Fabrication and Optimization of a 25µm Glucose Sensor

by

Mekala Sanathanie Pansalawatta
B.Sc. University of Peradeniya, 2003

Submitted to the Department of Chemistry and the
Faculty of the Graduate School of the University of Kansas
in partial fulfillment of the requirements for
The degree of Masters in Science

Prof. Michael A Johnson (Chair)

Prof. Carey K Johnson

Prof. Sue Lunte

Date defended: 6/5/2009

The Thesis Committee for Mekala Sanathanie Pansalawatta
certifies that this is the approved version of the following dissertation:

Fabrication and Optimization of a 25µm Glucose Sensor

Committee:

Prof. Michael A Johnson (Chair)

Prof. Carey K Johnson

Prof. Sue Lunte

Date approved: 6/5/2009

Dedication

To my loving husband, Roshan, and my kids, Vijini, and Themiya

ACKNOWLEDGEMENTS

First of all, I would like to thank my research adviser, Dr. Michael A. Johnson for his guidance and support throughout these years. I greatly appreciate his patience and the freedom he gave me in my degree. I would like to thank Dr. George S. Wilson for his helpful suggestions and allowing me to use his lab and equipments.

Special thanks go to my friends Chamini Perera and Sanjeewa Senadhera for their help and support I had during pyrrole distillation which I had to do at least twice a week. I wish to thank Dr. Richard Givens for allowing me to use his lab. My heartfelt appreciation goes to Dr Greg Osterhous, Dr. Na Feng, and Sam Kaplan for their support during the in vivo experiments. I would like to thank the Johnson group for their help and support throughout my graduate life. They stood by me and encouraged me whenever I needed a little encouragement. I would like to thank my thesis committee, Dr. Cary Johnson and Dr, Sue Lunte for their valuable comments about the thesis that helped me greatly to finish my thesis properly.

I wish to thank the University of Kansas and chemistry department for providing financial support. I would also like to thank the animal care unit of the University of Kansas for providing rats for in vivo experiments.

I would like to thank my parents, my sister, and my brother for their love and support throughout my life.

A big thank you to Roshan, my husband for taking care of the kids and everything around home, when, I was busy with my research and thesis writing. He

has been there for me when ever I needed. I'm sure my kids did not know why their mom was so busy lately. I greatly appreciate their contribution and sacrifice they made even without knowing what they were doing, to achieve this difficult task. This would not have been possible without the support of my husband and my kids. I want them to know that they mean everything to me.

ABSTRACT

Microelectrodes have been used for over 30 years of glucose biosensor history. Two different types of glucose biosensors were developed using 176μm and 25μm diameter Pt wire cylindrical microelectrodes. The 176 μm Pt wire type microelectrodes were modified with non-conductive polymers and glucose oxidase enzyme. The lifetime of biosensors was over 50 days and had negligible response to interference species such as ascorbate and urate. The 25μm Pt wire microelectrode was coated with glucose oxidase and conductive polymer, polypyrrole by chemical polymerization. Nafion® was used to eliminate interferences. The 25 μm polypyrrole based glucose sensor had the ability to measure *in vivo* glucose.

Table of Contents

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: Results and Discussion

Chapter 1: Introduction

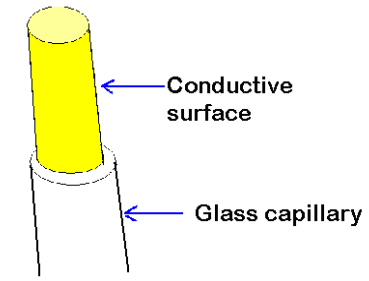
Section 1: Introduction to Microelectrodes

1.1 What is a microelectrode?

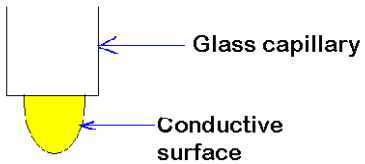
Microelectrodes are electrodes with submicron-micron or smaller dimensions. Since the late 1970's, electroanalytical chemists have given much attention to development of electrodes with critical dimensions ranging from 0.1μm up to 25μm [1-4]. The smaller size of microelectrodes makes them ideal for in vivo biological studies such as measuring concentrations of neurotransmitters inside brains of live rats and clinical applications for the detection of blood or brain glucose levels. In comparison to conventional electrodes, microelectrodes cause minimal tissue damage during implantation [3-7]. Furthermore, microelectrodes require only very small amounts of sample, making them useful in analyzing precious samples with limited volumes. Microelectrodes offer a number of advantages. First, the Ohmic drop, or IR drop, of microelectrode in non-steady state conditions is negligible compared to conventional electrodes [8; 10; 11]. Second, microelectrodes reach the limiting current quickly due to low capacitance of microscale electrode surfaces. Therefore, response time is in the millisecond-microsecond range. Because of these properties, microelectrodes often are used in high-speed voltametric methods such as fast-scan cyclic voltametry [1-5].

1.2 Types of Microelectrodes

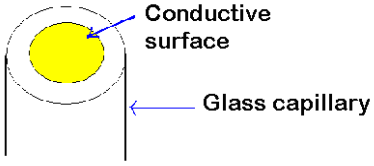
Microelectrodes are fabricated in several different geometries depending on the type of application and the materials being used. Commonly constructed microelectrodes are disk, cylinder, sphere/hemisphere, band and ring geometries [4; 8; 9; 12; 13]. Among these, disk and cylindrical microelectrodes are the most popular due to the relative ease of fabrication [4; 9; 13].



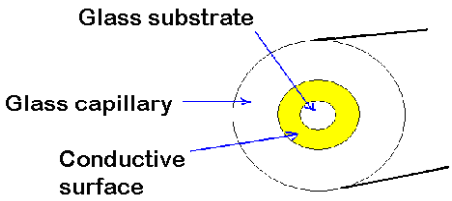
1. Cylindrical



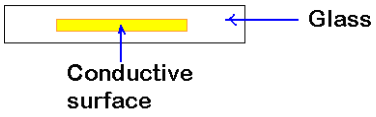
2.Spherical/Hemispherical



3. Disk



4. Ring



5. Band

Figure1: Schematic representation of cylindrical, disk, spherical, ring and band microelectrode geometries. [Adapted from 8]

1.3 Construction of Microelectrodes

Microelectrodes have been fabricated in a number of different ways as demanded by the objectives and conditions of experiments. Thus, the geometry and materials used to make microelectrodes vary from case to case. Carbon fibers and metal micro wires are widely used in construction of disk and cylindrical electrodes [1-5; 8; 9]. Microelectrodes that serve a commercial purpose must be easy to fabricate and the materials that are used should be inexpensive for mass production. Carbon fiber [4; 9;], ceramic [15; 16], and laminating foil have been employed in construction of inexpensive, reusable microelectrodes and arrays in cylindrical, band and disk geometries [12].

Most of the compounds present in biological samples and solvents are insoluble in glass and fused silica. Therefore, glass and fused silica capillaries are widely used to seal the excess metal wires or carbon fiber when making microelectrodes [5; 8; 11]. Carbon fiber, platinum, or gold micro wires can be inserted into glass capillaries by applying a vacuum. Then, the glass is sealed by melting and pulling using a heated coil microelectrode puller. The glass seal is often secured by applying epoxy containing m-phenylenediamine as the hardening component as described in Wightman *et* al. [4; 9]

Disk electrodes are constructed by grinding and polishing the micro wire-glass seal using a microelectrode beveler or coarse sandpaper [4]. These disk microelectrodes can be modified to make them hemispherical or spherical electrodes

by electrodeposition and progressive nucleation of mercury or platinum nano particles on the surface of the disk microelectrodes [8].

Microband electrodes have been constructed in dual-band or triple-band assembly by placing thin metal foil inside thin glass insulating plates [13]. Due to the practical difficulties of handling submicron dimension microbands, metal foil band microelectrode fabrication was limited to bandwidths of a few micrometers. To solve this problem, and construct even smaller micro bands, chemical sputtering of metal nano particles on a surface of a non-conductive polymer or on a thin epoxy layer followed by application of another insulating polymer coat has been studied [8].

When the electroactive surface is miniaturized, the current that can be genarated by a single microelectrode is small. In other words, if the electrochemical detector is not sensitive enough for nano-scale currents, taking measurements would be problematic. To overcome this problem and enhance the performance for neurochemical detection, a new approach that has been explored is to manufacture microelectrodes as arrays [14-17]. Lithographic techniques such as X- ray lithography, optical photolithography and electron-beam lithography are widely used in fabrication of commercially-available band and disk microelectrode arrays [8]. Gerhardt and co-workers have developed microelectrode arrays using photolithography for the detection of neurochemicals such as choline, acetylcholine and glutamate *in vivo* brain tissues in real time [15; 16]. The self-referencing nature of the electrode array allows for obtaining the signal from the analyte and from the background signal separately [16]. Therefore, the absolute signal due or to the analyte

of interest can be measured by subtracting the background from the signals. Yu and Wilson have described a method for developing microdisk sensor array comprising of different enzymes to monitor different analytes simultaneously in small sample volumes [17].

Electrodeposition of platinum or gold nanoparticles, carbon nanotubes on microelectrode surface is another method that can be used to enhance surface properties and improve sensitivity of the electrodes [19-21]. Tamiya and co-workers have developed platinized 7μm diameter disk type carbon fiber microelectrodes with improved activity compared to a regular carbon fiber electrode. These microelectrodes have further developed to a glutamate biosensor [1]. Ronalds and Yacynych developed a glucose sensor by platinizing 8μm carbon fiber [22; 23]. Platinum black or platinum nanoparticles have been used to roughen the surface of platinum/iridium microelectrodes and increase their electroactive surface area [18; 20].

In summary, microelectrodes are fabricated in number of different geometries using a wide variety of materials. Electrode construction techniques can be different from one another depending on the application. Considerable progress has been made in the construction of microelectrodes and their applications in biosensor studies. Microelectrodes offer a useful array of special characteristics compared to the conventional macroelectrodes, which make microelectrodes more suitable for *in vitro* and *in vivo* biosensors.

