter tubing. The microelectrode experiments used 100 um inner diameter capillary and alignment of the microelectrode was done using a stereomicroscope and an XYZ platform.
Chapter 3: Assembly and Characterization of Ferrocene poly(allylamine)-poly(styrenesulfonate) Layers by Electrostatics

3.1 Introduction

The first ionically-self-assembled monolayer (ISAM) was reported by Decher in 1997 using poly(allylamine) and poly(styrenesulfonate). Since then, ISAMs have become increasingly popular as a method for forming well-ordered surfaces with specific interfacial chemical and physical properties. ISAMs also have been used for a variety of purposes including: biosensing, chromatography and optical data storage. For this dissertation, electrostatically assembled polymers will be used to confine an electrochemically reversible redox couple and a biologically active enzyme at the interface of an electrode for use as a biosensor.

As a proof of principle, and to fully characterize the assembly process, ISAMs composed of ferrocene poly(allylamine) and poly(styrenesulfonate) ISAM were produced. During the ISAM assembly, different analytical techniques were used to characterize the assembly process. Once the ferrocene poly(allylamine)-poly(styrenesulfonate) ISAM was prepared and better understood then an enzymatic ISAM can be made.

3.2 Characterization of Thiol Deposition

Using the methods discussed in the experimental details section, a 3-mercapto-1-propanesulfonate or 11-mercaptoundecanoic acid self-assembled monolayer was prepared by
molecular self-assembly. To determine the surface coverage and to characterize the monolayer, reductive desorption and RAIRS measurements were performed.

Reductive desorption is a measurement that can characterize the strength of the lateral interactions between the chains. Shifts to a more negative desorption potential relative to a reference monolayer indicate stabilizing interactions within the monolayer. The reverse is also true, shifts to a more positive desorption potential can indicate destabilizing interactions in a chain. Reductive desorption can also be used to quantify the amount of material on the surface. RAIRS is a spectroscopic measurement that is used to understand the structural details of the monolayer.

3-mercapto-1-propane-sulfonate is a short chain mercaptan with a bulky head group and the monolayer is not expected to have dense packing. For this reason, RAIRS is not a suitable technique for 3-mercapto-1-propane-sulfonate characterization. From the low packing, it is likely that the spectral features of the monolayer would be below the signal to noise of the RAIRS measurement. This is what was observed.

Reductive desorption can be used, however, to characterize the 3-mercapto-1-propane-sulfonate surfaces and determine the surface coverage. An example of a reductive desorption voltammogram is shown in Figure 12.
Figure 12. Reductive desorption of (3-mercapto-1-propane-sulfonate) in 0.05 M KOH, 0.05 V/s

The surface coverage was calculated to be $3.04 \pm 0.71 \times 10^{-10}$ mol/cm$^2$ by integrating the area under the desorption curve. The low surface coverage is consistent with the inability of our RAIRS measurement to see spectral signatures above the signal-to-noise. The surface coverage value reported here is smaller than the other literature values reported for monolayers of 3-mercapto-1-propane-sulfonate$^{69}$ The difference in these values is possibly due to differences in preparation conditions. While the geometrically derived value for a densely packed monolayer is found to be $7.6 \times 10^{-10}$ mol/cm$^2$, the 3-mercapto-1-propane-sulfonate modified surfaces are going to be less tightly packed than long-chain n-alkanethiol modified surfaces. This difference in packing will be due to large steric repulsions of the sulfonate head groups. Additionally short alkyl chains will have lower coverages as they known to not form well-ordered monolayers.$^{67, 69}$
absence of significant van der Waals interactions prevents the formation of a well-ordered monolayer.

The reduction peak potential is at -0.838 V vs. Ag/AgCl. The positive value of the peak potential compared to that reported by Mokrani et al., is due to using a lower concentration of KOH. The lower surface coverage will also contribute to the positive shift in desorption peak potential. In work done by Widrig et al. they report the reductive potential of butanethiol, to be -0.90 V versus Ag/AgCl. This monolayer should have similar van der Waals interactions but has slightly more stabilization that the 3-mercapto-1-propane-sulfonate, and this is reflected in the more negative desorption potential.

Surfaces modified with 11-mercaptoundecanoic acid surfaces were also characterized by reductive desorption and RAIRS measurements. A representative reductive desorption voltammogram is shown in Figure 13.
The surface coverage was calculated by integration of the area under the desorption curve and determined to be $8.8 \times 10^{-10}$ mol/cm$^2$. The surface coverage found in this work corresponds well to reported literature values for 11-mercaptoundecanoic acid.$^{61, 67}$ The 11-mercaptoundecanoic acid layers will pack more tightly than the 3-mercaptopropane-sulfonate layers because of the increased stabilization given by the hydrogen bonding between the head groups and the increased van der Waals interactions among the longer alkyl chains.

The reduction peak potential for 11-mercaptoundecanoic acid is at -0.88 V vs. Ag/AgCl. Again, it is expected that these layers will be less stable than the counterpart n-alkanethiol monolayers, and would have a more positive reductive desorption peak potential. The reduction peak potential of 1-undecanethiol has been shown to be -1.0 V
versus Ag/AgCl. The carboxylic layer is destabilized by the unfavorable head group interactions, and has a more positive reduction peak potential.

RAIRS can also be used to characterize surfaces modified with 11-mercaptoundecanoic acid. Only vibrations that have a significant portion of their transition dipole moment perpendicular to the surface are observed in RAIRS. Figure 14 shows the RAIR spectrum of 11-mercaptoundecanoic acid adsorbed to a gold substrate.

![RAIR spectra of 11-mercaptoundecanoic acid on gold surface.](image)

**Figure 14.** RAIR spectra of 11-mercaptoundecanoic acid on gold surface. This spectrum is characteristic of a 11-mercaptoundecanoic acid monolayer with sharp peaks at 2926 and 2854 cm\(^{-1}\) for the asymmetric and symmetric methylene stretches. These peaks are shifted 4-8 cm\(^{-1}\) higher in energy from methylene stretches in a crystalline environment. This result indicates that the 11-mercaptoundecanoic acid
The 3-mercapto-1-propane-sulfonate was chosen as the thiol surface that would be chosen to build the ISAMs from as it is much easier to deprotonate than the 11-mercaptoundecanoic acid. The pKa of the 11-mercaptoundecanoic acid, 4.5, is higher than that of the 3-mercapto-1-propane-sulfonate is evidence to that fact. Creager et al. show that within the monolayer, however, the pKa for the 11-mercaptoundecanoic acid is even higher due to the formation of hydrogen bonds.

**3.3 Synthesis and Characterization of FePAA polymer**

The FePAA polymer was characterized using XPS, a method sensitive for both atomic composition and molecular environment. The primary features of an XPS survey scan, shown in Figure 15, are assigned to C (1s) at 285 eV, N (1s) at 401 eV, and Fe (2p) at 708 and 721 eV. These features are consistent with the poly(allylamine) backbone that contains a ferrocene modification.
Figure 15. XPS survey scan of FePAA ISAM.

There are also features from O (1s) at 481 eV, Cl (2p) at 1327 eV and O KVV Auger peaks at 1508 eV. These peaks are most likely due to water and electrolyte, and have been shown in other studies to be present during electrostatic assembly. The C (1s), N (1s), and Fe (2p) peaks are further investigated by collecting high-resolution spectra in the appropriate energy regions of the spectrum, and are shown in Figure 16.

The C (1s) peak at 285 eV is characteristic of the backbone of the polymer and is the major peak of the survey scan. The N (1s) peak is present at 401 eV, an energy characteristic of the quaternary amine in the poly(allylamine) backbone. Finally, the two Fe peaks (2p$_{1/2}$) and (2p$_{3/2}$) are found at 708.5 and 721 eV indicating that this is a ferrocene bound to the polymer.
The structure of the poly(allylamine) has 1 nitrogen on every monomer unit; therefore, taking the ratio of percent composition of the N (1s) to Fe (2p\textsubscript{1/2}) allows the number of ferrocenes per polymer monomer unit to be calculated. From the percent composition, it was determined that the ratio of N (1s) to Fe (2p\textsubscript{1/2}) was approximately 12:1, indicating that the structure of the FePAA is:

Figure 16. XPS high resolution scans of the C (1s), N (1s) and Fe (2p) regions of the FePAA ISAM.
Figure 17. Structure of FePAA.

This result corresponds well with established literature structure for FePAA.¹³,⁴¹

3.4 Characterization of Electrostatically Bound FePAA

Following electrostatic assembly, FePAA modified gold electrodes were characterized by cyclic voltammetry to (1) determine if the FePAA was bound to the surface and (2) to quantify the amount of Fe²⁺ confined to the surface of the electrode.

A layer of FePAA was electrostatically bound to 3-mercapto-1-propane-sulfonate and was characterized using cyclic voltammetry. The cyclic voltammogram of the modified electrode is shown in Figure 18.
Figure 18. a) Cyclic voltammogram of 3-mercapto-1-propane-sulfonate modified 2 mm gold electrode. b) Cyclic voltammogram of 3-mercapto-1-propane-sulfonate/FePAA modified 2 mm gold electrodes. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

The electrode modified with only 3-mercapto-1-propane-sulfonate shows only the capacitive current associated with charging of the electrochemical double layer. The FePAA modified electrode, however, has a peak at 0.427 V assigned to the oxidation of Fe$^{+2}$ on the forward scan and a peak on the reverse scan assigned to the reduction of Fe$^{+3}$ at 0.373 V. The oxidation to reduction peak separation is 0.054 V, which is larger than expected for a surface confined electroactive species. This peak separation is most likely due to the resistance toward charge transfer of the thiol monolayer under the FePAA layer.

The full-width at half peak height is 0.114 V, which is larger than the predicted value of 0.096 V for an ideal surface wave. These results suggest that the oxidation of the
confined ferrocene is subject to slower than expected heterogeneous kinetics. The slow heterogeneous kinetics of the system is likely due to resistance to electron tunneling through the insulating thiol layer.

Cyclic voltammetry also be used to determine if the FePAA polymer is bound to the surface. The peak current for the oxidation of FePAA will increase linearly with scan rate if the FePAA is confined to the electrode surface.\textsuperscript{58} Otherwise the peak current will vary linearly with the square root of the scan rate.\textsuperscript{58} After preparing an electrostatically assembled FePAA modified electrode, the oxidative peak current was plotted versus scan rate. As is graphically illustrated in Figure 19, the peak current increases linearly with scan rate for FePAA on a 3-mercapto-1-propane-sulfonate surface. This behavior is consistent with FePAA being bound to the surface. Also shown in Figure 19 is the peak current plotted versus square root of scan rate. This relationship is non-linear, which confirms that FePAA is surface bound.

![Figure 19. a) Linear relationship between peak current and scan rate b) Non-linear relationship between peak current and square root scan rate.](image-url)
FePAA polymer modified gold electrodes were also characterized to quantify the amount of Fe$^{+2}$ confined to the surface of the electrode. Figure 18 shows the current vs. potential curves for a 3-mercapto-1-propane-sulfonate/FePAA modified gold electrode. By integrating under the oxidative curve the surface coverage of adsorbed Fe$^{+2}$ can be determined.

FePAA layers were first made on gold slides (3-mercapto-1-propane-sulfonate underlayer) and the surface coverage was found to be 5.8 ($\pm$ 1.5) $\times$ 10$^{-11}$ mol/cm$^2$. For FePAA layers subsequently made on a 2 mm gold electrode (3-mercapto-1-propane-sulfonate underlayer), the surface coverage was found to be 6.3 ($\pm$ 2.6) $\times$ 10$^{-10}$ mol/cm$^2$. The FePAA surface coverage on the gold slide value corresponds well to literature reports of FePAA adsorbed on gold surfaces.$^{13}$ The 2 mm gold electrode surface coverage is higher than literature values.$^{13}$ This is due to the preparation method used for the modified 2 mm gold electrodes.$^{13}$ Unlike the experimental technique of Hodak et al., ISAM modified 2 mm gold electrodes were not rinsed with water to remove excess physisorbed FePAA from the ISAM.$^{13}$ In this case, the 2 mm gold electrodes were allowed to equilibrate in the electrolyte solution and electrochemically cycled between 0 and 0.6 V until three consecutive cycles exhibited identical current response. This technique was used as it was believed that this would make the most reproducible surface, based on the cyclic voltammograms being identical for three consecutive cycles.
3.5 Characterization of FePAA-poly(styrenesulfonate) ISAMs

Several different combinations of polyelectrolytes have been used to form bilayer ISAMs. Some of these are: poly(allylamine) and poly(styrenesulfonate),\(^4^7\) and FePAA and poly(vinyl sulfonate).\(^8^1\) A good model for demonstrating the ability to make ISAMs is to prepare ISAM bilayers composed of FePAA as the polycation and poly(styrenesulfonate) as the polyanion. The ISAM growth can then be characterized using cyclic voltammetry.