1.4 Special Features of Microelectrodes

1.4.1 Size

The small size of microelectrodes facilitates a wide range of applications due to the special features they offer compared to conventional macro electrodes. Microelectrodes make it possible to implant them inside live animal brains with minimal tissue damage [3-7]. Additionally, microelectrodes can be used for measuring small sample volumes present in biological systems, such as inside a single neuron or on the synaptic cleft [3]. Ultramicroelectrodes have gained a considerable interest due to capability of measuring currents generated by oxidation or reduction of neurotransmitters produced in the brain during signaling process [8; 19; 22].

Because of their smaller size, mass production of microelectrodes is possible and can bring the cost of manufacturing down significantly [12; 13]. Hence, production of disposable microsensors is possible [12]. Moreover, the portability of microelectrodes allows for developing sensors that can be used in field measurements. Microelectrodes can also be used as parts of other microelectronic devices that involve electrochemical detection. For example, White et al. developed a method to employ microelectrodes with open tubular liquid chromatographic (LC) columns to measure neurotransmitters [24]. Additionally, carbon fiber microelectrodes have been used in capillary electrophoresis (CE) as working electrodes [25]. The electrochemical response can be further enhanced several orders of magnitude by the employment of microelectrode arrays [14-17].

1.4.2 Diffusion properties

When a potential difference is applied between the working and the reference electrode, an electrochemical reaction occurs at the surface of the working electrode. The electroactive analyte molecules in the close proximity to the surface of electrode either oxidize or reduce generating a current signal. Due to this electrochemical reaction, the concentration of analyte close to the surface becomes depleted and creates a concentration gradient compared to that of bulk solution (Figure 2). Therefore, the analyte diffuses through this concentration gradient towards the electrode [8].

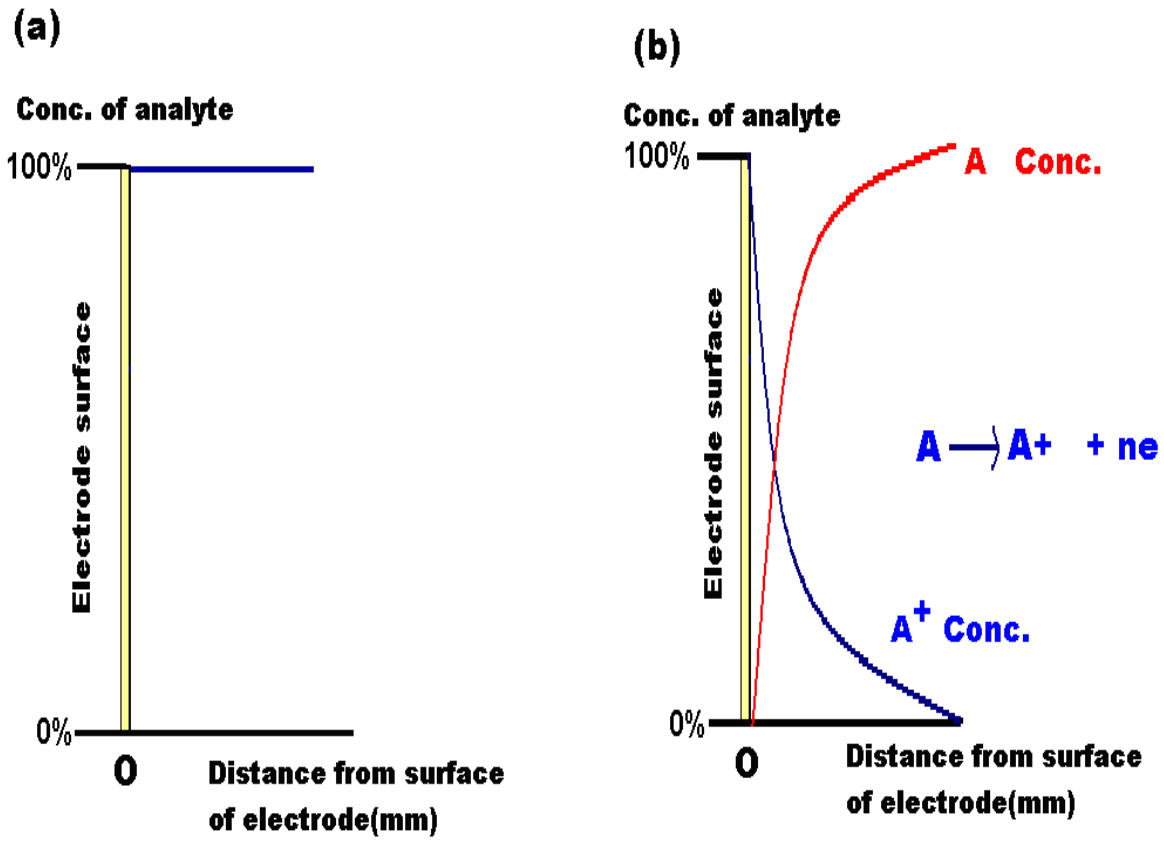


Figure 2: Concentration profiles of an analyte in the vicinity of a planar electrode (a) Concentration profile of an analyte (A) before a potential step is applied. (b) Concentration profiles of analyte (A) and oxidized form (A^+) of analyte during a potential step to peak potential (E_p).

The geometry of the diffusion field in the steady state at a macroelectrode is always linear regardless of the shape of the electrode. The analyte diffuses toward the electrode in a straight path and the mass transfer rate from the bulk solution to the electrode is dependent only on the distance from the electrode. Therefore, the current density of a planar electrode surface is same at each and every point [8; 10; 13]. However, disk, sphere and hemisphere microelectrodes show spherical diffusion fields in the steady state [8]. These microelectrodes show non-uniform current densities on the electrode surfaces due to their non-linear mass transfer paths. For instance, spherical microelectrodes have higher current density close to their glass seals compared to the middle. The non-linear diffusion characteristics of microelectrodes result in significantly higher current densities and larger mass transport rate constants compared to macroelectrodes. This greatly minimizes the time required for a microelectrode to reach the steady state conditions. On the other hand, if the electrode is so small that it can fit in the diffusion layer, the steady state approaches instantaneously. The size of the electrode is inversely proportional to the time it takes for the electrode to reach the steady state [8].

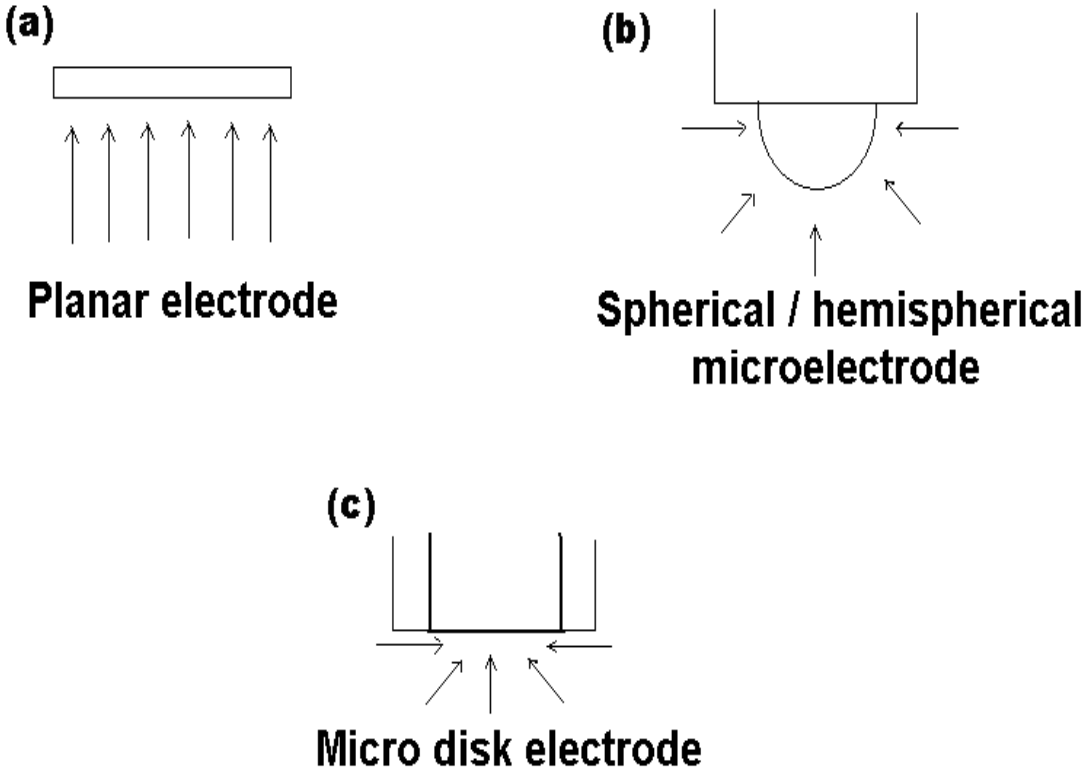


Figure 3: Illustration of analyte diffusion fields. (a) Linear diffusion field at a planar electrode, (b) Spherical diffusion field at a spherical or hemispherical microelectrode and (c) Spherical diffusion field at a disk microelectrode.

1.4.3 Charging Current (ic)

At the beginning of an electrochemical experiment, a potential difference is applied between the working and the reference electrodes. The electrons generated by a redox reaction will move towards the electrode or away from the electrode depending on whether it is an oxidation or reduction reaction. The current generated by the movement of electrons will pass through the system. Initially, the interphace of electrode surface-electrolyte solution creates an electrical double layer that works as a capacitor and requires some current in order to be discharged. This current is called charging current (i_c). Thus, the electrode uses a part of the current generated by the electrochemical reaction, to discharge the surface. Therefore, a high charging current could distort the experimental data that involve potential sweep method. The charging current due to the electrical double layer is directly proportional to the active area of the electrode. Hence, macro-electrodes have higher charging currents compared to microelectrodes. At the steady state, the faradaic current is proportional to the radius of the electrode. As a result, microelectrodes have favorable faradaic and charging currents especially for experiments that produce nano scale electron flows [8; 11].

1.4.4 Limiting Current (i_L)

The limiting current is the current required for an electrode to reach the steady state where there is no more increase in current even the potential is increased. The small surface area and low double layer capacitance of microelectrodes makes it possible to reach the steady state more rapidly than macroelectrodes resulting faster

response times for microelectrodes. Microelectrodes can provide responses in micro or sub-microsecond time scales, which allow measuring virtually real time *in vivo* electrochemistry [26; 27].

1.4.5 iR drop / Ohmic drop

When a current passes through a conductive medium such as a solution or a wire, a fraction of potential is lost due to resistance of the medium. This potential loss is called iR drop or Ohmic drop.

According to the Ohm's law;

$$E = iR \qquad (1)$$

where E is potential, i is current and R is resistance.

If the current that passes through a medium or the resistance of the medium is high, the iR drop is high. In an electrochemical experiment where the current passes through a liquid or solid medium, iR drop cannot be avoided. Hence, the iR drop needs to be controlled to minimize the distortion of the data. When using a regular macroelectrode, the iR drop can be minimized using a three electrode system consists of a counter electrode with reference and working electrode. Keeping the reference electrode close to the working electrode also helps decrease the potential loss during an experiment [11]. The iR drop can be problematic for experiments in highly resistive media or voltammetry experiments at fast scan rates [5; 11].

Under non-steady state conditions, current is directly proportional to the surface area of the electrode. Therefore, the iR drop is also proportional to the area of

the electrode. Electrodes made in the micro or nano scale exhibit very small iR drops under the non-steady state, improving the data significantly. Whereas, under the steady state conditions, the iR drop does not depend on the size of the electrode. Other factors such as resistance of media and the scan rate can change in potential. However, having a smaller surface area has the advantage of more accurate results over the conventional macroelectrode.

Since the uncorrected iR drop is insignificant when using a microelectrode, a three electrode system is not necessary. This provides an additional advantage for *in vivo* experiments because only the working electrode and the reference electrode need to be implanted inside the living animal, thereby resulting in minimal tissue damage while having a less iR distorted current [26; 27]. These special features of microelectrodes have facilitated a wide variety of applications that were not feasible before the development of microelectrodes.

1.5 Applications of Microelectrodes in Biosensor Development

Microelectrodes have emerged as a major area of research based on the special characteristics provided by their small dimensions. In neurological sciences, microelectrodes have been used since early seventies by the Nobel Prize nominee Ralph N. Adams for studying basic neurotransmitter signaling in animal brain tissues [28]. Microelectrodes made up of 5μm -10μm diameter carbon fibers are widely used in electrochemical measurements of neurochemicals in brain [1; 2; 5; 22; 23]. Techniques such as fast scan cyclic voltammetry (FSCV) and constant potential

amperometry are important tools for measuring catecholamine release in disease state animal models [5; 22; 23].

Chemically modified microelectrodes have been used as microsensors to detect various chemicals *in vitro* and *in vivo*. Meyerhoff and coworkers have developed nitric oxide sensors using platinized platinum microelectrodes [18]. Due to the additional surface area of the electrode, sensor performance was improved by 10 fold in sensitivity and had about a 10 fold lower detection limit.

Microelectrodes can be assembled in arrays to enhance the signal. These arrays can be used for measure multiple analytes simultaneously by coating appropriate enzymes and polymers [15-17].Gerhardt and co-workers have constructed microelectrode arrays using ceramic materials to minimize cross talk between microelectrodes [15; 16].

Implantable enzyme coated amperometric biosensors suitable for clinical applications have also been thoroughly investigated. Glucose oxidase immobilized microbiosensors have a leading role in biosensor research [27; 29; 30]. The increasing demand for more effective glucose sensors for the detection of blood and brain glucose levels has broadened the interest of sensor studies.

Section (2) Introduction to Biosensors

1.2 Biosensor

A biosensor is an analytical device that can be used to quantitatively measure a specific analyte(s) that combines a biological element with a physiochemical detector component [31-33].

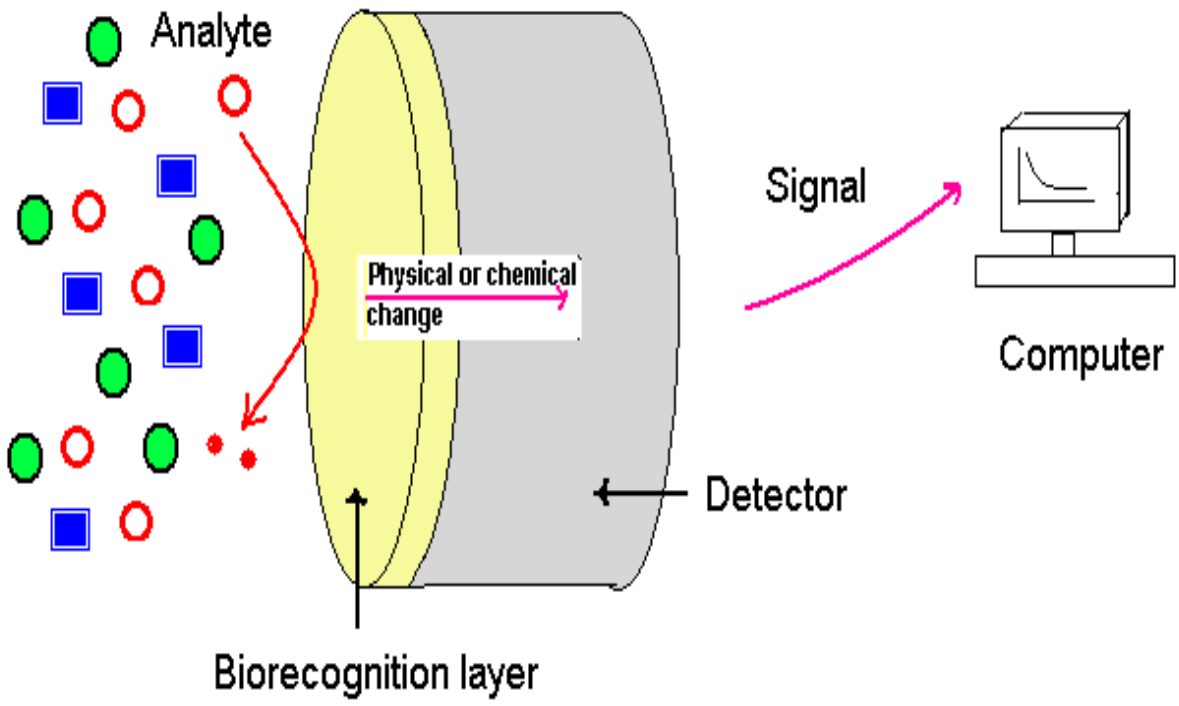


Figure 4: Schematic representation of a biosensor.

The biosensors have biological layers that recognize, communicate or react with a specific analyte in the presence of other interfering elements. The biological element or the bio-recognition layer is typically composed of enzymes, antibodies, ligands, single cells or tissues [31; 32]. The bio-recognition layer is adsorbed, covalently attached or electrochemically immobilized onto the surface of a detector or a transducer. Specific interactions between the biological layer and the analyte generate a chemical or a physical change. This physiochemical change is recognized by the detector and the signal is amplified or directly displayed on a computer screen. The intensity of the signal is proportional to the concentration of the analyte.

The detectors can be categorized into electrochemical, optical, thermal, or piezoelectric by the type of signal that is produced by the analyte and bio-recognition layer interaction. Electrochemical detectors can further be divided into potentiometric, voltammetric and amperometric sub-categories by considering the techniques that used to measure the signal [31-33].

1.3 Glucose Sensors

Diabetes is a common disease that is caused by the body's poor production or inability to use insulin hormone properly. According to the American Diabetes Association's statistics, 7.8% of the total population of America, or about 23.6 million people, have diabetes [34].

The first written evidence for the medical treatment of diabetes is found in famous ancient Egyptian "Ebers Papyrus" documents dated back to 1500BC [35].

Since then, much research has been conducted on developing various techniques to diagnose and treat glucose related complications [34; 36]. For more than 30 years, electroenzymatic glucose sensors have served the need to detect blood glucose levels in patients. So far, a variety of electrochemical glucose sensors with improved properties have been developed to fulfill the increasing demand [20; 21; 27; 29]. However, it would be beneficial to improve precision, reproducibility, selectivity, production costs and biocompatibility for biosensors used *in-vivo*.

1.3.1 The Role of Glucose Oxidase (GOx)

Glucose oxidase is the most widely used enzyme in biosensor development. It is a 160KDa dimeric protein with a cofactor, flavin adenine dinucleotide (FAD), bound in each monomer [68]. The commercially available GOx enzyme used in biosensors is often extracted from *Aspergillus niger* [15-17], which is highly specific for β-D glucose.

Glucose oxidase catalyses the oxidation reaction of β-D glucose to D-glucono-1,5-lactone that further hydrolyze to gluconic acid. During the redox reaction, FAD is reduced to $FADH_2$ and re-oxidized to FAD through the reduction of O_2 into H_2O_2 (Equation 2, 3) [37].

$$\beta\text{-D glucose} + FAD \xrightarrow{GOx} FADH_2 + \text{D-glucono-1,5-lactone} \quad (2)$$

$$FADH_2 + O_2 \longrightarrow FAD + H_2O_2 \quad (3)$$

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e \qquad (4)$$

Enzymatically produced H_2O_2 can then be electrochemically converted to oxygen and water at the surface of an electrode (Equation 4). Therefore, the current measured is equal to the H_2O_2 flux, which is directly proportional to the concentration of glucose.

1.3.2 Methods of Enzyme Immobilization

With the increased use of enzymatic biosensors a variety of approaches have been explored for the immobilization of enzyme on to the surfaces of electrodes [27; 29-33]. Physical adsorption of an enzyme at a metal surface is a simple technique that can be performed on a variety of electrode materials. The sensors are manually dropped or dipped in an enzymatic solution for a given period of time and air dried. In this method, the amount of chemical disruption to enzyme is minimal compared to other methods that use harsh chemicals. However, this method has a couple of drawbacks: (1) the thickness of the enzyme layer is very small due to the monolayer formation [8] and (2) the stability of enzyme and metal surface interaction tends to change with conditions of analyte medium such as pH and temperature [20; 21; 33].

Co-deposition of glucose oxidase with another supporting protein such as bovine serum albumin (BSA) or collagen is another conventional approach for enzyme immobilization [33; 38-40]. These proteins form a densely packed self-assembled monolayer on the metal surface. The proteins are energy-wise stable on

the surface of the electrode compared to that of the aqueous solution. The disulfides thiol groups on the protein act as chemisorption agents and form a highly ordered monolayer on the surface of electrodes [11; 41].