Bilayer modified electrodes were constructed and characterized by cyclic voltammetry. The amount of FePAA on the electrode was determined after addition of each subsequent bilayer by integrating the charge under the oxidative curve. Figure 20 shows the results using both 3-mercapto-1-propane-sulfonate (MPS) and 11-mercaptoundecanoic acid (MUA) as the thiol underlayer.
As is illustrated in Figure 20, the 11-mercaptoundecanoic acid is able to electrostatically bind more of the FePAA than the 3-mercapto-1-propane-sulfonate. This is expected as the 11-mercaptoundecanoic acid surface coverage is greater than the 3-mercapto-1-propane-sulfonate surface coverage. A greater surface coverage results in additional surface charge, allowing more FePAA to assemble to the surface.

In spite of the differences in surface coverage, both surfaces show the same trend of a linear increase in integrated charge with increasing number of bilayers through the first 7 bilayers. After 7 bilayers have been electrostatically assembled, the integrated charge of the assembly reaches a constant value. This result suggests that the ferrocene-modified polymer in the outermost layers can no longer be oxidized by the heterogeneous reaction. With each addition electrostatically assembled layer, the newly deposited ferrocene
modified polymer is farther away from the surface. Based on work done by Caruso, the 7th bilayer may be as far away from the electrode as 209 Å. At this distance electron transfer less efficient and it is not unexpected that the integrated charge will level off. The result that the integrated charge approaches a constant level after 7 bilayers is consistent with the work reported on by Lui et al. They also reported slow electron transfer with increasing number of bilayers.

3.6 Summary

As indicated by reductive desorption and RAIRS measurements, both 3-mercapto-1-propane-sulfonate and 11-mercaptopoundecanoic acid spontaneously form loosely-packed monolayers on gold. The negatively charged terminal groups for each monolayer subsequently served as a base for the ISAM construction. FePAA, the positively-charged ISAM component, was synthesized using poly(allylamine) and ferrocene carboxaldehyde and was found by XPS to have an approximate N:Fe ratio of 12:1.

After exposing the 3-mercapto-1-propane-sulfonate monolayer to a solution of FePAA, cyclic voltammetry was used to characterize the film. The initial ISAM formation was evidenced by the Fe$^{+2}$ oxidization and reduction peak having a small peak separation. Additional evidence of ISAM formation was the Fe$^{+2}$ peak current scaling linearly with scan rate. Further cyclic voltammetry analysis determined a FePAA density of 5.8 (± 1.5) x 10$^{-11}$mol/cm$^2$ for the gold slide and 6.3 (± 2.6) x 10$^{-10}$ mol/cm$^2$ for the 2 mm gold electrode indicating a larger than expected surface coverage.
Building on the FePAA success, negatively-charged poly(styrenesulfonate) was electrostatically attached, making the first bilayer. These bilayers were electrochemically characterized to determine amount of charge contributed by the FePAA with each bilayer. From these experiments it was confirmed that the 11-mercaptoundecanoic acid has a greater surface coverage than the 3-mercapto-1-propanesulfonate as the FePAA density was greater on the 11-mercaptoundecanoic acid surfaces. Regardless, these surfaces behaved the same with increasing integrated charge with increasing number of bilayers. This demonstrates that the bilayers form in a reproducible and orderly fashion regardless of the thiol underlayer. The increase in the integrated charge continued until the 7th layer at which point the integrated charge leveled off. At distances beyond the seventh bilayer, electron transport becomes very inefficient and it is not unexpected that these layers will not contribute to the total integrated charge of the ISAM. All further experiments will be limited to less than 7 bilayers. In the following chapters, ISAMs are assembled by substituting GluOx for poly(styrenesulfonate) and cyclic voltammetry experiments will be carried out to determine if mediated electron transfer is occurring at the film interface.
4.1 Introduction

Glucose biosensors were among the first biosensors developed that made use of an enzyme reaction for detection of analytes. The first amperometric glucose sensors were designed by Clark to monitor hydrogen peroxide produced from glucose reacting with glucose oxidase. Since the development of these first biosensors, many different types of amperometric biosensors have been developed. Most of these are designed around a hydrogen peroxide independent reaction.

One type of biosensor that is finding the largest application in biological sensing is an amperometric biosensor. This type of sensor typically is designed with a thin, immobilized layer confined to the electrode surface that contains a redox mediator and enzyme. These biosensors use small electron transfer distances between the mediator and the enzyme to oxidize the enzyme and transport the electrons to the electrode surface. Ionically self-assembled monolayers are a simple way to make these kinds of mediated electron films.

In this section, the development of an ISAM that incorporates FePAA and GluOx for the successful mediation of GluOx will be discussed. These ISAMs will be used to oxidize glucose in solution. The development of the sensing layer, a description of the enzyme homogeneous reaction and flow-injection experiments of modified 2 mm electrodes will be presented.
4.2 Assembling the GluOx ISAM Biosensor

ISAMs formed from FePAA and poly(styrenesulfonate) have been shown to make bilayers that assemble in a linear fashion. Based on these results, an ISAM prepared from FePAA and glucose oxidase (GluOx) can be assembled. GluOx is a large globular polyanion that is easily formed into layers for electrostatic assembly.\textsuperscript{85}

Transitioning from the FePAA–poly(styrenesulfonate) ISAM to a FePAA–GluOx biosensor requires careful control of two important parameters; namely the GluOx must be negatively charged to electrostatically assemble to the FePAA, and the enzyme must have a mediator incorporated into the ISAM. GluOx can replace poly(styrenesulfonate) at pH 5 because its pI (isoelectric point) is 4.05, meaning that at pH 5 GluOx has a net negative charge.\textsuperscript{86} Also, FePAA can act as a mediator for the oxidation of the enzyme, ultimately allowing for mediated electron transfer. FePAA is a polycation that has the mediator designed into the polymer. This will allow the mediation to take place at the enzyme-polymer interface. Bartlett and Pratt have completed a comprehensive theoretical study on confined ferrocene-mediators and GluOx at an electrode surface.\textsuperscript{84, 87} They have shown that mediated electron transfer will occur from the ferrocene to the GluOx in the presence of glucose.\textsuperscript{84, 87} In addition many groups have successfully used confined ferrocene mediators to oxidize GluOx in a film.\textsuperscript{4, 6-8, 10, 12, 13, 15, 28, 34, 41, 42, 45, 88-91}

Using electrostatic assembly to oxidize GluOx at the surface of an electrode will be a simple and reproducible method to make a glucose biosensor.
The GluOx biosensors are being developed for three main reasons. The first reason is to determine if these ISAMs can be assembled, and the conditions for assembly. Secondly, they are being designed to test ISAM stability and detection limits in flow-injection experiments. Finally, once the method of assembling ISAMs with GluOx incorporated in the film, this method of mediated electron transfer to a FAD containing enzyme confined in an electrostatic film can be applied to other FAD enzymes, such as glutamate oxidase. These proof-of-principle experiments are very important to developing a method that has simple to adjust parameters and can be easily applied to other FAD containing enzymes.

4.3 Cyclic Voltammetry of the GluOx ISAMs

Cyclic voltammetry was used to follow the assembly of the FePAA and GluOx bilayers and the oxidation of the glucose by the GluOx. A 3-mercaptop-1-propane-sulfonate/FePAA ISAM was made and characterized using cyclic voltammetry, as is seen in Figure 21. When GluOx is adsorbed to the FePAA the cyclic voltammogram does not change dramatically from that seen prior to the adsorption of the GluOx. This result is expected, and demonstrates that the redox center of GluOx is not accessible to the electrode.
Figure 21. Cyclic voltammogram of a) 3-mercapto-1-propane-sulfonate/FePAA modified 2 mm gold electrode. b) 3-mercapto-1-propane-sulfonate/FePAA/GluOx modified 2 mm gold electrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

There is a decrease in the peak height the Fe$^{+2}$ oxidation after the electrostatic deposition of GluOx. This result has previously been reported by Hodak et al.$^{13}$ The decrease in the peak height is probably due to loss of physisorbed polymer to the aqueous buffer.

When glucose is added to the solution adjacent to the modified electrode the cyclic voltammogram changes dramatically from what was shown in the absence of glucose. This is shown in Figure 22. The cyclic voltammogram is showing a steady state behavior, meaning that the flux of electrons to the electrode reaches a constant rate at the more positive potentials. This is in contrast to the cyclic voltammogram in the absence of glucose, also shown in Figure 25. In that voltammogram, the current diminishes to zero at increasing potential as all of the Fe$^{+2}$ at the surface of the electrode has been oxidized.
to Fe$^{+3}$. In the steady state cyclic voltammogram, glucose is in excess and the Fe$^{+2}$ is constantly being regenerated with the ISAM giving a constant current response.

Figure 22. Cyclic voltammogram of a) 3-mercapto-1-propane-sulfonate/FePAA/GluOx modified 2 mm gold electrode and b) in the presence of 4 mM glucose. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

The steady state response can be explained by the following catalytic cycle:

Figure 23. Catalytic cycle for the oxidation of glucose by the GluOx and FePAA in the ISAM.
Both the FePAA and the GluOx are confined at the interface within the ISAM matrix, while the glucose is present in the adjacent aqueous phase. During the catalytic cycle, solution-phase glucose is oxidized by GluOx(FAD), producing glucono-d-lactone and GluOx(FADH$_2$). GluOx(FADH$_2$) is subsequently re-oxidized by the Fe$^{+3}$ in the FePAA, generating Fe$^{+2}$ in the polymer. Finally, the Fe$^{+2}$ is oxidized back to Fe$^{+3}$ by the heterogeneous reaction. The observed current is due to the oxidation of the Fe$^{+2}$ and is proportional to the glucose concentration by the mediated electron transport process.

Once it was established that the 3-mercapto-1-propane-sulfonate/FePAA/GluOx ISAM will oxidize glucose, two objectives were set. First several bilayers were made to determine if additional bilayers of FePAA/GluOx added linearly to the interfacial assembly. A second objective was to determine if the addition of multiple GluOx layers to the ISAM would increase the steady state current of the ISAM to glucose in the solution. It is known that the maximum steady state current the presence of glucose is directly related to the enzyme concentration in the ISAM.$^9, 13, 42, 84, 92$ Therefore, it is expected that as the enzyme concentration in the ISAM increased, the maximum steady state current for each bilayer would also increase.

Multiple FePAA/GluOx bilayers were made and the amount of FePAA on the electrode was determined after each addition using cyclic voltammetry. By integrating under the Fe$^{+2}$ oxidative curve the charge could be determined and therefore the FePAA amount on the surface. Figure 24 shows the results.
As is shown in Figure 24, the layers do add in a linear fashion, indicating that electrostatic assembly is occurring in a regular fashion as was expected. This linear assembly was shown in the FePAA/poly(styrenesulfonate) and has been shown in other ISAM systems.\textsuperscript{13,90,91}

The previous system of FePAA/poly(styrenesulfonate) ISAMs showed increasing integrated charge with increasing number of bilayers through the 7\textsuperscript{th} bilayer. The FePAA/GluOx system shows increasing integrated charge per area with increasing bilayers until the 3\textsuperscript{rd} bilayer when the integrated charge starts to level off. This means that past the 3\textsuperscript{rd} layer, these additional layers are not contributing to the total signal. This result is different than the FePAA/poly(styrenesulfonate) system where the FePAA could
be oxidized by the heterogeneous reaction out to the 7th bilayer. A large difference between the FePAA/poly(styrenesulfonate) ISAMs and the FePAA/GluOx system is the size of the GluOx molecule. GluOx is thought to have a slightly larger hydrodynamic diameter, 86 Å,\textsuperscript{22} than poly(styrenesulfonate), and therefore will take up a larger part of the film. With each addition of a FePAA/GluOx layer, the FePAA is farther away from the surface. Caruso et al. measured the thickness of an individual poly(allylamine) layer, to be 5 Å. Using that thicknesses to calculate the dimensions of three FePAA/GluOx bilayers, the film thickness will be approximately 273 Å.\textsuperscript{22, 82} Comparing that to the 276 Å for 7 bilayers of poly(allylamine)/poly(styrenesulfonate), measured by Caruso et al., helps explain why FePAA/GluOx bilayers are similar in thickness after 3 bilayers to 7 bilayers of FePAA/poly(styrenesulfonate) ISAMs.\textsuperscript{82}

The second objective for the FePAA/GluOx ISAMs was to determine if increasing the number of bilayers would result in a linear increase in the maximum steady state current. The expectation was that it would, as the maximum steady state current and total enzyme concentration in the ISAM are proportional. The steady state currents for the multiple bilayers were measured in the presence of constant glucose concentration and these are shown Figure 25.

For increasing number of bilayers the maximum steady state current increases in the presence of constant glucose concentration. Beginning with 4 bilayers, however, the steady state current decreases with each subsequent bilayer added to the assembly. The reason for this decrease is that even though there is more GluOx in the ISAM, the GluOx
cannot be regenerated by the Fe$^{+2}$/Fe$^{+3}$ heterogeneous reaction. It has been shown that increasing the number of bilayers beyond 3rd produced assemblies in which the outer portion is too far away from the surface to be regenerated. For this reason it is not unreasonable that the glucose oxidation is not efficient when using the catalytic cycle with these outermost layers. The glucose is able to be oxidized by the GluOx, but if mediated electron transfer does not occur through all of the inner layers, the outer GluOx will not contribute to a larger steady state signal proportional to the amount of GluOx in the ISAM. This is most likely what is happening in the 4th and 5th bilayers. The outermost GluOx is not being regenerated to make the steady state signal larger than the 3rd bilayer as would be expected. In fact, the 5th bilayer is characterized by a decrease in the steady state current, indicating that this layer is much too far away for the mediated electron transport to be efficient.

![Graph](image)

**Figure 25.** Steady state glucose oxidation by 1, 2, 3, 4 or 5 bilayers on 2 mm FePAA/GluOx modified gold electrodes. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl, 4 mM glucose.
4.4 Limit of Detection and Limit of Linearity for the GluOx ISAMs

Studies were then done on the FePAA/GluOx ISAM to determine the limit of detection for glucose using 1, 2 and 3 FePAA/GluOx bilayers. The limit of detection is a good method to compare these FePAA/GluOx ISAMs to other GluOx biosensors. As demonstrated in the previous section, using more than 3 bilayers does not improve the sensitivity of the measurement, as was illustrated from the steady state experiments. Three and four bilayers have the same steady state response to glucose concentrations, even though four bilayers FePAA/GluOx ISAM has a greater amount of GluOx in the ISAM.

Figure 23 shows a plot of the limiting current for 1, 2 and 3 bilayers for different concentrations of glucose. From this plot the limits of detection (LOD) are determined.
Figure 26. Calibration curves for detection of glucose using 1, 2 or 3 bilayers. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

From Figure 26, normalizing by the area of the electrode surface, it was determined that the LOD for the 2 mm gold electrode ISAMs is 30 µM for 1 bilayer, 10 µM for 2 bilayers and 10 µM for 3 bilayers (n = 6). It is expected that the LOD will decrease with increasing enzyme concentration, indicating that the biosensor is becoming more sensitive for glucose. LOD for glucose biosensors are well established in the literature. They range from 80 µM to 0.5 µM. The LOD determined for the FePAA/GluOx ISAM compares well with the literature LOD. This result illustrates that the ISAM assemblies are an effective method for detecting glucose and are comparable to other glucose detection methods.

The limit of linearity (LOL) was also determined. From Figure 26, the limit of linearity (LOL) for all bilayers is shown to be around 2 mM (n = 6). LOL has also been
investigated by other groups and ranges from only 300 µM up to 30 mM. For practical commercial use, the LOL should be linear up to 15 mM, which is the glucose concentration in the body. These FePAA/GluOx ISAMs, however, do fall within the literature LOL indicating that they are efficiently oxidizing glucose with a linear steady state response with increasing glucose concentrations. These ISAMs, however, are not practical for in vivo glucose detection.

4.5 Evaluating the Enzyme Catalysis for the GluOx ISAMs

Once it was determined that FePAA-GluOx ISAMs were successful at oxidizing glucose in solution and increasing enzyme load shows an increase in maximum steady state current, a further investigation into the enzymatic catalytic cycle was undertaken. During the enzyme catalytic cycle, shown in Figure 23, oxidation of the glucose out in solution is controlled by the kinetics of the redox mediation within the ISAM. Bartlett and Pratt have extensively studied this system and have shown that this type of ISAM will have kinetics that can be modeled by Case I and V. In these cases, the mediator and enzyme are at constant amounts and it is expected that for increasing GluOx concentration, the catalytic current will increase. To calculate this catalytic current and the Michaelis-Menten constant for the overall biosensor, Hanes-Woolf plots were constructed. The apparent Michaelis-Menten constant (K$_m'$) gives an estimate of the biosensor affinity for the glucose and will be compared to other GluOx biosensors and to the K$_m$ of unbound GluOx. K$_m'$ is not an intrinsic property of the enzyme but of the system.
Hanes-Woolf plots determines the Michaelis-Menten constant ($K_{m}'$) and the maximum catalytic current ($I_{cat}$) for the different bilayers.$^{59,92}$ Figure 27 is an example of a Hanes-Woolf plot for 1, 2 and 3 bilayers.

![Hanes-Woolf plot for 1, 2 and 3 bilayers](image)

**Figure 27.** Hanes-Woolf plots determining $K_{m}'$ and $I_{cat}$ for 1, 2 or 3 bilayers 2 mm FePAA/GluOx modified 2 mm gold electrodes. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

In a Hanes-Woolf plot, $I_{cat}$ is the inverse of the slope and the x-intercept is the apparent Michaelis-Menten constant, $K_{m}'$. Since the GluOx is bound within a matrix and not in a free solution, the apparent Michaelis-Menten constant, $K_{m}'$ is reported. From the Hanes-Woolf plot, it was determined that the $K_{m}'$ for the 2 mm modified gold electrodes was 0.21 (± 0.04) mM ($n = 6$). Comparing the Michaelis-Menten constant of GluOx in solution, 25 mM,$^{9,13}$ to the $K_{m}'$ of the ISAM assemblies on the 2 mm gold electrodes (0.21 (± 0.04) mM), the lower $K_{m}'$ shows that the ISAM GluOx biosensor is better at oxidizing the glucose. This comes from classic Michaelis-Menten kinetics where the faster the overall forward reaction, the lower the Michaelis-Menten constant. The $K_{m}'$ for
all the FePAA/GluOx ISAMs are close to other reported $K_m$’s for GluOx biosensors.$^{41, 42}$ This result shows that using electrostatic assembly for biosensor preparation can be used for glucose detection.

$I_{\text{catalytic current}} (I_{\text{cat}})$ is a measure of the dependence of the catalytic current with substrate concentration.$^{13, 41}$ For this system, the $I_{\text{cat}}$ for 1, 2 and 3 bilayers was 0.125 $\mu$A, 0.25 $\mu$A and 0.375 $\mu$A ($n = 6$). The $I_{\text{cat}}$ will increase with increasing number of bilayers as it is directly related to the concentration of enzyme in the ISAM $^{9, 41, 84, 87, 100, 101}$ These values are approximately 100 times lower than for other reported systems, but in line for having a small $K_m$. $^{41, 42, 88}$ This result demonstrates that each additional layer of enzyme is making good contact with the layers below to increase the catalytic current.

### 4.6 Flow – injection Experiments for the GluOx ISAMs

After charactering the ISAM modified 2 mm gold electrode, flow-injection experiments were performed to characterize the response of the detection system to transient analyte concentration. These kinds of experiments are ideal to determine the ability of the electrode to detect glucose under sheer stress conditions. For these experiments the ISAM modified 2 mm gold electrode was used in a flow cell that passed glucose over the ISAM modified surface. The electrode was held at a constant potential beyond the oxidation potential of the FePAA. This potential was chosen to ensure mediated electron transport would occur. Injections of different glucose concentrations were introduced into the flowing system as 20 $\mu$L plugs. One bilayer was constructed and the electrode was inserted into the flow cell. One bilayer was chosen as the subject for the flow-
injection experiments as it is a better comparison to other flow-injection experiments in the literature. The glucose was injected into the capillary and the glucose emerges at the ISAM modified electrode 2-3 minutes later using a flow rate of 0.5 mL/min. The amounts of glucose that were introduced are: 20 µmole, 10 µmole, 5.0 µmole and 2.5 µmole. Figure 28 shows the current response to these amounts of glucose.

![Figure 28](image_url)

**Figure 28. Flow injection peaks for 1 bilayer FePAA/GluOx ISAM on 2 mm gold electrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.600 V vs. Ag/AgCl, 0.5 mL/min, 20 µL injections.**

All of the current responses are indistinguishable. This result was obtained with all of the trials using an ISAM modified 2 mm gold electrode as the detection system in the flow-injection experiments. The amount of glucose detected reported for this kind of flow-injection experiment with other glucose sensors are in the range of 0.1 - 5 µmole. The amounts of glucose introduced were chosen to be in this range of detection, but the ISAM modified electrode was unable to distinguish between any of the amounts injected.
The FePAA/GluOx ISAMs electrode was also not stable over long amounts of time in the flow-injection experiments. This was determined by continually introducing different amounts of glucose and monitoring the current response. After 30-45 minutes had passed, there no longer was a current response to the glucose. Following this, the electrode would be placed into a static solution and the response to glucose using cyclic voltammetry determined. For some electrodes, there was evidence that FePAA was still present on the surface, but the modified electrode had no enzymatic activity. For the majority of electrodes tested there was no evidence that FePAA was still attached to the electrode following long exposure to applied potential and sheer flow. An example of the cyclic voltammogram taken before and after the flow-injection experiments is shown in Figure 29.

![Figure 29. FePAA/GluOx modified 2 mm gold electrode a) before and b) after flow-injection experiment. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.](image_url)
Shown in Figure 29 is a cyclic voltammogram of a modified FePAA/GluOx electrode before the flow-injection experiment and then the same electrode after the flow-injection experiment. The electrode does not have any oxidative or reductive peaks indicating that there is not a measurable amount of polymer remaining on the surface. This would seem to indicate that the ISAM structure is not stable in the shear flow and is falling apart under the stress.

Since these flow-injection experiments hold the ISAMs at a constant potential for long periods, a constant potential experiment was performed to determine if the prolonged oxidation-reduction cycles in the ISAM was decreasing the FePAA redox charge in the ISAM. One bilayer of FePAA was constructed and placed in a static solution. The ISAM would be held at a constant potential beyond the oxidation potential of the FePAA for increasing increments of time. This would ensure that the mediator would constantly be in similar conditions to the flow-injection experiments. After the constant potential had been applied for the predetermined time, a cyclic voltammetry scan was applied and the relative amount of FePAA still on the surface was determined. The results of this experiment are show in Figure 30.
As is illustrated in Figure 30, after several minutes of being held at a constant potential approximately 85% of the film is still intact. These films are also still able to oxidize glucose. This result indicates holding the ISAM at a constant potential does not cause the total loss of sensitivity to glucose. This result suggests that the stress from the sheer flow is the cause of the loss of polymer assembly from the electrode.

4.7 Summary

GluOx ISAMs were assembled and characterized on 2 mm gold electrodes. The purpose behind choosing GluOx biosensors was to determine if ionic-self-assembly could be used to make enzymatic biosensors and to determine their stability under sheer stress. Cyclic voltammetry experiments demonstrated the FePAA/GluOx ISAMs ability to detect glucose in solution. The steady state curve was characteristic of the detection of glucose.
Additional bilayers of FePAA/GluOx were assembled and electrochemically characterized. These layers were shown to assemble in a linear fashion, demonstrating the orderly way in that ionic-self-assembly occurs. The FePAA/GluOx bilayers showed an increase in integrated charge until the third bilayer, after which the integrated charge of the subsequent bilayers leveled off. This indicated that the FePAA in these outer layers is probably too far away from the surface to be regenerated by the heterogeneous reaction at the surface.

The LOD and LOL were determined for the first three bilayers, once it was determined that these were the analytically most sensitive from the steady state data. The LOD decreases with increasing layers as expected and compares well to other glucose biosensors. The LOL for all layers was shown to be within the literature LOL indicating that these FePAA/GluOx ISAMs are efficiently oxidizing glucose.

Once it was determined that FePAA-GluOx ISAMs were successful at oxidizing glucose in solution and increasing enzyme load shows an increase in maximum steady state current, the $K_m'$ and $I_{cat}$ were determined. The $K_m'$ is a measure of the affinity of the biosensor for the glucose. The lower $K_m'$ compared to literature values means that the ISAM GluOx biosensor is better at oxidizing the glucose. $I_{cat}$ was also determined and $I_{cat}$ was shown to increase with increasing layers as was expected. Although $I_{cat}$ was smaller than literature, it is in line with having a small $K_m'$. These kinetic parameters characterize the GluOx biosensor as a good sensor for detection of glucose.
These favorable results in the building of the GluOx ISAM led to the testing of the ISAM in the flow-injection system. This experiment though was unable to show any differentiation between the amounts of glucose injected. It was also noted that the ISAM lost sensitivity to the glucose injections over the time. A subsequent study was performed to determine if holding the ISAM at a constant potential was causing the loss in enzymatic activity at the ISAM interface. This experiment showed that approximately 85% of the FePAA was still on the surface after the experiment and enzymatic activity was still there. It would seem to be that the sheer stress of being in the flow-injection experiment is the larger problem in this experiment.