Covalent attachment of enzymes is a widely used immobilization approach. As the first discovery of covalent attachment of monolayers on electrodes, Murray and co-workers applied a silanization reaction on silane to attach hydroxyl groups on the surfaces of various metal electrodes [42]. Enzymes have also been co-immobilized with BSA in a sol-gel medium to produce a thin, uniform multilayer of enzyme with enhanced enzyme activity. The stability sensor could be improved by cross linking with glutaraldehyde [27]. However, the main drawbacks of entrapment of enzyme in a gel matrix are the analyte could not pass through the gel easily and the enzyme tends to leak out due to large pores on gel matrix [33].

Encapsulation of enzyme in multilamellar vesicles is a newly developing method that provides improved sensitivity and long term stability of polypyrrole based glucose sensors [43-45]. As Olea and co-workers have observed, the entrapment of glucose oxidase in multilamellar vesicles can cause a 200-fold increase in apparent maximal current of the sensor [43; 44]. Covalent cross-linking enzyme with a suitable cross-linker is also a successfully used enzyme immobilization technique [46]. One method of cross-linking of enzyme makes use of glutaraldehyde, which reacts with free amino groups of lysine residue in the enzyme and forms a stable attachment [47]. (3-aminopropyl)trimethoxysiline has also been used in

glucose sensors to form electrochemically assisted cross-linking between polymer and enzyme layers [48].

In addition to proteins, Pt black or nanoparticles, carbon nanotubes, and non ionic detergents such as Triton-X-100 have been co-deposited with the enzyme to enhance the amount of enzyme coated on the electrode surface and increase the electroactive surface area of the electrode [39; 49]. Incorporation of multilayer polymer films in biosensor designing has become popular in the last two decades. Polymers provide many advantages that described in details on next section.

1.4 The use of polymer films

Over the past 30 years, the modification of electrode surfaces to convert bare electrodes into sensors that could measure a particular electrochemical reaction has been the subject of intense investigation. It has been demonstrated that sensors coated with polymers and other protective coatings generally have a longer life time compared to sensors without these coatings. Likewise, the long-term stability of sensors have been tremendously increased with polymer coatings [33; 43-55].

A general advantage of many polymer films is the prevention of electrode fouling. When the enzyme layer of a sensor is directly exposed to analyte solution, molecular species in the sample are adsorbed onto the surface of electrode. This prevents measurement of the analyte and causes reversible fouling of sensor. A variety of protective coatings have proven to improve anti-fouling properties of microsensors and increase long-term stability of sensor by preventing enzyme leaking

out to the biological fluid or sample solution. These coating includes polyphenol, poly(o-phenylenediamine), polyaniline, and polypyrrole [43;49-55].

Another use of polymeric coatings is to control mass transfer of interferents to the electrode surface. One method of achieving this goal is to control pore size in polymers such as polyurethane, so that they act like a sieve. These polymers allow only small molecules to pass through and prevent larger molecules such as proteins, DNA, antibodies getting to the electrode surface. Some polymers and gels provide a matrix for the enzyme entrapment without denaturation. For example, polyphenol and sol-gel provides a matrix such that the enzyme is more stable on the electrode surface than enzyme in solution. The presence of polymer films minimizes the leaching out of enzyme to solution [39; 45].

Polymers that are used in sensor fabrication could be categorized as non-conductive polymers [29; 33; 39] or conductive polymers [43; 44]. A large number of polymers have been employed in a number of different sensor applications [43-55]. Non-conducting polymers such as poly (o-aminophenol) or phenol have been shown to produce self-limiting thin layers that entrap enzymes. Thin films allow analytes to diffuse towards the electrode surface more rapidly compared to thicker films. This improves electrode response times and limits of detection, key factors to consider for *in-vivo* studies [39; 49]. Another advantage of using non-conducting polymers is that the ability to reject interfering species present in biological samples [49]. These polymers acts as impervious layers to electroactive species present in biological sample that interfere with the analyte signal. For example, Nafion® can be applied on

an electrode to prevent negatively charged uric acid and ascorbic acid from entering and reacting with the electrode [56; 57]. The application of Nafion®, and its role as a barrier for cationic electroactive interference species, is further discussed below.

On the other hand, some of the conductive polymers have electrochemically active functional groups that can undergo redox reaction. The electroactivity of most polymers is typically confined to a highly localized electrochemically active functional group area. Therefore, a functional group site that facilitates electron transfer towards the electrode must be present [33]. Poly(o-phenylenediamine) (PPD), polypyrrole (PPy), polyaniline polymers can be applied to metal surfaces either by dip-coating or by electrochemical depositon. It is easy to prepare multilayer films under mild chemical conditions and the thickness of polymer film can be controlled by controlling the polymerization time [54; 55; 58; 59]. Polypyrrole has been used on the construction of the glucose sensor discussed in this thesis and is discussed in more details in this section.

1.4.1 Nafion® / Perfluorosulfonate Ionomer

Nafion® is an ion containing polymer of poly(tetrafluoroethylene). Nafion® is a clear liquid that is insoluble in water and has a complex chemical structure (Figure 5). Key advantages of Nafion® include high thermal stability and high ionic selectivity. Due to the electrostatic interactions between ionic functional groups, Nafion® molecules tend to aggregate forming tightly packed regions called “clusters”. In the presence of aqueous solutions, Nafion® could absorb water by increasing the

size of "cluster". The sulfonate group on Nafion® interacts with positively charged ions via a cation exchange type reaction. Therefore, Nafion® acts as a cation exchanger that excludes anions such as urate and ascorbate. The ion-exchange properties of Nafion® serve to exclude anionic species that interfere with the analyte signal before entering the electrode.

Nafion® has been used in a number of applications. Nafion® can be used as an effective diffusion barrier by preventing negatively charged electroactive species contacting electrode surface. Michael and co-workers have observed that anti-fouling ability of glutamate sensors coated with 0.5% Nafion® solution and glutamate oxidase has significantly higher biocompatibility than sensors without Nafion® [7]. Additionally, Nafion® has been used on glucose oxidase coated Pt electrodes to measure *in-vitro* glucose in whole blood [56]. In addition to the resistance to interferences, pre-concentration of trace compounds within Nafion® film in stripping voltametric studies have greatly improved the limits of detection of sensors [59]. Nafion® coated mercury electrodes have been developed by S.Daniele et.al for the detection of trace amounts of heavy metals [57].

Even though Nafion® does not dissolve in water, commercially available 5% aliphatic Nafion® is readily soluble in water. The film thickness can be controlled by changing the Nafion® concentration and the number of times the electrodes were dip coated. Nafion® can be applied to electrodes by dip or drop evaporating method which is a fast and convenient coating method.

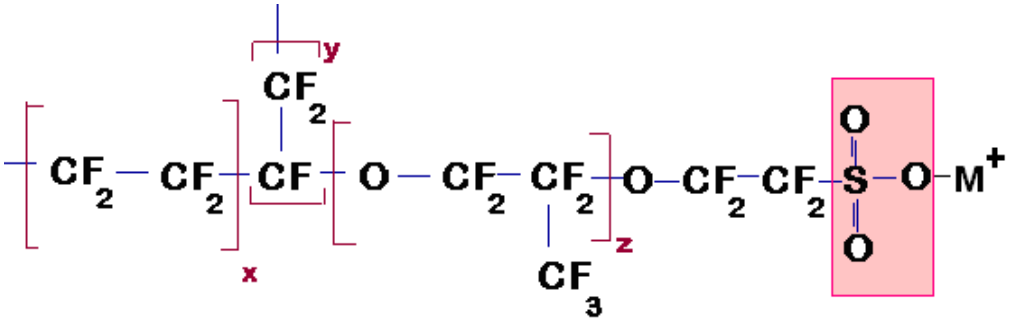


Figure 5: Chemical structure of Nafion® where, selected group is either carboxylic acid or sulfonic acid group and M+ is a metal ion in the neutral form or H+ ion in the acidic form.

1.4.2 Polypyrrole

Pyrrole is a simple, slightly basic five membered unsaturated heterocyclic aromatic ring compound that can polymerize to form polypyrrole (PPy), a highly stable conductive polymer (Figure 6). Natural polypyrrole can be found in plants and animals skin cell pigment called melanin [60]. Typically pyrrole turns into a dark yellow colored solution when exposed to air/O_2 and it polymerizes naturally when exposed to light. Additionally, pyrrole can be polymerized by chemical oxidation [44; 61; 62] or electrochemical polymerization [64; 18] to produce thin, transparent films on metal surfaces. Polymerized pyrrole mainly forms linear polymer chains [62]. However, branched polypyrrole is also found in the literature [61].

The conductivity of polypyrrole can be improved by electrochemical oxidation or over-oxidation using cyclic voltametric potential sweep. Once the appropriate oxidation potential is attained, the pyrrole molecules at the electrode surface oxidize and polymerizes. N-functionalized pyrrole derivatives can be used in various applications [63].

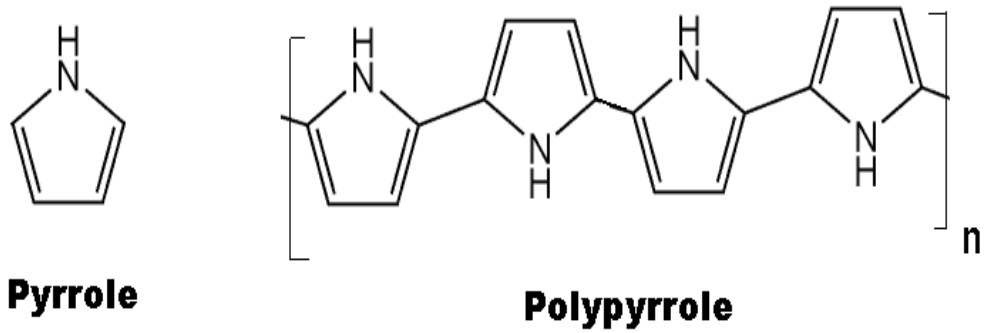


Figure 6: Structures of pyrrole and polymerized pyrrole (PPy)

Polypyrrole is stable in aqueous buffer solutions and in complex biological environments and is non-reactive with biological compounds under physiological conditions. Thus, the lifetime of sensor is usually not dependent on the breakdown of polymer, but rather the loss of enzyme activity. Polypyrrole is among the most common conductive polymer used in biosensor fabrication due to its biocompatibility [44; 45; 56; 64]. The characteristics and thickness of the polymer film can be easily controlled by changing the electrochemical polymerization time or the number of times the electrodes dip-coat in polymer solution [64]. Therefore, the sensors can be made with thin polymer films that allow analyte an easy access to the enzyme. (Figure 7).