This chapter has shown that FePAA/GluOx ISAMs can be constructed on a 2 mm gold electrode. In order to be able to use them effectively in flow-injection experiments at the end of a capillary, a microelectrode would be much more useful. The next chapter will discuss assembling and characterization of the FePAA/GluOx ISAMs on microelectrodes.
Chapter 5: Characterization of an Electrostatically Assembled Glucose Biosensor on a Gold Fiber Microelectrode

5.1 Introduction

FePAA/GluOx ISAMs have now been assembled onto 2 mm gold electrodes. They are not, however, as stable in flow-injection experiments as desired. It is thought that using a microelectrode with FePAA/GluOx ISAM bilayers would provide advantages that the 2 mm gold electrode could not.

The microelectrode (diameter of less than 100 µm) has several advantages over a 2 mm gold electrode. The most important advantage to this system is the small size so the microelectrode will be able to fit into the end of the capillary for flow-injection experiments. This should help with the ISAM stability, as the laminar flow inside the capillary should be less turbulent than the flow inside the 2 mm flow cell. Additionally, microelectrodes have a smaller surface area so that capacitive current is going to be smaller than the larger electrodes. The result is systems can be studied using faster scan rates without the capacitive current becoming large.

Microelectrodes do have the disadvantage of being more difficult to fabricate than other electrodes. The diameter of the gold wire for the microelectrode that was used for this study is 50 µm, the lengths varied. Since microelectrode gold fiber was a smaller diameter than the capillary used for the flow-injection experiments, they will be able to fit inside the capillary during the flow-injection measurements.
In this section, the development of a modified microelectrode with a FePAA/GluOx ISAM will be discussed. These ISAMs will be used to oxidize glucose in solution. The preparation and characterization, evaluating the homogeneous enzyme reaction and flow-injection experiments of microelectrodes will be presented.

5.2 Gold Fiber Microelectrodes

ISAMs formed from FePAA and GluOx have been shown to make bilayers that assemble in a linear fashion on a 2 mm gold electrode. Based on these results, an ISAM prepared from FePAA and glucose oxidase (GluOx) can be assembled on a microelectrode.

Microelectrodes were prepared as described in the experimental section. Since there is no difference between the types of surfaces being modified, it is expected that mediated electron transport will occur as well on the microelectrode surfaces as was found on the 2 mm gold electrodes. These electrodes should also be better in the flow-injection experiments as they will fit into the capillary and are expected to experience less sheer flow as a result. This is due to the difference between the types of flow cells used for the microelectrode and the 2 mm gold electrodes. The 2 mm gold electrode uses a large bore capillary that has random turbulent flow pushing the analyte through. The microelectrode, however, uses a small-bore capillary and as a result will have laminar flow. Laminar flow is much less turbulent and so should help keep the ISAM assembled on the microelectrode surface. These electrodes will also be the style of electrodes used when developing the glutamate oxidase biosensors.
5.3 Cyclic Voltammetry of GluOx ISAMs

Cyclic voltammetry was used to follow the assembly of the FePAA and GluOx bilayers and the oxidation of the glucose by the GluOx. A 3-mercaptop-1-propane-sulfonate/FePAA ISAM was made and characterized using cyclic voltammetry, as is seen in Figure 31. When GluOx is adsorbed to the FePAA the cyclic voltammogram does not change dramatically from that seen prior to the adsorption of the GluOx. This result is expected, and demonstrates that the redox center of GluOx is not accessible to the electrode. There again seems to be a decrease in the peak height of the Fe$^{+2}$ oxidation peak, but this is thought to be loss of the physisorbed polymer to the aqueous buffer and has been seen previously in work done by Calvo and in the 2 mm gold electrode.$^{13}$

![Figure 31. Cyclic voltammogram of a) 3-mercaptop-1-propane-sulfonate/FePAA gold fiber microelectrode. b) 3-mercaptop-1-propane-sulfonate/FePAA/GluOx gold fiber microelectrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.](image)
Glucose is added to the solution and steady state response is obtained, shown in Figure 32. This result indicates that the ISAM has been successfully been made. This result is comparable to the ISAM modified 2 mm gold electrode by taking the average steady state current for 1 bilayer at 4 mM and finding the steady state current density (µA/cm²). With the modified 2 mm gold electrodes it was determined that the steady state density for 1 bilayer was 5.8 (±1.3) µA/cm² \((n = 6)\). For the ISAM modified microelectrode it was determined that the steady state density for 1 bilayer was 12.7 (±7.0) µA/cm² \((n = 9)\). This can be compared to Hodak’s work where for 1 bilayer, the steady state density was 0.5 µA/cm².\textsuperscript{13} As the steady state current is a measure of the ability of the electrode to oxidize the glucose, it is reasonable that the microelectrode has the largest steady state density. The microelectrode has increased mass transport compared to the larger electrodes and will be able to oxidize more glucose per area than the other electrodes. The FePAA/GluOx ISAMs have a higher steady state current for both the microelectrode and the 2 mm gold electrode compared to Hodak’s work.\textsuperscript{13} This could be due to more oxidase on the surface or better contact to the FePAA on the layer below.
Figure 32. Cyclic voltammogram of a) 3-mercapto-1-propane-sulfonate/FePAA/GluOx gold fiber microelectrode and b) in the presence of 4 mM glucose. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

Once it was established that the 3-mercapto-1-propane-sulfonate/FePAA/GluOx ISAM can be used to monitor the oxidation of glucose, then several bilayers were made to compare to the ISAM modified 2 mm gold electrode. The bilayers were made and the amount of FePAA on the electrode was determined after each addition using cyclic voltammetry and determining the charge by integrating under the $\text{Fe}^{+2}$ oxidative curve and normalizing for the electrode area. This data is shown in Figure 33.
Figure 33. Effect of number of FePAA/GluOx bilayers vs. integrated charge per area for gold fiber microelectrodes. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

The integrated charge per area increases in a linear fashion, which indicates that the layers are forming in an organized, self-limiting fashion. This indicates that these ISAMs are forming in a similar fashion to the ISAM modified 2 mm gold electrodes. The integrated charge per area increases with increasing numbers of bilayers through the second bilayer, after which the integrated charge levels off. This result indicates that the FePAA cannot be oxidized by the heterogeneous reaction in the bilayers past the second bilayer. This is similar to what was shown in the ISAM modified 2 mm gold electrodes, indicating that the ISAM bilayers on the microelectrodes are formed in a similar fashion to the ISAMs formed on the 2 mm gold electrodes.
5.4 Limit of Detection and Limit of Linearity for the GluOx ISAMs

Once it was determined that multiple layers could be made, limits of detection (LOD) were determined for the individual bilayers. These values were calculated accounting for the area of the gold fiber. The LOD was calculated only for the first and second bilayers as additional bilayers did not demonstrate increased response to glucose. The LOD is expected to be lower for a microelectrode as they have the advantage of enhanced mass transport in the absence of convection. The LOD was determined to be 1.0 µM for 1 ISAM and 0.7 µM for 2 ISAMs (n = 9). These LOD are much lower than the ISAM modified 2 mm gold electrode, indicating that the ISAM modified microelectrode is successful in detecting lower amounts. On the other hand, this result seems to indicate that there is not much enhanced sensitivity between the first and second layers even though there is additional FePAA on the surface of the ISAM. This is very different from the ISAM modified 2 mm gold electrode results.

The limit of linearity (LOL) for the 1 and 2 bilayer modified ISAM microelectrode was also determined from Figure 34. It was shown to be only 0.7 mM for both the layers (n = 9). This is much lower range than the ISAM modified 2 mm gold electrodes, and the reported literature values. This indicates that the microelectrode is getting saturated with glucose at a very low concentration of glucose compared to the ISAM modified 2 mm gold electrode. This, however, may be due to the enhanced mass transport of glucose to the FePAA/GluOx microelectrodes, where the GluOx is becoming saturated at a lower concentration than expected. Nevertheless, this does demonstrate that the ISAM modified microelectrodes detect lower concentrations of glucose in
solution then the ISAM modified 2 mm gold electrodes, and lower than most of the literature values for glucose biosensors that are reported.\textsuperscript{37, 93, 94, 96, 97}

Figure 34. Calibration curves for detection of glucose using 1 bilayer FePAA/GluOx on a gold fiber microelectrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

5.5 Evaluating the Enzyme Catalysis for the GluOx ISAMs

Once it was determined that FePAA/GluOx is able to oxidize glucose in solution and that increasing the number of bilayers does show some increase in the sensitivity of the biosensor, a subsequent study of the enzymatic catalysis was undertaken. The catalytic current and Michaelis-Menten constant were determined from Hanes-Woolf plots. It is expected that the catalytic current will increase with increasing number of bilayers, although the catalytic current will be smaller than that found for the ISAM modified 2 mm gold electrode. The Michaelis-Menten constant most likely will be smaller than found for freely diffusing GluOx; yet, should be the same as found for the ISAM
modified 2 mm gold electrode as the ISAMs should have the same affinity for the glucose in solution. Figure 35 is an example of a Hanes-Woolf plot for 1 and 2 bilayers.

![Hanes-Woolf plot](image)

**Figure 35.** Hanes-Woolf plots determining $K_m'$ and $I_{cat}$ for 1 and 2 bilayers of FePAA/GluOx on a gold fiber microelectrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

The Hanes-Woolf plots can be used to determine the $K_m'$ and $I_{cat}$. From the Hanes-Woolf plots, it was found that the $K_m'$ for the ISAM modified microelectrode system was 0.25 (± 0.15) mM ($n = 9$). Solution state $K_m$ is 25 mM,\textsuperscript{13} so this is 100 times below the freely diffusing GluOx, indicating that the ISAM biosensor is making the oxidation of glucose very favorable. This $K_m'$ is also very close to what was observed for the ISAM modified 2 mm gold electrode (0.21 (± 0.04) mM).\textsuperscript{102-104} It is expected that the biosensors will behave similarly as they are assembled in the same way and the $K_m'$ demonstrates this.
The $I_{cat}$ was also determined for the microelectrode GluOx biosensors. It is expected that the $I_{cat}$ is going to increase with increasing number of bilayers as it is directly related to the enzyme concentration in the ISAM.\textsuperscript{9, 41, 84, 87, 100, 101} For this system, the $I_{cat}$ for 1 and 2 bilayers was $0.20 \, \mu A$ and $0.29 \, \mu A$ ($n = 9$). These values are almost identical, and the first and second bilayers do not show the increased response that would be expected. This value is in line with the results shown in the LOD results where there was little change between the first and second layer. These $I_{cat}$ values demonstrate that the first bilayer of enzyme is experiencing efficient heterogeneous kinetics by the FePAA, however the second bilayer does not seem to be experiencing the same efficient electron transfer. This is evidenced by the $I_{cat}$ is lower than expected, it should be doubling in value as the GluOx has doubled in concentration, as was observed for the ISAM modified 2 mm gold electrode. The $I_{cat}$ values, in general, are approximately 100 times lower than for other reported systems, but in line for having a small $K_m'$ and very similar to what was shown in the 2 mm gold electrode.\textsuperscript{41, 42, 88} These $I_{cat}$ and $K_m'$ results further demonstrates that the ISAM modified 2 mm gold electrode and ISAM modified microelectrodes are being assembled in a similar fashion.

### 5.6 Flow – injection Experiments for the GluOx ISAMs

The purpose of constructing the FePAA/GluOx microelectrode was to use it in the flow-injection system. The microelectrode should be able to detect lower concentrations than the 2 mm gold electrode as microelectrodes gains sensitivity from the high mass transport to the surface of the electrode, even in the absence of convection.\textsuperscript{58} So when there is convection, the amounts that the GluOx modified microelectrode should be even lower.\textsuperscript{58}
The size of the microelectrode is also very advantageous to these flow-injection experiments as the electrode can sit right inside the end of capillary. This should also help in being able to detect lower amount of glucose as less of the glucose plug will bypass the electrode on the way out of the capillary. Sitting in the end of the capillary should also help with the ISAM remaining attached to the surface of the electrode, as the laminar flow more ordered than the turbulent flow experienced by the ISAM modified 2 mm gold electrode.

For these experiments the ISAM modified microelectrode was placed at the opening of a 100 µm capillary. The electrode was held at a constant potential beyond the oxidation potential of the FePAA. This potential was chosen to ensure mediated electron transport would occur. Injections of different glucose concentrations were introduced onto the capillary as 20 µL plugs. One bilayer was constructed and the electrode was inserted into the end of the capillary. One bilayer was chosen as the subject for the flow-injection experiments as it is a better comparison to other flow-injection experiments in the literature and to the ISAM modified 2 mm gold electrode. The glucose was injected into the capillary and the glucose sample emerged at the ISAM modified microelectrode approximately 2-3 minutes later. Figure 36 is the resulting current-time recording.
As is seen in Figure 36 the current responses for the different amounts of glucose were impossible to differentiate. This is similar to what was observed with the ISAM modified 2 mm gold electrode. The amounts detected were not as low as could were detected in a static system. This was an unexpected result. Microelectrodes have several advantages for this type of experiment; however, these flow-injection experiments did not demonstrate these advantages, as the amounts detected were indistinguishable.

In addition, as with the 2 mm gold electrode, the electrode was unable to withstand the sheer stress of the flow of the mobile phase. After 15-20 minutes had passed, there was no longer a current response to the glucose introduced to the capillary. These ISAM modified electrodes were no longer detecting glucose. To determine if the enzyme or the polymer had been removed, the electrode was placed back into a static system for testing.
For all electrodes tested there was no response to glucose, indicating that there was no GluOx on the surface. Additionally, for most electrodes there was no FePAA signal, meaning that the ISAM had been removed from the surface. This is shown in Figure 37.

Figure 37. FePAA/GluOx modified golf fiber electrode a) before and b) after flow-injection experiment. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

As is illustrated in the cyclic voltammograms, the ISAM before the flow-injection experiment has FePAA on the surface, while after the constant potential experiment, there is no oxidative or reductive signal characteristic of the FePAA. This result indicates that the ISAM has been stripped from the surface. The pure ISAM seems to be unable to withstand the sheer stress of the flowing system even when placed in the laminar flow-injection experiment. This would seem to show that purely ionically self-assembled monolayers are not the optimal system for these kinds of tests.
5.7 Summary

Microelectrodes were modified with FePAA and GluOx and characterized using cyclic voltammetry. This system was shown to be able to oxidize glucose in solution as evidenced by the steady state curve produced in the presence of glucose. This was evidence that the ISAM was being formed on the surface and that mediated electron transport was taking place successfully in the film.

Once one bilayer was assembled, multiple bilayers were assembled to determine if they would add in a linear fashion, and if adding additional layers would increase sensitivity to glucose in solution. It appears that a linear increase of the integrated charge (calculated per unit area of the electrode) occurs, demonstrating that these ISAMs form in a similar way that they do on the ISAM modified 2 mm gold electrode, which was expected. The linear increase in integrated charge per unit area increased for the first 2 bilayers, after which it leveled off. This phenomenon is similar to the formation of the FePAA/GluOx ISAMs on the ISAM modified 2 mm gold electrodes, providing additional evidence that the electrostatic assembly is occurring in a similar fashion. The steady state density was also shown to be similar between the ISAM modified 2 mm gold electrode and the modified microelectrode. The higher density compared to Hodak’s work is likely due to a higher enzyme concentration in the first bilayer.13

Once it was determined that bilayers could be made, the LOD and LOL were calculated to compare to other glucose biosensors. The LOD is expected to be lower for a microelectrode, which it is. It was shown, however, that there is not much of a difference
between the LOD of the two layers. The LOL was also determined to be lower than found with the ISAM modified 2 mm gold electrode, indicating that the ISAM modified microelectrode is becoming saturated especially at higher concentration ranges.

To get additional information about the similarities between the ISAM modified 2 mm gold electrode and the ISAM modified microelectrode, the $K_{m}'$ and $I_{\text{cat}}$ were investigated. Using Hanes-Woolf plots, the $K_{m}'$ calculated for the FePAA/GluOx microelectrode and shown to be very close to the $K_{m}'$ of the FePAA/GluOx 2 mm gold electrode. This is expected as the type of electrode modification is identical, so the affinity of the biosensor for the glucose should be the same. $I_{\text{cat}}$ was also calculated and was expected to increase with increasing number of bilayers. It was shown that while similar to the behavior of ISAM modified 2 mm gold electrode, the first and second bilayer did not have much difference in the $I_{\text{cat}}$. The same was observed for the LOD, indicating that the 2nd bilayer was not adsorbing as much GluOx as possible. This is most likely due to the irreproducibility inherent in the microelectrode experiments.

Finally the flow-injection experiments were carried out and it was expected that the microelectrodes would be able to detect lower amounts of glucose than the 2 mm gold electrodes, due to the enhanced mass transport from the convection. This was not the case as it was shown that the ISAM modified microelectrode was unable to distinguish any of the glucose amounts from each other. These ISAMs also fell apart under the sheer stress of the flow-injection experiments, much like the 2 mm modified gold electrodes. Clearly while the FePAA/GluOx ISAMs have many advantages, for the purpose of being
able to detect in a flow-inject experiment, electrostatically assembled electron transfer mediation is not going to work.

In the following section the development of a hybrid ISAM is going to be discussed. In these systems, a layer of FePAA is covalently bound to the thiol-underlayer through an amide bound. This should give the advantage of covalently coupling the mediator-containing polymer to the electrode and provide more control over the amount of polymer on the surface. The FePAA will still have amine sites available for ionic-self-assembly of the GluOx. These layers will be characterized to determine if mediated electron transfer will occur and if these layers will be more successful in flow-injection experiments.
Chapter 6: Design and Characterization of Covalently Modified ISAM Glucose Biosensor

6.1 Introduction

ISAM modified electrodes seem to be unable to withstand the turbulent flow of the flow-injection experiment. As a solution to this problem, a modified ISAM with covalent character was devised. The method developed to couple the FePAA to the thiol-underlayer will provide a simple and reproducible method to make a redox underlayer to which the GluOx can be ionically self-assembled. This method should also provide more control over the amount of polymer attached to the surface and eventually the amount of enzyme attached to the surface.

This section will describe the design and characterization of the covalently modified surface. These surfaces will then have GluOx ionically self-assembled to them and determine if mediated electron transport can still occur with these films. Once the ability to oxidize glucose is determined, then these films will be used in flow-injection experiments to determine if these are a better film testing in flowing systems.

6.2 Coupling of FePAA to Electrode Surface

To couple the FePAA to the surface of the SAM, in this case 11-mercaptoundecanoic acid, 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride was used. The proposed reaction is shown in Figure 38.
Figure 38. 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride reaction between 11-mercaptoundecanoic acid and FePAA.

The 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride is used as a coupling agent to promote the formation of the amide bonds between the acid and the amine. It is most commonly used for biological work using proteins and amino acids. The detailed reaction of 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride with 11-mercaptoundecanoic acid and FePAA is shown in Figure 39.

1) \[
R_1-N=C=N-R_2 + O=C-OH \xrightleftharpoons{H^+} H-N-R_1 \overset{R_3-C-O}{\rightarrow} C=N-R_2
\]

2) \[
R_3-C-O \xrightarrow{R_4-NH_2} \overset{R_3-C-N-R_4}{\rightarrow} + \overset{R_1-N-C-N-R_2}{\rightarrow} N
\]

Figure 39. Two step reaction of 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride reaction between 11-mercaptoundecanoic acid and FePAA.

1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride reacts with carboxylic acid group on 11-mercaptoundecanoic acid and activates the carboxyl group to form an
active $O$-acylisourea intermediate. This allows the intermediate to be coupled to the amino group on the FePAA. The by-product of the reaction is a soluble urea and the FePAA is bound to the 11-mercaptoundecanoic acid via a new amide bond. This reaction has been used to successfully couple amines to acid terminated monolayers by Anderson and Baltzersen. At pH 5 the remaining unreacted amine groups on the FePAA can then be used for ionic-self-assembly with GluOx.

6.2.1 Characterizing the Covalently Modified FePAA Film

To determine if the 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride reaction was taking place on the surface of the electrode, IR and XPS measurements were performed. Figure 40 shows the RAIRS spectrum of the 11-mercaptoundecanoic acid surface before and after the reaction.

![RAIRS spectrum](image)

**Figure 40.** RAIRS spectrum of a) 11-mercaptoundecanoic acid and b) covalently modified film.
In the spectrum, scissoring vibrations of the methylene are apparent (1464 cm$^{-1}$) as well as the more diagnostic carboxylic acid stretch (1740 cm$^{-1}$) and carboxylate stretch (1377 cm$^{-1}$) from the 11-mercaptoundecanoic acid. This carboxylate stretch indicates that at least some of the monolayer is in the deprotonated form. Once the 11-mercaptoundecanoic acid film is reacted with FePAA and the 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride, it is expected that the carboxylic acid peak will disappear and new amide and amine peaks will grow in. This is shown to be true in the RAIRS spectra in Figure 40. The carboxylic acid carbonyl peak at 1740 cm$^{-1}$ is no longer apparent while several new peaks have appeared. There is an amide carbonyl stretch (Amide I) at 1652 cm$^{-1}$ and an amine N-H bend at 1635 cm$^{-1}$. A protonated amine peak at 1540 cm$^{-1}$ and a small amide C-N stretch at 1397 cm$^{-1}$ is also apparent. This indicates that the reaction goes to completion.

The covalently modified surfaces were also characterized using XPS. The main differences that are expected are: shifts in the nitrogen peaks and additional peaks in the carbon region. The primary features of the XPS survey scan, seen in Figure 41, shows C (1s) at 285 - 289 eV, and N (1s) at 399.6 eV. For all covalently modified surfaces studied, the Fe (2p) was unable to be distinguished from the noise as there is not enough polymer on the surface to get a strong signal. There is also a very prevalent O (1s) peak at 532 eV, due to amide. There are several peaks due to the gold under layer, Au (4f 5/2) at 85, Au (4f 5/2) at 87 eV, Au (4d 5/2) at 335 eV, Au (4d 3/2) at 352 eV and Au (4p 3/2) at 547 eV.
A high resolution scan was obtained to determine if the different carbon peaks could be distinguished. This is shown in Figure 42. The C (1s) peak at 285.2 eV is characteristic of the backbone of the polymer and is a major peak of the survey scan. There is a secondary peak on the C (1s) at 288.8 eV which is due to the carboxylic carbon.\textsuperscript{108, 109}

The N (1s) peak is present at 399.6 eV, an energy characteristic of an amide.\textsuperscript{110} This is a different peak than what was observed in the FePAA ISAM surface, where the N (1s) peak was at 401.5 eV, indicative of a quaternary amine in the polymer backbone.\textsuperscript{77}

Here, the amine peak is impossible to distinguish apart from the noise. This was observed for all of the covalently modified samples studied. This is most likely due to the small number of amines available on the surface after the reaction.
6.2.2 Cyclic Voltammetry of the Covalently Modified FePAA Film

Cyclic voltammetry was used to determine if the covalently bound FePAA would influence the ability of the sensor to detect glucose. It was expected that GluOx would still be able to electrostatically bind to the FePAA surface and mediated electron transfer would still occur.

A cyclic voltammogram of the covalently modified FePAA 2 mm gold electrode is shown in Figure 43. Based on the XPS data, it was known that the FePAA was bound to
the surface, and from the cyclic voltammetry data the amount on the electrodes was
determined. The average amount of FePAA bound to the surface using the covalent
method was $2.6 \pm 0.5 \times 10^{-10}$ mol/cm$^2$, compared to $6.3 \pm 2.6 \times 10^{-10}$ mol/cm$^2$ using a
pure ISAM. The lower surface coverage for the covalently modified electrodes indicates
that, once one amine binds, FePAA must space itself out before binding again. This is
unlike in the electrostatic binding where only through space interactions are considered,
in the covalently modified surface, steric play a role in the reaction, and may lead to a
lower surface coverage.

The GluOx, which is in pH 5 buffer, is then adsorbed to the covalently bound FePAA
through electrostatic interactions. Upon addition of GluOx, the cyclic voltammogram
does not change dramatically from the cyclic voltammogram of the covalently bound
FePAA. This is to be expected since the redox center of the GluOx is not easily
oxidizable and has been seen previously.$^{13}$
There is a decrease in the oxidative Fe$^{+2}$ peak height from the polymer terminated layer and the oxidase terminated layer. This is most likely loss of any remaining unreacted physisorbed polymer into the aqueous solution.

Upon addition of glucose to the solution, a steady state response is observed, consistent with the catalytic oxidation of the glucose, shown in Figure 44. This seems to indicate that the GluOx has self-assembled to the remaining protonated amine sites on the FePAA and is able to oxidize the glucose in solution. The steady state current density for these modified electrodes is 13.9 (± 1.9) µA/cm$^2$ ($n = 3$). This is larger than the ISAM modified 2 mm gold electrodes, and nearly the same as observed for the ISAM modified microelectrodes. Even though the surface coverage is low, the covalently modified electrodes is much more efficient at the mediated electron transfer. There is no evidence
that there is more GluOx on the surface, but that the catalytic cycle is apparently much more efficient. This may be because the direct wiring of the polymer to the surface of the electrode improves the heterogeneous electron transfer kinetics to the Fe$^{+2}$.

![Cyclic voltammogram](image)

**Figure 44.** Cyclic voltammogram of a) covalent modification/GluOx 2 mm gold electrode and b) in the presence of 4 mM glucose. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

### 6.2.3 Limits of Detection and Limit of Linearity

The limit of detection was also determined for the covalently modified 2 mm gold electrodes once it was determined that mediated electron transport was taking place. It is expected that the LOD should be lower than the 2 mm gold electrode as the steady state current density seems to indicate that the electrode is very efficient at oxidizing the glucose in solution. The LOD was calculated taking into account the area of the electrode and determined to be 10 µM ($n = 3$). This is lower than the 1 bilayer LOD for the 2 mm gold electrode (30 µM). This lower LOD is expected with faster heterogeneous
kinetics on the covalently modified electrode which will allow faster oxidization of glucose and therefore a lower LOD.