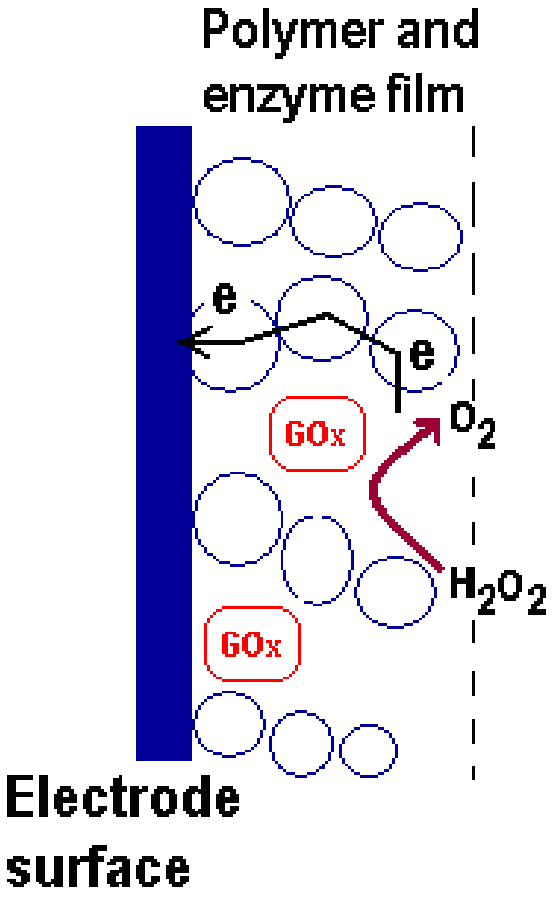


Figure 7: Schematic diagram of electrode modified with conducting polymer film

1.5 Amperometry to Detect Glucose

There are several types of controlled-potential electrochemical techniques Depending on the type of measurement, it could be categorized as chronoamperometry, which measures current verses time, chronocoulometry, which measures charge, or chronoabsorptometry which measures optical absorbance or concentration of absorbing analyte species [11; 67]. Controlled potential amperometric techniques are based on application of a single or multiple potential steps between the working and the reference electrodes. Diffusion is the major mass transport process for a non-stirred solution, which controls oxidation or reduction rate of the electroactive species. If a potential step is applied to a non-stirred solution, electroactive species that are in close proximity to the electrode are oxidized or reduced. Due to the oxidation or reduction of these electroactive species a concentration gradient forms between bulk solution and solution close to the electrode. The electroactive species is transported towards the electrode through the concentration gradient by diffusion. The current generated by the oxidation or reduction of electroactive species is measured over a period of time (Figure 8).

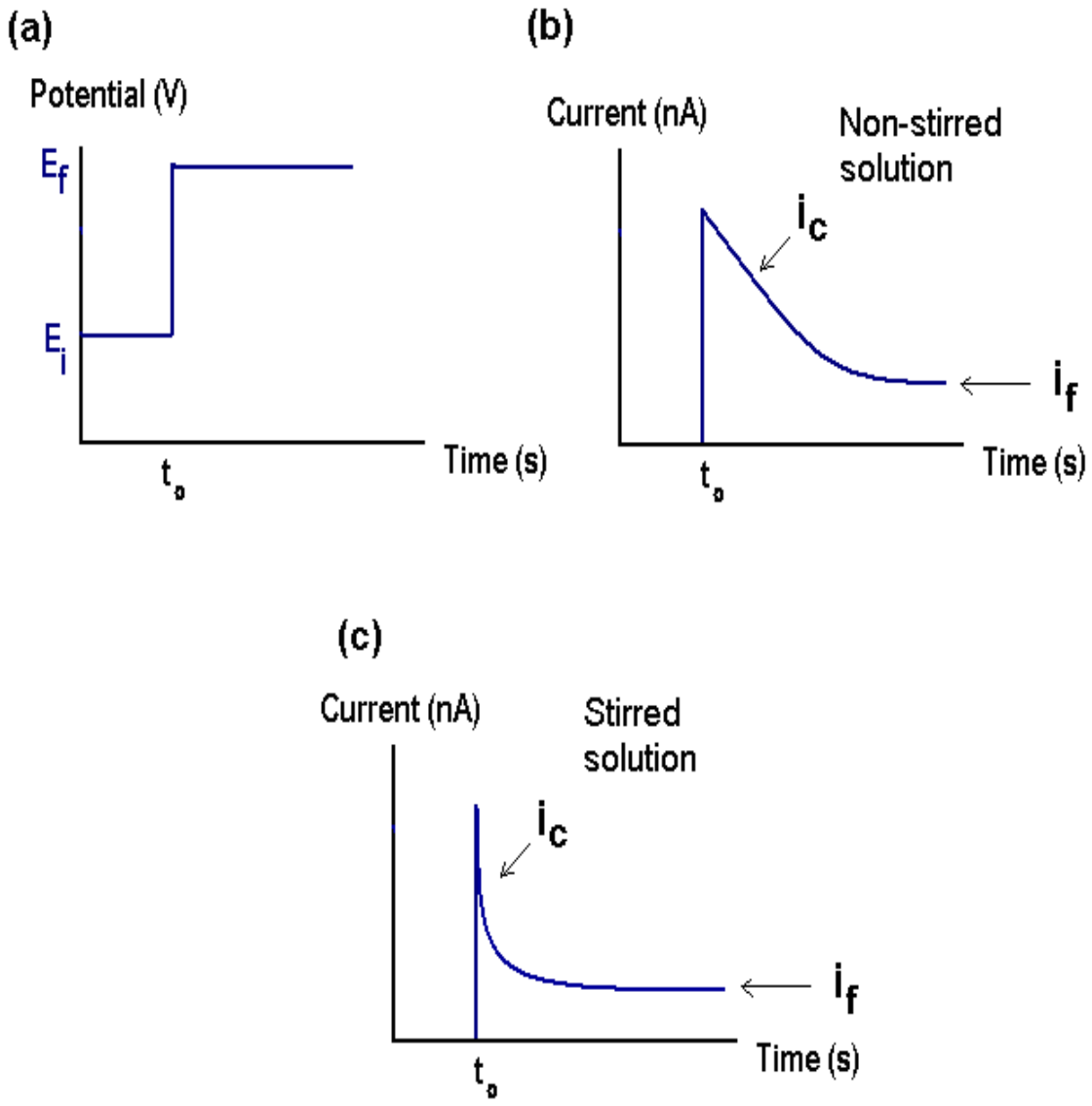


Figure 8: Illustration of the current change (b), (c) measured due to the oxidation or reduction of an analyte during a potential step (a) (i_c – charging current, i_f – faradaic current)

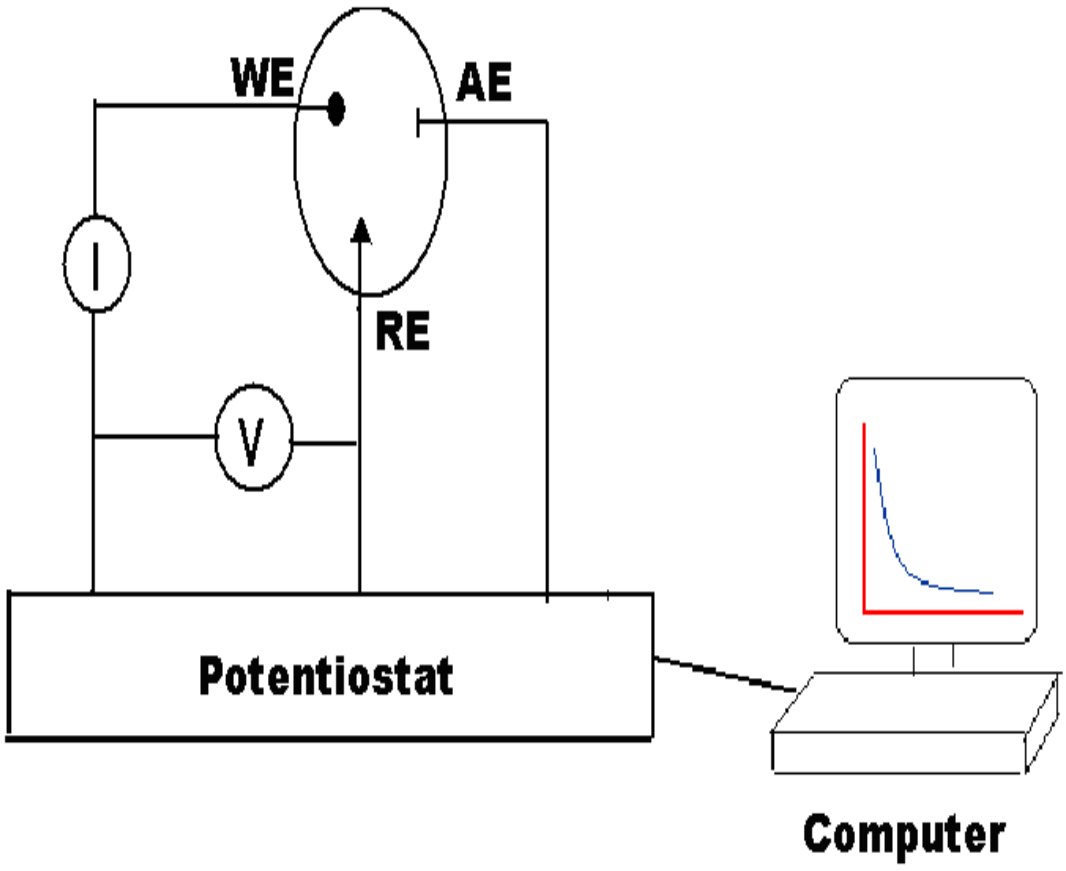


Figure 9: Basic components of the *in-vitro* amperometric experimental set up used in this research.