The limit of linearity (LOL) for the covalently modified electrode was also determined from Figure 45. It was shown to be 1.5 mM, which is the same range as the ISAM modified 2 mm electrode \( (n = 3) \). This demonstrates that these electrodes will work well for flow-injection experiments over a wide range of concentrations.

![Figure 45. Calibration curve for detection of glucose using covalently modified ISAM on 2 mm gold electrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.](image)

6.2.4 Evaluating the Enzyme Catalysis of the Covalently Modified ISAM Biosensor

The covalently modified electrodes have been shown to be able to oxidize glucose as well as the ionically self-assembled monolayers. To determine how the enzyme catalysis in the film were influenced by the covalent coupling, the \( K_m' \) and the \( I_{cat} \) of the covalently
modified system were determined. Figure 46 is an example of a Hanes-Woolf plot for the covalently modified biosensor.

![Hanes-Woolf plot](image)

**Figure 46.** Hanes-Woolf plots determining $K_m'$ and $I_{cat}$ for covalently modified ISAM on 2 mm gold electrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

From the Hanes-Woolf plot, it was found that the $K_m'$ for the system was 0.47 (± 0.55) mM ($n = 3$). This is close, including the error, to the $K_m'$ of the ISAM modified 2 mm gold electrode. This again indicates that covalently modifying the FePAA to the surface of the electrode has not changed the ability of the GluOx to oxidize the glucose in solution. The large error associated with this measurement is due to the small sample size, only 3 samples. With more trials, the error is expected to decrease.

For this system, the $I_{cat}$ was 0.20 (± 0.17) µA, ($n = 3$). This favorably compares with the $I_{cat}$ of the ISAM modified 2 mm gold electrode, indicating that covalently binding the FePAA to the surface does not negatively affect the ability of the GluOx to oxidize the
glucose. Since $I_{\text{cat}}$ is a measure of the enzyme concentration in the bilayer, having a similar $I_{\text{cat}}$ to the 1 bilayer 2 mm gold modified electrode indicates that both theses systems have a similar amount of GluOx on the surface of the electrode.

### 6.2.5 Flow – Injection Experiments of the Covalently Modified ISAM biosensor

The covalently modified electrodes show similar ability to oxidize glucose in solution and are just as simple to assemble. The homogeneous reaction of the enzyme was also very close to that of the FePAA/GluOx modified 2 mm gold electrode, so this system seems to be ideal to replace the pure ISAM for the flow-injection experiments.

Since these electrodes were designed to be more robust, the covalently modified electrodes were tested in the flow-injection system. For these experiments, the 2 mm gold electrode was covalently modified and then GluOx was ionically adsorbed to the surface. This electrode was then used in a flow cell with different concentrations of glucose introduced. The amounts of glucose that were detected were: 2.0 μmole, 1.0 μmole, 0.5 μmole, 0.25 μmole, 0.12 μmole, and 0.032 μmole. The current response is shown in Figure 47.
The different amounts of glucose are distinguishable in these measurements, and much lower amounts of glucose were detected than was found when using the pure ISAM in the flow-injection system. This result was consistent for all trials when using the covalently modified electrode in the flow-injection measurement. As is shown in Figure 48, 2.0 μmole of glucose is beginning to reach the limit of linearity for this type of electrode in the flowing system, indicated by the leveling off of the integrated peak areas, but the biosensor had not reached its saturation point of glucose detection.
The electrode modification was also stable over a much longer period than the pure ISAM modified surface. A covalently modified electrode was able to detect in a flowing system five times longer than a pure ISAM, based on the time that the flow-injection experiments lasted. This result indicates that the covalently modified electrode works well for stabilizing the FePAA/GluOx assembly to the electrode in a sheer flow while not affecting the ability of the GluOx to interact with the polymer or the glucose. Additional experiments need to be done to determine the lifetime of these covalently modified electrodes.

To determine if the constant potential affected the covalently modified ISAMs in the same way that it affected the pure ISAMs, the electrodes was held at a constant potential for a period of time. This will help determine if just the sheer flow of the flow-injection
experiment is the cause of the degradation of the pure ISAMs in the flow-injection experiments. A covalently modified electrode was constructed and placed in a static solution. The covalently modified electrode was held at a constant potential beyond the oxidation potential of the FePAA for increasing increments of time. This would ensure that the mediator would be in similar conditions to the flow-injection experiments. After the constant potential had been applied for the predetermined time, a cyclic voltammetry measurement was conducted and the relative amount of FePAA still on the surface determined. The results of this experiment are show in Figure 49.

Figure 49. Degradation of covalently modified FePAA/GluOx 2 mm gold electrode held at constant potential. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.06 V vs. Ag/AgCl.

As is illustrated in Figure 49, after several minutes of being held at a constant potential over 85 % of the film is still intact. These films are also still able to oxidize glucose. This indicates that being held at a constant potential effects the FePAA in the covalently modified films in the same way as it does in the pure ISAM films. This result
demonstrates that the sheer stress of the flow-injection experiment that causes the ISAM to fall apart in those experiments.

6.3 Covalently Modified ISAM on a Gold Fiber Microelectrode

Using a microelectrode with a covalently modified surface was also tested to determine if the advantages demonstrated in the 2 mm covalently modified electrode could be scaled down to the microelectrode. The covalently modified microelectrode was assembled in the same way as the 2 mm gold electrode. 11-mercaptoundecanoic acid, 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride were reacted with FePAA to make a covalently modified layer on the gold surface. To this layer, GluOx was ionically-self-assembled. The microelectrodes were characterized with cyclic voltammetry to determine if the covalent modification and ionic-self-assembly had occurred on the surface.

6.3.1 Cyclic Voltammetry of the Covalently Modified FePAA Film on a Gold Fiber Microelectrode

Cyclic voltammetry was used to characterize the FePAA covalently bound to the surface of the electrode, as well as the oxidization of the glucose by GluOx. FePAA was covalently modified and the cyclic voltammogram of that surface is shown in Figure 50. When GluOx is adsorbed to the FePAA the cyclic voltammogram does not change dramatically from the cyclic voltammogram prior to the adsorption of the GluOx. This result is expected, and demonstrates that the redox center of GluOx is not accessible to the electrode. There is a decrease in the peak height of the Fe$^{3+}$ oxidative peak on the
polymer layer to the oxidative peak on the oxidase layer. Again, this is most likely from loss of the non-reacted physisorbed polymer to aqueous solution.

![Cyclic voltammogram](image)

**Figure 50. Cyclic voltammogram of a) covalent attachment gold fiber microelectrode. b) covalent attachment/GluOx gold fiber microelectrode.** 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

Glucose is added to the solution and a steady state response is observed, Figure 51. This result indicates that the GluOx has been successfully been electrostatically adsorbed.
Figure 51. Cyclic voltammogram of a) Covalent attachment/GluOx gold fiber microelectrode and b) in the presence of 4 mM glucose. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

6.3.2 Limits of Detection and Limit of Linearity

Once it was determined that covalently modified microelectrodes were able to detect glucose, the limit of detection and limit of linearity were determined. The LOD is expected to be lower for the covalently modified microelectrode than for the 2 mm covalently modified electrode. This was shown to be true, as the LOD, accounting for the area of the microelectrode, was 2.0 µM, lower than the covalently modified 2 mm gold electrode. The LOD is in the same range as the pure ISAM microelectrode. Any difference in the LOD between the covalently modified microelectrodes and the pure ISAM microelectrodes is most likely due to differences in the amount of GluOx assembled to the surface of the FePAA on the covalently modified microelectrodes. This is a reasonable assumption as there will not be as many amine sites for the GluOx to ionically self-assemble to.
The LOL was shown to be linear over the same range as the 2 mm covalently modified electrode which was expected as they are made in the same way, so they should detect glucose linearly over the same range.

![Figure 52](image_url)

**Figure 52.** Calibration curves for detection of glucose using Covalent attachment/GluOx gold fiber microelectrode. 0.01 M acetate buffer, 0.05 M KCl, pH 5, 0.005 V/s vs. Ag/AgCl.

### 6.3.3 Flow – Injection Experiments of the Covalently Modified ISAM biosensor on a Gold Fiber Microelectrode

The purpose of making the covalently modified microelectrode was to determine if it would have a better response to the glucose in the flow-injection experiments than the 2 mm covalently modified gold electrode. The microelectrode has the advantage of enhanced mass transport, so the thought that it would be able to detect lower amounts than the 2 mm covalently modified gold electrode.
For these experiments, the covalently modified microelectrode was used in the flow cell that placed the modified microelectrode in the end of the capillary. The electrode was held at a constant potential beyond the oxidation potential of the FePAA. This potential was chosen to ensure mediated electron transport would occur. Injections of different glucose concentrations were introduced onto the capillary as 20 µL plugs. The glucose was injected into the capillary and the elution time was approximately 2-3 minutes. Figure 53 is the resulting current time recording.

![Figure 53. Flow injection peaks for covalently modified ISAM on gold fiber microelectrode biosensor. 0.01 M acetate buffer, 0.14 M KCl, pH 5, 0.600 V vs. Ag/AgCl, 0.5 mL/min, 20 mL injections.](image)

As is seen in Figure 53, the amounts that the covalently modified microelectrode were able to distinguishable are: 2.0 µmole, 1.0 µmole 0.5 µmole and 0.25 µmole. These are smaller amounts than the pure ISAM microelectrode, however, these are not as low as the
2 mm covalently modified gold electrode. A possible reason for this is a low coverage of GluOx on these electrodes, which would explain why the LOD is higher than expected. Shown in Figure 54 the integrated peak areas are starting to level off at around 2.0 µmole indicating that this is the limit of linearity for this type of electrode.

![Figure 54](image.png)

**Figure 54.** Integrated peak areas of flow – injection peaks for gold fiber covalently modified microelectrode detecting glucose.

The covalently modified microelectrode was also able to withstand the sheer flow of the mobile phase, unlike the pure ISAM microelectrode counterpart. This shows again that using the covalent modification is a feasible way of immobilizing the redox polymer to the surface of the electrode.
6.4 Summary

Covalently modified electrodes were made to address the stability the ISAM modified electrodes under the sheer stress of the flow-injection experiments. The covalent reaction on the surface was designed to take place between 11-mercaptoundecanoic acid and FePAA in the presence of 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride. This reaction was shown to go to completion using RAIRS and XPS. The RAIRS data show the carboxylic acid on the 11-mercaptoundecanoic acid disappearing and the amide and amine peaks from the covalent product appearing. The XPS data confirmed this data by having O (1s) at 532 eV, N (1s) at 399.6 eV and C (1s) at 288 eV from the amide bond being formed. Cyclic voltammetry then used to determine the surface coverage of the FePAA. This was determined to be $2.6 \pm 0.5 \times 10^{-10}$ mol/cm$^2$. The surface coverage on the covalent surfaces is smaller than on the pure ISAM modified 2 mm gold electrodes, and this is most likely due to the steric hindrance when binding a polymer to the self-assembled layer preventing more FePAA from binding. Electrostatic assembly is not subject to the same limitation as they only use through space interactions that allow for a higher surface coverage. The steady state current density was also shown to be higher on a covalently modified surface than for a pure ISAM indicating that the heterogeneous electron transfer reaction at the surface was very efficient, which would be good for flow-injection experiments. While this is not a sign that there is more GluOx on there surface, this does not mean that the homogeneous reaction will not limit the reaction.
Once it was determined that the covalently modified electrode was able to oxidize glucose, the LOD and LOL were calculated. The LOD was shown to be lower than the 2 mm ISAM modified gold electrode. This indicates that the fast heterogeneous kinetics leads to very favorable glucose oxidation and a low LOD. The LOL was also shown to be the same for both the pure ISAM and covalently modified 2 mm gold electrodes.

It was expected that the $K_{m'}$ and the $I_{cat}$ would be lower for the covalently modified glucose biosensors, but with the small data set, this is hard to say for certain. The $K_{m'}$ being slightly higher than the pure ISAM modified 2 mm gold electrode, but with a large enough error to be considered equal. Since the $I_{cat}$ was close, this would indicate that the enzyme concentrations were similar, but with better heterogeneous kinetics, the covalently modified electrode should have a higher catalytic current for the same amount of enzyme. This is evidenced by the steady state current density.