A typical electrochemical cell consists of three electrodes (Figure :9):

1. The working electrode (WE) is the electrode at which electrochemical oxidation or reduction reaction takes place.

2. The reference (RE) electrode has a constant potential against which the potential at the working electrode is measured

3. The counter or auxiliary electrode (AE) works as an electron sink that allows excess current generated at the working electrode to pass through. This helps to achieve an over all current balance.

Experiments are performed by immersing the three electrodes in an ionic buffer solution as electrolyte. The potentiostat is used to control the applied potential between the working and the reference electrodes and measure current as it passes through the auxiliary and the working electrodes during an electrochemical reaction. The potentiostat is connected to a computer where the amplified signal is displayed and data is recorded rapidly and efficiently.

1.6 Glucose Sensor for in vivo studies

For many years glucose sensors have been utilized to detect blood glucose levels in animals and humans [33; 49; 51; 56; 64]. The present study was based on the performance of glucose sensor in brain to quantify extracellular glucose concentrations.

Brain uses glucose and lactate as energy sources. But, glucose is the major source of energy in the brain. The brain does not have stored glucose as muscles do. Therefore, glucose is supplied from liver through the blood [65]. Other than being an energy source, glucose plays an important role that it regulates neuronal processing. Recent studies show that chronic starvation for glucose in brain could trigger degenerative brain disorders such as Alzheimer's disease [65; 66]. Extracellular glucose levels in a normal animal brain have been reported as 2-3 mM range. However, literature can be found that reports glucose levels higher than 5mM on brain regions that glucosensing neurons (the neurons sensitive to glucose) are located such as the arcuate nucleus [66].

In this study, Pt microelectrodes were constructed and developed implantable glucose biosensors using non-conductive and conductive polymers and glucose oxidase enzyme. These biosensors were tested and optimized *in vitro* and *in vivo* for glucose measurements.

Chapter 2: Materials and Methods

2.1 Materials used

2.1.1 Construction of Pt wire type electrode

Bare diameter 0.007" Teflon (PTFE) coated Pt/Ir wire (90% Pt and 10% Ir) was obtained from Medwire (Vernon, NY). Colloidal silver liquid (type D550) was obtained from Fujikura Kasei Co. Ltd, (Tokyo, Japan). Heat shrinkable plastic tubes (Polyolefin) were purchased from 3M Electronics/Electrical, (Austin, TX). Curing agents Epi-cure3234 and EPON 815C were purchased from Miller Stephenson Chemical Co.

2.1.2 Construction of Pt –fused silica electrode

Pt/ Ir wire (90% Pt and 10% Ir) (25µm diameter) was purchased from Goodfellow Cambridge Limited (Huntingdon,UK). Outer diameter 104µm-105µm and Inner diameter 42µm fused silica capillaries purchased from Polymicro Technologies. Hypo Tubes (outer diameter 0.02 inches and inner diameter 0.010 inches) were purchased from Small Parts Inc. (Miramar, FL)

2.1.3 Material used for enzyme immobilization and electrode testing

The enzyme glucose oxidase from *Aspergillus niger* (EC1.1.3.4, 277 units/ mg) was obtained from Biozyme International (San Diego, CA). Triton-X-100, (3-aminopropyl) trimethoxysilane and analytical grade d-glucose, uric acid and ascorbic acid were purchased from Sigma-Aldrich Inc., (St. Louis, MO). Oxygen permeable

polyurethane (BBF 11910/S.5) was obtained from The Polymer Technology Group Inc. (Berkeley; CA). K_2PtCl_6 (98% FCC grade) pyrrole and 5% aliphatic Nafion® were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Analytical grade 30% H_2O_2 solution purchased from Fisher Chemicals, (Pittsburgh, PA). Larconco- UV ultrapure water was used to prepare all the aqueous solutions.

2.1.4 Apparatus

A CH Instruments (Austin, TX, USA) model CHI814 electrochemical analyzer was used for all of the electrochemical experiments in this study. The electrochemical data provided by the CH Instrument was displayed, recorded and analyzed by using IZArc software.

2.2 Electrode construction

2.2.1 Construction of 176µm diameter Pt-Wire type microelectrodes

Teflon coating of one side of the Pt/Ir wire was exposed and glued to a standard wire using colloidal silver liquid. The connection was secured using heat shrinkable plastic tubes. Next, the other side of the Pt/Ir wire was cut and the coating was removed 300µm +/- 50 µm to expose the wire.

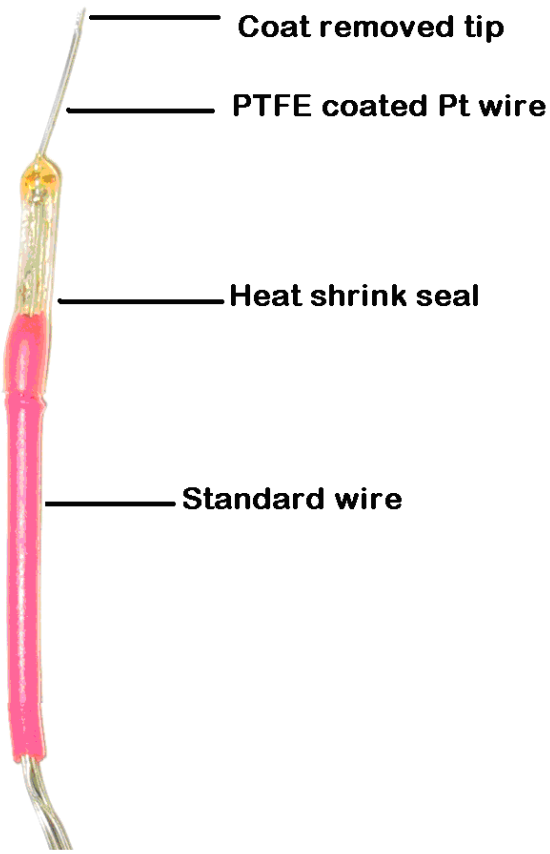


Figure 10: Pt wire type electrode (176μm diameter)

2.2.2 Construction of 25µm diameter Pt-Fused silica microelectrodes

Pt wire (25µm) was manually inserted into a fused silica capillary tube (ID 42µm). Then, one side of the fused silica tube with excess Pt wire was inserted into a 1cm long Hypo tube and the connection secured with super glue. The other side of the fused silica with excess Pt wire was cut while been magnified with a microscope to make the cylindrical electrode 300nM +/- 50 nM long. The tip of the Pt cylindrical electrode was sealed with epoxy as describe below.

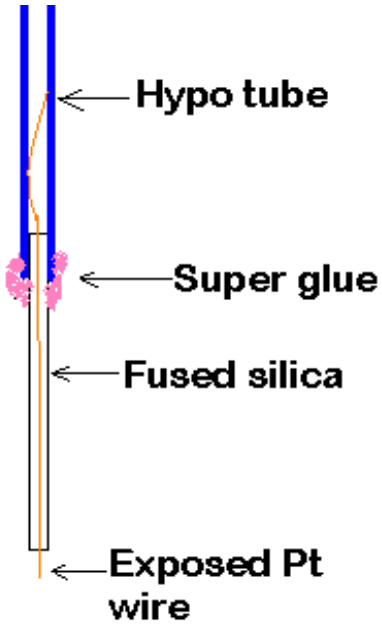


Figure 11: A schematic representation of 25µm diameter Pt- fused silica microelectrode

2.2.3 Microelectrode Curing

A curing solution was made by mixing 100 parts of EPON 815 (2.0g) with 12 parts of Epi-cure 3234 (0.24 g). The tip of the electrodes was dipped in the solution for 5 seconds and excess epoxy was washed off with Toluene. Then, the electrodes were kept at room temperature for 5 days to complete curing process. To seal the tip of the Pt wire with silica tube 25μm Pt-fused silica electrodes were coated epoxy with this method.

2.2.4 Phosphate Buffer Saline (PBS) 50mM Solution

PBS solution was made by dissolving 12.3 g of $Na_2HPO_4.7H_2O$, 2.2 g of KH_2PO_4 and 5.85g of NaCL in 1.0L of doubly distilled water. The pH of the solution was adjusted to 7.4 by adding either 2.0M HCl or 2.0M NaOH. All the chemicals that used in this study are analytical grade. All of the in-vitro experiments in the present study were carried out in a standard three electrode electrochemical cell.

2.2.5 Testing electrodes in PBS solution to evaluate connection

Electrodes were tested in constantly stirred PBS solution using amperometry i-t curve mode using a CHI instrument 814 (6A829) to test them for a good electrical connection. A potential of +0.6 V was applied against Ag/AgCl reference (3 M NaOH, BAS model MW 2030) and a Pt wire was used in all experiments as counter electrode. If the electrodes had a stable steady state background current, then they had a good connection between Pt wire and the outer circuit. If the epoxy seal was

compromised or the gap between the Pt wire and fused silica tube is not sealed, this could allow solvents to pass into silica tube resulting a current overflow indicating a bad electrode. Only the electrodes with stable steady state currents were used.

2.2.6 Microelectrode Cleaning:

The microelectrode Pt tips were cleaned [29; 49] by consecutively soaking for 15 minutes in the following solutions at room temperature:

(1) A aqueous 1:1 mixture of 1M HNO_3 and 1M H_2SO_4 solution,

(2) A aqueous solution of 1M NaOH

(3) Polished (18.3MΩ) water

2.3 Fabrication of Glucose Sensor

2.3.1 Non-conductive Polymer based glucose sensor

Phosphate buffer solution (at pH 7) was prepared by mixing 17.5ml of 27.6g/L mono-basic sodium phosphate-monohydrate solution and 30.5mL of 28.4g/L di-basic sodium phosphate solution. Then, distilled water was added until the liquid level is 200mL and the pH was adjusted to 7.