Finally, the covalently modified 2 mm gold electrode was used in the flow-injection experiment. This glucose biosensor was shown to detect smaller amounts and be stable over a longer time period than the pure ISAM 2 mm gold electrode. The same constant potential experiments were performed to determine if prolonged oxidation-reduction cycles affected the covalent films in the same way as the pure ISAM films. It was determined that they did, so the ISAMs are falling apart during the flow-injection experiments because of the sheer stress, not because of the applied constant potential.
Microelectrodes were also prepared with covalent modification. It was thought that they would be able to perform better in the flow-injection experiments as microelectrodes benefit from enhanced sensitivity. Cyclic voltammetry experiments determined that mediated electron transport occurred and these covalently modified microelectrodes were able to detect glucose in solution. LOD and LOL were then calculated. It was determined that the LOD was lower for the covalently modified microelectrode as compared to the 2 mm covalently modified electrode, although it was not as low as a pure ISAM microelectrode. This is most likely due to not having as much GluOx on the surface of the electrode. The LOL was shown to be the same as for the covalently modified 2 mm gold electrode, which was expected. This demonstrates that these covalently modified electrodes will be suitable for detection in a flowing system over the range of concentrations used.

The covalently modified microelectrodes were then tested in the flowing system and demonstrated that they were able to withstand the sheer flow. While they did not detect concentrations lower than the 2 mm covalently modified gold electrode, which was unexpected, the covalently modified microelectrodes did detect much lower concentration than the pure ISAM modified microelectrodes. This seems to confirm that the covalent modified with the GluOx electrostatically assembled to the surface is the better than a pure ISAM electrode for detection of bioanalytes.
In the subsequent section, the development of the glutamate biosensor using a pure ISAM is discussed. This biosensor builds on the techniques developed in all the previous sections for the detection of glutamate.
Chapter 7: Assembly and Characterization of an electrostatically assembled glutamate biosensor

7.1 Introduction

Only a few methods for determining glutamate in analytical samples have been reported.\textsuperscript{49, 50, 55, 56} There are currently two methods for determining glutamate concentrations using electrochemistry. The first method is to detect the hydrogen peroxide produced from the reaction of glutamate oxidase in the presence of glutamate. This method is limited, however, because the oxidation of hydrogen peroxide requires a large positive overpotential. This is a problem when analyzing biological samples because of the interference from the oxidation of many other species in the sample that will occur at the large, positive overpotential.\textsuperscript{55, 56, 111, 112} The second most common method for glutamate detection is to trap hydroxyl radicals produced in the presence of excess glutamate. The excess glutamate initiates a production of reactive oxygen species, such as hydroxyl radicals. Salicylic acid can be used to react with these oxygen radical to form 2,3-dihydroxybenzoic acid.\textsuperscript{55, 111, 112} This product can then be quantified by either electrochemistry or by spectroscopy. While this is an effective measurement of glutamate concentrations, it is not a direct measurement of the glutamate in the system.

For a direct measurement of glutamate, mediated electron transport can be used. Since glutamate oxidase (GlutOx) is a FAD enzyme, it should be able to use the same mediation scheme that was used for GluOx. This application, however, has not been reported. To show that electrostatically assembled monolayers can be used for the detection of glutamate, an ISAM of FePAA/GlutOx was made and characterized.
7.2 Assembling the GlutOx ISAM Biosensor

The GlutOx containing ISAMs will be prepared as described in the experimental section. To transition from GluOx to GlutOx a change in the pH of the deposition solution must be made. The pI (isoelectric point) of GlutOx is 6.2, meaning that at pH 6.6 GlutOx has a net negative charge. Using the same method electrostatic assembly that has been previously used, at pH 6.6 GlutOx will electrostatically assemble onto the FePAA. There is some concern that FePAA will not be as protonated at this pH, meaning that the coverage of GlutOx will be lower than expected. The use of GlutOx in the ISAM should be able to use mediated electron transfer to oxidize the glutamate out in the solution, and catalytically regenerate the GlutOx and FePAA.

7.3 Cyclic Voltammetry of the GlutOx ISAMs

Cyclic voltammetry was used to follow the assembly of the FePAA and GlutOx. A 3-mercapto-1-propane-sulfonate/FePAA ISAM was made and characterized to determine the amount of polymer on the surface of the electrode. This cyclic voltammogram is shown in Figure 55. When the GlutOx is adsorbed to the FePAA the cyclic voltammogram shape did not significantly change and demonstrates that the FAD center of the GlutOx is not accessible to the electrode surface. This was an expected result as GlutOx is a FAD enzyme, and although the structure has not yet been determined, the FAD is probably buried in the center and inaccessible to oxidation by the electrode.
There also seems to be an apparent decrease in peak height of the Fe$^{2+}$-oxidation peak after the electrostatic deposition of the GlutOx. This again is most likely from loss of the polymer to the aqueous buffer. This is similar to what has been seen previously in the GluOx electrodes.

![Cyclic voltammogram](image)

**Figure 55.** Cyclic voltammogram of a) 3-mercapto-1-propane-sulfonate/FePAA gold fiber microelectrode. b) 3-mercapto-1-propane-sulfonate/FePAA/GlutOx gold fiber microelectrode. 0.01 M acetate buffer, 0.05 M KCl, pH 6.6, 0.1 V/s vs. Ag/AgCl.

When glutamate is added to the adjacent solution, an increased current response is observed, as is shown in Figure 56. A true steady state response is not observed and this is due to the fast scan rate that was chosen for these experiments. The faster scan rates were chosen to determine if these biosensors were going to be able to detect glutamate in flow-injection experiments. Microelectrodes should be in theory able to use faster scan rates and not have the analyte in the solution around the tip depleted. Using a faster scan rate will give an indication of how quickly the microelectrode will respond to transient...
concentration pulses of glutamate. At faster scan rates, however, the steady state was not achieved. Instead only an increase in the current was observed, suggesting that redox catalysis was occurring. This also indicates that the mediated electron transport is occurring, but the cyclic voltammetry experiment is occurring too quickly to let the heterogeneous reaction reoxidize the GlutOx in the film. Despite not being at steady state, the catalytic current is still greater than found with just the Fe$^{+2}$ oxidative peak in the absence of glutamate. The increase in the catalytic current is what will be used in the analysis of the FePAA/GlutOx ISAMs and the detection of glutamate.

**Figure 56.** Cyclic voltammogram of a) 3-mercapto-1-propanesulfonate/FePAA/GlutOx gold fiber microelectrode and b) in the presence of 4 mM glutamate. 0.01 M acetate buffer, 0.05 M KCl, pH 6.6, 0.1 V/s vs. Ag/AgCl.

Figure 57 illustrates the response trend found as the number of FePAA/GlutOx bilayers increase. The catalytic current increases with increasing GlutOx concentration in the bilayers. This is an expected result as the catalytic current is directly related to the GlutOx concentration in the bilayers. The catalytic current also seems to increase in a
fairly linear fashion, which would be expected for a linear assembly process like ionic-self-assembly. The same phenomenon was shown in the FePAA/GluOx ISAMs.

![Graph](image)

**Figure 57.** Steady state glutamate oxidation by 1, 2 or 3 bilayers of FePAA/GlutOx for gold fiber microelectrodes. 0.01 M acetate buffer, 0.05 M KCl, pH 6.6, 0.1 V/s vs. Ag/AgCl, 4 mM glutamate.

### 7.4 Limits of Detection and Limit of Linearity for the GlutOx ISAM

Once it was determined that the FePAA/GlutOx ISAM was responding to glutamate, the limits of detection (LOD) were determined. The LOD values were corrected for the area of the electrodes and the LOD were determined to be 30 μM, 2 μM and 0.3 μM for 1, 2 and 3 bilayers of FePAA/GlutOx respectively. This is on the order of the detection limits for the other glutamate biosensors in the literature. These are reported as being 0.5 and 3.9 μM.\(^{50, 114}\) These are lower than the corresponding GluOx ISAM modified microelectrode LOD values, and this may be due to the lower than expected GlutOx coverage.
The limit of linearity for the bilayers were also determined from Figure 58, and is found to be around 5 mM all of the bilayers. This is larger than for other GlutOx biosensors, indicating that this style of biosensor will be able to detect over glutamate a larger range of concentrations.\textsuperscript{50, 114} This again may be due to the lower than expected GlutOx coverage, as it will take more glutamate to saturate the enzyme at the surface.

![Figure 58](image.png)

**Figure 58.** Calibration curves for detection of glutamate using 1, 2 and 3 bilayers of FePAA/GlutOx for gold fiber microelectrodes. 0.01 M acetate buffer, 0.05 M KCl, pH 6.6, 0.1 V/s vs. Ag/AgCl.

7.5 Evaluating the Enzyme Catalysis for the GlutOx ISAMs

Since these biosensors are ultimately being designed to replace the current glutamate biosensors, it is important to determine how well the enzyme is functioning in the film. Hanes-Woolf plots were made for each of the bilayers and from these plots the K\textsubscript{m} and I\textsubscript{cat} were determined. The K\textsubscript{m} was determined to be 0.40 (± 0.13) mM. This value is compared to the K\textsubscript{m} for similar films, reported as between 3-7 mM.\textsuperscript{114} Compared to the
Kₘ of freely diffusing GlutOx in solution, as both 1.1 mM\textsuperscript{115} and 0.29 mM, the GlutOx in
the film is oxidizing the glutamate at a comparable rate to that reported for redox
catalysis systems that are freely diffusing.\textsuperscript{113}

The I\textsubscript{cat} for the increasing number of bilayers are 0.02 (± 0.01) µA, 0.04 (± 0.01) µA and
0.05 (± 0.02) µA are for 1, 2 and 3 bilayers respectively. This result makes sense as one
would expect that the catalytic current should increase as the enzyme concentration
increases. The error on these values, however, does not allow one to state with certainty
that these values are different from each other. There have not been any I\textsubscript{cat} reported for
other GlutOx sensors.

![](image)

**Figure 59.** Hanes-Woolf plots determining K\textsubscript{m}' and I\textsubscript{cat} for 1, 2 and 3 bilayers of
FePAA/GlutOx for gold fiber microelectrodes. 0.01 M acetate buffer, 0.05 M KCl,
pH 6.6, 0.1V/s vs. Ag/AgCl.
7.6 Summary

FePAA/GlutOx ISAMs were assembled as an alternative method to using salicylic acid or oxidizing hydrogen peroxide to detect glutamate. These films were shown to use mediated electron transfer much like the FePAA/GluOx films and detect glutamate in solution. It was expected that GlutOx would be able to be mediated by FePAA as GlutOx is a FAD enzyme. This was shown to be true and glutamate was able to be detected in solution.

The detection of glutamate was performed using faster scan rates than had been done previously, as fast-scan experiments are a more realistic diagnostic for a flowing-injection experiment. As a drawback to the faster scan rates, steady state was not achieved in the films, however, a greater catalytic current was observed in the presence of glutamate than without. The enhanced catalytic current was used in the same manner as the steady state current for the previous analyses.

Multiple layers were made of the FePAA/GlutOx and a linear increase in the catalytic current was observed. This was expected as the bilayer ISAMs have been shown to increase in a linear manner. Once it was determined that multiple layers could be made, the LOD and LOL were calculated. The LOD was determined to be 30 µM, 2 µM and 0.3 µM for 1, 2 and 3 bilayers respectively. These LOD compare well to the literature LOD of 0.5 and 3.9 µM for similar films. The LOL was also determined to be 5 mM for all of the films.
Finally the $K_m$' and the $I_{cat}$ of the GlutOx films were determined. For these glutamate biosensors to replace the current glutamate biosensors, the $K_m$' should be comparable. It was determined that the $K_m$' for the FePAA/GlutOx ISAM film was 0.40 (± 0.13) mM. This is lower than the $K_m$' of other glutamate biosensors and intermediate to some of the literature values for the freely diffusing enzyme.\textsuperscript{113-115} The $I_{cat}$ was shown to increase with increasing numbers of bilayers as expected, confirming that GlutOx is adding with each additional layer. These results tell us that the ISAM modified GlutOx biosensor is able to oxidize the glutamate as efficiently as the current glutamate biosensors found in the literature.

This ISAM method of building a glutamate biosensor seems to be an easy and efficient way to detect glutamate in solution. More work is needed to determine if, at slower scan rates the steady state be achieved. Similarly, additional work needs to be conducted to determine if these glutamate biosensors be used in the flow-injection experiments. In general the ISAM FePAA/GlutOx biosensors are a viable option for the detection of glutamate.
Chapter 8: Summary and Future Work

8.1 Summary

The main goal of this research was to develop an easy to prepare and sensitive biosensor that would be able to detect glutamate in solution using ionic-self-assembly methods. In order to accomplish this goal, it had to be proven that electrostatic assembly could be used to prepare an ISAM that included an electron transport mediator and an enzyme, and to show that it would be able to generate a current that was proportional to the concentration of analyte in solution. Much of the development work was performed using glucose oxidase (GluOx), but the procedure was general enough so that it could easily be applied to other FAD containing enzymes.

A cationic polymer of ferrocene poly(allylamine) (FePAA) was assembled on both 3-mercaptopropane-sulfonate and 11-mercaptoundecanoic acid modified gold surfaces. Surface confining of the FePAA was confirmed by cyclic voltammetry. This was an expected result as the exposed terminal group of these thiols is negatively charged and the FePAA is positively charged – conditions required to form an electrostatic assembly.

Poly(styrenesulfonate) was used with FePAA to determine if multiple bilayers of the FePAA could be made using electrostatic assembly and if each additional layer was electrochemically accessible. These bilayers were shown to assemble in a linear fashion, a result expected for a linear process like ionic-self-assembly. With the assembly, however, it was found that there was a maximum number of bilayers that could be assembled before the outer layers of the FePAA/poly(styrenesulfonate) assembly were
not oxidizable. From these results, the development of the glucose oxidase (GluOx) containing ISAMs followed a similar protocol.

The enzyme containing ISAM required deposition of the GluOx onto the ISAM. As enzymes are large polyions, the substitution of GluOx for poly(styrenesulfonate) was straightforward. In addition, since both of GluOx is a FAD containing enzymes, FePAA should be able to mediate the electron transport for both enzymes and facilitate the oxidation of glucose. An ISAM was assembled using FePAA and GluOx and shown to detect glucose in solution based on the steady state current response in the presence of glucose. Once it was determined that one bilayer could be made with FePAA and GluOx, multiple bilayers were made and these were also shown to also assemble in a linear fashion. This was the expected result based on the FePAA/poly(styrenesulfonate) ISAMs as ionic-self-assembly is a linear assembly process. It was shown that FePAA/GluOx assemblies have a maximum of 3 bilayers until the integrated charge per area levels off, characteristic of a lack of communication between the electrode surface and the bilayers furthest from the substrate. This difference between the FePAA/GluOx ISAM and the FePAA/poly(styrenesulfonate) ISAM was consistent with the size of the GluOx on the surface of the electrode.

The FePAA/GluOx ISAMs were further characterized by their LOD and LOL. It was shown that the LOD for the FePAA compared well to values reported for other GluOx sensors in the literature. This demonstrates that the ISAMs assemblies are a good alternative to other methods of confining the sensing components to the electrode.
interface. The GluOx ISAM biosensor also compares favorably with regard to the enzyme kinetics reported in the literature.\textsuperscript{41, 42} The $K_m'$ was found to be smaller than other glucose biosensors, indicating that the FePAA/GluOx ISAM is more efficient than other glucose biosensors. The $I_{cat}$ was found to be increasing with increasing number of layers. This shows that the GluOx is making good contact with the layer below.

The FePAA/GluOx modified 2 mm gold electrodes were then used in flow-injection experiments. It was expected that detection limits similar to those found with static solutions would be observed, however, this was not the case. The FePAA/GluOx modified 2 mm gold electrodes was unable to distinguish between injected amounts of glucose. This is most likely because the enzyme is unable to oxidize the glucose fast enough to detect the transient analyte zones. These films were also found to be unstable of the flow experiments. To determine if the constant potential was a possible cause of the degradation of the film, the FePAA/GluOx films were tested in conditions similar to the flow experiments, but in a static solution. It was determined that the film was able to maintain its stability, indicating that it is most likely the flow that is causing the films to degrade over time.

Scaling FePAA/GluOx ISAMs down for use on microelectrodes should have several advantages. The microelectrode will be able to fit into the end of the capillary. This means that the amounts detected should be lower for the microelectrode. It was also felt that the laminar flow will not disturb the surface of the ISAM as much. Microelectrodes can also be used at faster scan rates without the capacitance increasing dramatically and
possibly providing better transient response. These are important advantages and for these reasons, a FePAA/GluOx ISAM modified microelectrode was prepared.

The FePAA/GluOx ISAM modified microelectrode detected glucose in solution as shown by the steady state response in the presence of glucose. The steady state current density for these systems was also calculated and found to be higher for the FePAA/GluOx ISAM modified microelectrode than for the FePAA/GluOx ISAM modified 2 mm gold electrode. This is most likely due to increased mass transport to the microelectrode. The steady state current density was shown to be larger than both the FePAA/GluOx ISAM modified 2 mm electrode in this research and Hodak’s work, this is most likely due to more enzyme on the surface of the electrode.\(^\text{13}\) Once it was determined that one bilayer could be made with FePAA and GluOx on the microelectrode, multiple bilayers were made and these were also shown to assemble in a linear fashion. The maximum number of bilayers that assembled in a linear fashion before the outer layers of the FePAA/GluOx assembly could no longer be detected electrochemically is found to be two bilayers. This is similar to the 2 mm ISAM modified gold electrode.

The LOD and LOL were calculated, accounting for the area for the FePAA/GluOx ISAM modified microelectrode. The LOD was expected to be lower for the FePAA/GluOx ISAM modified microelectrode and this is shown to be true. This is due to the increased mass transport expected for microelectrodes. The LOD is also smaller than many of the other glucose biosensors in the literature.\(^\text{37, 93, 94, 96, 97}\) The LOL, however, was lower than found with the 2 mm ISAM modified gold electrode. This means that the FePAA/GluOx
ISAM modified microelectrode will not be usable over as large of a concentration range as the FePAA/GluOx ISAM modified 2 mm gold electrode.

To compare the FePAA/GluOx ISAM modified microelectrode further to the FePAA/GluOx ISAM 2 mm gold electrode, the $K_{m'}$ and the $I_{cat}$ were calculated. The $K_{m'}$ of the FePAA/GluOx ISAM modified microelectrode was shown to be very close to the $K_{m'}$ of the FePAA/GluOx ISAM modified 2 mm gold electrode. This was expected as the modification of the electrode is identical. The $I_{cat}$ was also calculated for the FePAA/GluOx ISAM modified microelectrode. It was determined that while the $I_{cat}$ increased for an increasing number of bilayers, the enzyme kinetics of the first and second bilayer did not have much difference between them. This corresponds to the lower than expected LOD for the 2$^{nd}$ bilayer.

Finally, the flow-injection experiments were conducted to determine if ISAM modified microelectrodes could be used in flowing systems. It was found that, as with the FePAA/GluOx ISAM modified 2 mm gold electrode, the FePAA/GluOx ISAM modified microelectrode was unable to distinguish any of the glucose amounts introduced to the flowing stream. This was the same as was observed for the FePAA/GluOx ISAM modified 2 mm gold electrode. The FePAA/GluOx ISAM modified microelectrodes also fell apart in the capillary flow-injection system, which was unexpected as the sheer stress under laminar flow in these systems is thought to be less than in the larger capillary flow-injection systems. This seems to indicate that the ISAM modified electrodes are not going to be the optimum system for use in flowing systems.
As a solution to the ISAM modified electrodes degrading in the flowing system, a covalently modified surface was developed. The covalently modified surface should be more stable in the flow-injection experiments as this surface covalently binds the FePAA to the surface of the electrode then ionically self-assembles the GluOx to the remaining amines of the FePAA. FePAA was bound to the 11-mercaptoundecanoic acid using 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride. This reaction was characterized using RAIRS and XPS and was shown to form an amide bond. The RAIRS data showed the formation of the amide bond and the disappearance of the carboxylic acid. The XPS data confirmed this by showing the amide N (1s) peak at 399.6 eV, a C (1s) peak at 288 eV for the carboxylic amide peak and the O (1s) peak at 532 eV from the carboxylic amide.

These films were then characterized by cyclic voltammetry to determine if they would be able to detect glucose using the same mediated electron transport scheme. FePAA was shown to have a lower surface coverage when covalently bound than when ionically self-assembled, but the system was able to support electron transport mediation having a relatively high current density. GluOx was ionically self-assembled to the surface and glucose was detected as evidenced by the resulting steady state curve in the presence of glucose. This is evidence that mediated electron transport is still occurring even though FePAA is covalently bound to the surface. The LOD was calculated and shown to be lower than the ISAM modified 2 mm gold electrode. This is most likely because the steady state density is higher for the covalently modified electrodes, indicating that the
glucose is able to be oxidized more efficiently giving a lower LOD. The LOL came out to be same for the covalently modified 2 mm electrode and the ISAM modified 2 mm electrode.

The \( K_{m}' \) and \( I_{cat} \) were calculated to further compare the covalently modified and ISAM modified 2 mm gold electrodes. It was shown that the \( K_{m}' \) and the \( I_{cat} \) were the same for these modifications. This is an indication that even though the FePAA is covalently attached to the surface of the electrode, the oxidation of the glucose by the film is not being affected. This demonstrated that the covalently modified electrodes could be used to replace the pure ISAM films in the flow-injection experiments.

The flow-injection experiments demonstrated that the covalently modified electrode also detected much lower amounts than the ISAM modified electrode. The covalently modified electrodes were also much more stable than the FePAA/GluOx ISAM electrodes. To determine if the constant potential affected the covalent films in the same way as the ISAMs a constant potential experiment was conducted, and it was shown that both films behaved in that same way. This illustrated that the ISAMs are being affected by the sheer stress of the flowing systems, while the covalently modified electrodes do not seem to have the same problems.

The covalently modified electrodes were scaled down to microelectrodes to determine if smaller amounts could be detected. The FePAA was covalently attached to the 11-mercaptopoundecanoic acid in the same way using 1-[3-(dimethylamino)propyl]3-
ethylcarbodiimide hydrochloride. GluOx was ionically self-assembled to the remaining amine groups on the FePAA. These films also detected glucose as evidenced by a steady state response in the presence glucose. The LOD for the covalently modified microelectrodes was determined to be lower than the covalently modified 2 mm gold electrode. The LOL was shown to be the same as the covalently modified 2 mm gold electrode though which was expected.

The covalently modified microelectrodes were then tested in the flow-injection system and it was determined that these films were able to detect lower concentrations than the corresponding ISAM modified microelectrodes. They, however, were not able to detect the same concentrations that the covalently modified 2 mm gold electrodes were. This result is in line with the higher than expected LOD. These results taken together mean that the covalently modified electrodes are a good replacement for the ISAMs and will be a good system for detection of bioanalytes in flowing systems.

The development of all of these different biosensor systems was for proof of principle so that the system could be extended to other FAD containing enzymes. Since it was demonstrated that an ionically self-assembled monolayer using FePAA could be used with GluOx for the detection of glucose, it seemed logical that the preparation procedure could be extended to other enzymes containing an FAD active site. In this case, the procedure was applied to GlutOx for the detection of glutamate. Both GluOx and GlutOx are FAD enzymes and FePAA should be able to mediate the electron transfer for oxidation of both enzymes. An ISAM was constructed using FePAA and GlutOx and the
detection of glutamate was observed by the increase in the catalytic current. For this system, true steady state was not observed as fast scan rates were used that prohibited the achievement of steady state.

Multiple bilayers were made once it was determined that a bilayer of FePAA and GlutOx could be assembled. It was shown that these layers increase in catalytic current with increasing number of bilayers. This was expected as ionic-self-assembly is a linear process and is the same phenomenon was observed for bilayers of FePAA and GluOx. The LOD and LOL were calculated and shown to be on the order of other glutamate biosensors.$^{50, 114, 117}$ The $K_m$ and $I_{cat}$ were then calculated to determine if these glutamate biosensors could be developed to replace the current types of glutamate biosensors. The $K_m$ was shown to be comparable,$^{50, 114, 117}$ while the $I_{cat}$ increased with increasing bilayers as was expected.

In this work, ISAMs have been shown to build up bilayers in a linear fashion with control over the amount adsorbed. The GluOx and GlutOx biosensor have enzyme activity in each layer and by adding layers, the biosensors becomes more sensitive, up to a point. Beyond this point, additional bilayers actually lower the efficiency of the measurement. This is likely due to the inefficiency of the heterogeneous electron transfer as the mediator polymer is placed further from the substrate. These biosensors are comparable to other glucose and glutamate biosensors found in the literature and are very simple to make. While the pure ISAM does not seem to be a practical method for detection in a flowing system, the covalently modified ISAM works very well under both turbulent and
laminar flow conditions. In addition, these results demonstrate that an ISAM constructed using FePAA/GlutOx is a feasible way to detect glutamate in a system.

8.2 Future Work

Future work for this project would include further investigation of the covalently modified ISAM electrodes. Several areas of investigation would be to determine if covalently attaching GluOx to the FePAA would affect the ability of the enzyme to oxidize the analyte in solution. Additionally if the enzyme is still active, then determining the stability of these new covalently bound FePAA/GluOx in the flow – injection experiments.

The long-term stability of these electrodes also needs to be investigated further. All of these studies were conducted in one day, so it is not know what the stability of the enzyme on the surface of the electrode is over the long-term.

Another set of experiments would be to look at the other FAD enzymes to incorporate into the ISAMs and determine if they can be mediated with the FePAA. Cholesterol oxidase would be of interest because of its ability to oxidize cholesterol. Cholesterol monitoring being a great concern to the average person, being able to accurately monitor cholesterol levels would be a great advance.
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Vita

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