Glucose oxidase electrodeposition solution [49] was freshly prepared each time by mixing together 20mg of glucose oxidase enzyme, 200μL of 8mM triton-X-100 and 1.8mL of (pH 7) phosphate buffer solution. The enzyme is readily soluble in phosphate buffer and forms a clear yellow colored solution. Triton-X-100 was added

to the aqueous enzyme solution and stirred until fully dissolved. The working electrode was held at +1.3V constant potential vs a Ag/AgCl reference electrode for one hour to deposit the enzyme directly on the surface of the Pt microelectrode. A conventional three electrode system was used. After deposition of enzyme, the electrodes were stored dry in a refrigerator at 4^0C overnight before the polymerization of phenol step.

A stock phenol solution was prepared by dissolving 0.0423g of phenol in 30mL of distilled water, 0.2238g of KCl dissolved in 30mL of distilled water and mixing the two solutions with 30mL of pH 7 phosphate buffer. The phenol solution was refrigerated at 4^0C and used for electropolymerization. The solution was degassed with argon or nitrogen for 20 minutes prior to use and an argon saturated environment was maintained throughout the experiment. A constant potential of +0.9V (versus Ag/AgCl reference electrode) was applied for 15 minutes to apply a thin layer of polyphenol on the glucose oxidase enzyme coated microelectrode.

Right after the phenol coating was deposited, electrodes were electrochemically coated with (3-aminopropyl) trimethoxysilane for 15 minutes by applying a +0.6V potential against a Ag/AgCl reference. Next, polyurethane solution was prepared by dissolving 0.15mL of polyurethane in 4.9mL of tetrahydrofuran (THF) and 0.1mL of diethylflouramine (DMF). The electrodes were dipped three times in the solution and left to air dry. The electrodes were refrigerated at 4^0C overnight prior to being tested.

2.3.2 Conductive polymer based Glucose Sensor

The electrochemical cell was prepared with 5.0 mL of phosphate buffer saline (PBS) as the electrolyte solution in contact with three electrodes, a Ag/AgCl reference electrode, a Pt wire as the counter electrode, and a Pt wire type microelectrode as the working electrode. The electrodes were tested by amperometry i-t curve mode using CHI detector. A constant potential of +0.6 V was applied on the working electrode against the Ag/AgCl reference for 30 seconds. The solution was constantly stirred with a Teflon coated magnetic stirring bar throughout the experiment. The electrodes that had a diffusion controlled and steady state currents were chosen to develop as a glucose sensor.

2.3.3 Vacuum Distillation of Pyrrole

Pyrrole was fractionally distilled immediately before the electrodes were fabricated. Calcium hydride was used as a drying agent. Pyrolle and calcium hydride solutions were heated in a oil bath at medium high temperature (about 40 ^{0}C) while the solution was stirring. Reduced pressure was applied. Distilled pyrrole was stored in a nitrogen saturated container until use it.

Immobilization was accomplished by following a previously described method [64]. Immobilization solution was made with 0.5mM (0.003g) K_2PtCl_6, 0.2M (0.206g) of freshly distilled pyrrole and 1mg/mL (0.015g) of glucose oxidase enzyme were dissolved in15.0mL of ultrapure water.

Electrodes were cleaned and the tips of electrodes were dipped in freshly prepared immobilization solution for few seconds and left air dry. The dip evaporating procedure was repeated twice.

2.4 Electrode testing and Calibration procedures

2.4.1 Pt-wire type glucose sensor

A concentration of 250mM glucose solution was prepared by dissolving analytical grade (1.125g) D-glucose in 250mL of Ultra-pure water. The stock solution was kept in a refrigerator for about 24 hours to allow equilibration of the anomers. [49].

To evaluate sensor performance, the Pt wire-type glucose sensor was kept under +0.6V constant potential against a Ag/AgCl reference electrode. A 176μm diameter Pt wire was used as the counter electrode. Electrodes were equilibrated in phosphate buffered saline (PBS) until a stable baseline current, lasting for a few minutes, was measured. Then, the electrodes were tested in 5mL of PBS by adding 100μL of 250mM glucose to the electrode solution to a final concentration of 5mM glucose. Glucose was then added two more times in 100μL aliquots at 100 second-intervals. The solution was stirred throughout the experiment. Unless otherwise noted all experiments were performed inside a Faraday cage to minimize external electrical noise.

2.4.2 Linearity of Sensor

The linear range of the response to glucose was determined by successive addition of glucose to the cell and detecting current signals corresponding to the concentrations of glucose in the solution. A series of glucose solutions (5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM and 45mM) were prepared using analytical grade D-glucose. A volume of 5.0mL PBS solution was used in three electrode electrochemical cell. Electrodes were kept in a constant potential of +0.6V potential and the electrodes were allowed to reach the steady state. A volume of 100μL from 5mM glucose solution was injected and allowed the current to be stable. The final concentration of glucose in the cell was 0.1mM. The solution was stirred during the experiment.

Then, another 100μL volume of 10mM glucose was added to the electrochemical cell. This procedure was repeated for other glucose solutions and recorded the current increase due to the different concentrations of glucose.

2.4.3 Selectivity of Sensor

The electrodes were tested with potential electroactive compounds that interfere with the signal produced by oxidation of glucose. Ascorbic acid and uric acid are known to present in the blood and the brain in large quantities that possibly interfere with the signal.

Solutions of 250mM glucose, 5.0mM ascorbic acid, and 5.0mM uric acid were freshly prepared before the experiments. A volume of 5.0mL PBS solution was

used as the conducting electrolyte in the electrochemical cell. A constant potential of +0.6V on the amperometry i-t curve mode was applied to glucose sensors versus a Ag/AgCl reference electrode while constantly stirring the solution. A Pt wire was used as counter electrode. Electrodes were allowed to reach steady state baseline currents for several minutes. A 100µL aliquot from the 250mM glucose solution was injected into the cell after the electrode has reached its steady state. The final concentration of glucose in the cell was 5mM. After 100 seconds 100µL of ascorbic acid was injected. This procedure was repeated for two more injections of glucose and uric acid. The final concentration of uric acid and ascorbic acid after injection was 0.1mM. The solution was constantly stirred through out the experiment using a Teflon® micro stir bar.

2.4.4 Stability of Sensor

Seven wire-type electrodes were freshly prepared and developed into glucose sensors by coating enzyme and other three coatings. After 24 hours in a refrigerator at 4^0C, the glucose sensors were tested with 250mM of glucose using amperometry. A volume of 5.0mL PBS solution was used as the electrolyte in the electrochemical cell. A constant potential of +0.6V vs. Ag/AgCl was applied. A standard three electrode electrochemical cell was made by adding a Pt wire as the counter electrode. Sensors were allowed to reach the steady state baseline currents for several minutes and a 100µL volume of 250mM glucose solution was injected while the solution is constantly stirring. After the glucose test, sensors were stored dry in a refrigerator at

4^0C until testing them the next day at about the same time. The stability test was performed repeatedly for 50 days at about the same time continually for these seven electrodes.

2.4.5 Sensor Response Time

Biosensors were tested in a flow cell with a sample flow rate of 2mL/min. The flow path of the cell was adjusted to 0.1mM KCl buffer (pH=7) and biosensors were kept in a +0.6V vs Ag/AgCl. A Pt wire was used as the counter electrode. The flow path was changed to 250mM glucose to pass through the channel after the sensor has reached a steady background signal. Sensor response for 250mM glucose was recorded. The temporal resolution was defined here as the time it takes to reach 90% of the peak signal from 10% of the peak signal, and was calculated for five glucose sensors. The average temporal resolution time of the glucose sensor was 5± 1.3 seconds.

2.5 Pt-Fused silica conductive polymer based glucose sensor

2.5.1 Polymerization of Pyrrole

To determine whether electrochemical or chemical polymerization resulted in the best electrode performance, a set of freshly prepared Pt/PPy/GOx modified electrodes were polymerized electrochemically and a second set of electrodes were allowed to polymerize chemically.

2.5.2 Electrochemical Polymerization

$LiClO_4$ was purchased from Sigma-Aldrich Inc. (St. Louis, MO). A set of electrodes were coated with Pt/PPy/GOx as described previously.0.1M $LiClO_4$ solution was prepared by dissolving 0.0532g of dry $LiClO_4$ in 5mL of ultra pure water. Constant potential of + 0.75 V vs Ag/AgCl was applied for 100s. A Pt wire was used as the counter electrode. Pyrrole on the electrode surface was electrochemically polymerized. The electrodes were kept in a refrigerator at 4^0C for 24 hours before testing them.

2.5.3 Chemical Polymerization

A set of freshly prepared clean electrodes were dip coated with immobilization solution three times as described previously. Then, the electrodes were kept in a refrigerator at 4^0C for 24 hours allowing for K_2PtCl_6 catalyzed chemically driven polymerization.

2.5.4 Sensor Calibration with glucose

0.1M KCl phosphate buffer was prepared by mixing 0.0459 M (12.3 g) of Na_2HPO_4, 0.0162 M (2.235g) of NaH_2PO_4 and 0.10 M (7.456g) of KCl in1L of ultra-pure water. The pH of buffer was adjusted to 7.0 by either adding 2.0M HCl or 2.0M NaOH.1.0M D-glucose solution was made and kept in a refrigerator at 4^0C for 24 hours to equilibrate.

A potential of +0.7 V vs Ag/ AgCl reference was applied to both types of electrodes when reached a stable baseline current then 20μL of 1.0M glucose was injected in two intervals and current response was recorded. The experiment was repeated for 3 days.

2.5.5 Application of Nafion® on Sensors

An experiment was performed to determine if Nafion® should be applied inside the GOx/Pt/PPy layer which means directly on the electrode surface or outside.

Two sets of new electrodes were made.

Electrode set (1): 5% Nafion® was dip coated on clean electrode surface and left air dry for few minutes. Then, Pt/PPy/GOx immobilization solution was dip evaporated three times. Electrodes were stored dry in 4^0C for 24 hours before testing them.

Electrode set (2): Pt/PPy/GOx immobilization solution was dip coated on clean electrodes as described previously. 5% Nafion® was dip-evaporated and left air dry at 4^0C for 24 hours.

Concentrations of 250mM glucose, 5.0mM ascorbic acid, and 5.0mM uric acid solutions were freshly prepared using analytical grade compounds purchased by Fisher Chemicals, New Jersey. A volume of 5.0 mL, 0.1M KCl phosphate buffer was used in the electrochemical cell. A constant potential of +0.6V (versus Ag/AgCl reference) was applied on Nafion® coated Pt/PPy/GOx electrodes while stirring the

solution. The electrodes were allowed to reach steady state baseline currents for several minutes and 100µL of ascorbic acid solutions was injected so the final concentration of analyte in the cell was 0.1mM. The currents were recorded. The same procedure was repeated with fresh different set of Nafion® coated Pt/PPy/GOx electrodes with uric acid and glucose. The change in current was recorded.

2.5.6 Optimization of Nafion® Concentration

A series of aqueous Nafion® solutions were prepared from 5% aliphatic Nafion® solution [22]. Newly prepared Pt/PPy/GOx electrodes were dip-coated with 0.25%, 0.50%, 0.75% and 5.0% Nafion® solutions. Electrodes were kept in 4^0C for 24 hours before testing them. Bare Pt electrodes were also tested with H_2O_2 for comparison.

2.5.7 Performance of sensor with H_2O_2

A solution of 5.0 mM H_2O_2 was freshly prepared by dissolving 0.0567g of stock solution in 100mL of ultrapure water. Inner and outer Nafion® coated Pt/PPy/GOx electrodes were freshly prepared. A volume of 5.0 mL of phosphate buffer with 0.1M KCl was used as electrolyte in the electrochemical cell. A constant potential of +0.6V vs Ag/AgCl reference was applied while stirring the solution. Electrodes were allowed stable baseline currents for several minutes before injecting analyte. 100µL sample of H_2O_2 solution was injected and created a concentration of 0.1mM within the cell. The current increases due to H_2O_2 injections were recorded.

Bare pt electrodes were also tested with H_2O_2 solution to compare data as the same procedure described above.

2.5.8 Sensor performance on glucose

Freshly prepared 0.75% Nafion® coated electrodes were used as working electrodes. A potential of +0.6V was applied to working electrode against Ag/AgCl reference. A series of D-glucose solutions (0.25M, 0.5M, 0.75M, 1.0M, 1.25M, 1.5M, 1.75M and 2.0M) were prepared. PBS buffer, 5.0mL in volume, was used as the supporting electrolyte in the three electrode electrochemical cell. A 100µL volume of each glucose solution was injected after the electrode has reached its steady state. The final concentration of glucose in cell was 5.0mM. The solution was stirred during the experiment.

2.5.9 Response Time of Pt/PPy/GOx sensor

Pt/PPy/GOx sensors were tested in a flow cell with a sample flow rate of 2mL/min. At the beginning of the experiment, the flow cell was perfused with 0.1mM KCl buffer (pH=7) while a +0.6V constant potential was applied to the biosensors. Biosensors were allowed to reach the steady state before 250mM glucose was introduced through a 3-way valve. Sensor response for 250mM glucose was recorded. The temporal resolution was defined as the time required for the signal to increase from 10% to 90% of the total response. Pt/PPy/GOx sensors had a response time of 1.4s ±0.5s.

2.6 In-vivo Procedures

2.6.1 Animal handling

Sprague Dawley rats, 4-5 months old were purchased from Charles River. Animals were housed at the University of Kansas Animal Care Unit. Rats were kept in standard plastic cages containing non-aromatic wood chip bedding. Food and water were supplied adlibitum. These animals were housed under a 12 hour light/dark cycle at the temperature of 75^0F and 75 ± 25% humidity. All animal procedures were approved by The University of Kansas Institutional Animal Care and Use Committee.

2.6.2 Stereotaxic Surgery for Implantation of Glucose Sensor

Sprague Dawley rats weighing 250-500g were anesthetized by ip injection of urethane at a dosage of 1.5 g/kg animal weight prior to surgery. The top of the head area was shaved and the rats were placed in a stereotaxic apparatus purchased from David Kopf Instruments, Tujunga, CA, USA. Rats were kept on a heating pad during the experiment to keep the body temperature stable. Care was taken to minimize the pain and suffering of the animal during the surgery and experiments.

An incision was made along the center of scalp using a clean scalp and the skin and the tissues underneath it were removed to expose the skull. Three holes were drilled using a Dremel drill following stereotaxic coordinates AP -1.0, -2.0 ML, and -1.3 DV for the Ag/ AgCl reference electrode, +1.0 AP, +2.0 ML, and -1.3 DV for the glucose biosensor and the Ag wire auxiliary electrode at -1.0AP, +2.0 ML and -1.3 DV.

2.6.3 Glucose containing artificial cerebral spinal fluid (aCSF) Buffer

Artificial cerebral spinal fluid contain 7.36g of NaCl, 5.206g HEPES sodium salt,0.187g of $NaH_2PO_4.H_2O$, 0.186g of KCl,4.10g of $NaHCO_3$,9.0 g of glucose, 0.753g of $CaCl_2$ and 0.244g of $MgCl_2$ dissolved in 1L of ultra-pure water. The pH of solution was adjusted to 7.4 by addition of either 2.0M NaOH or 2.0M HCl.

2.6.4 Microinjection Procedures

Glucose sensors were pre-calibrated with 250mM glucose using a flow cell. Electrodes were connected to CHI instrument and +0.6 V was applied to working electrode/ glucose sensor against Ag/AgCl reference electrode.

After the pre calibration, the glucose sensor was attached to a micro syringe containing 5µL of 25mM glucose in aCSF solution. The distance between the glucose sensor and the microsyringe tip was about 100-200µm. The glucose sensor and micro syringe were lowered 2mm from the skull to reach the cortex. The reference electrode and the counter electrodes were positioned on the same level.

The electrodes were held at a constant +0.6V potential against the Ag/AgCl reference and glucose sensors were allowed to achieve steady state for 15-30 minutes. Once the electrodes were reached a stable back ground current signal, a 5µL volume of 25 mM d-glucose in aCSF buffer was injected to the close proximity of the sensor by gently pushing down the micro syringe. The current increase was recorded. After the experiment was completed the glucose sensors were post-calibrated with glucose.

Chapter 3: Results and Discussion

3.1 Non conductive polymer based glucose sensor

Our initial studies were aimed at the development and optimization of 176μm diameter glucose biosensors. These sensors were tested with 5.0mM glucose additions to PBS solution and had well-defined response to glucose as the electrode is comparatively larger than 25μm Pt/PPy/GOx based biosensors (Figure 12). The flow cell analysis of the glucose sensor has reported an average time it takes to increase the signal from 10% to 90% of peak current signal was 5 ± 1.3 seconds (Figure 13). The response time depends on the thickness of polymer layers and the time it takes for the analyte to pass through.

The sensors were tested in different concentration of glucose by successive additions to the same electrochemical cell. The current response for different glucose concentrations was recorded. As presented in Figure 14, glucose sensors had linear response to glucose at 0.4-1.0 mM ($R^2 = 0.9969$)

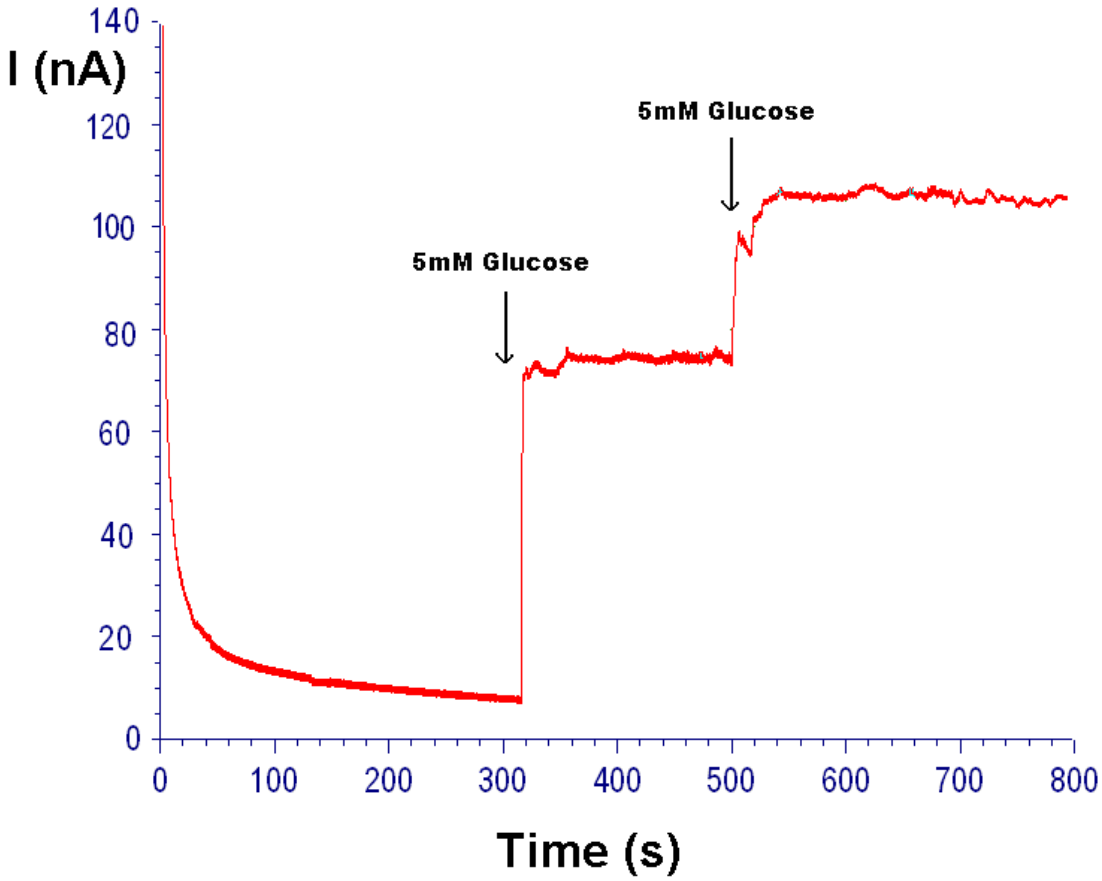


Figure 12: Typical current – time curve of 176µm non-conductive polymer based glucose sensor for two successive additions of 5.0mM glucose in PBS (pH 7.4, 50mM). Sensors were kept at +0.6V constant potential (vs. Ag/AgCl).

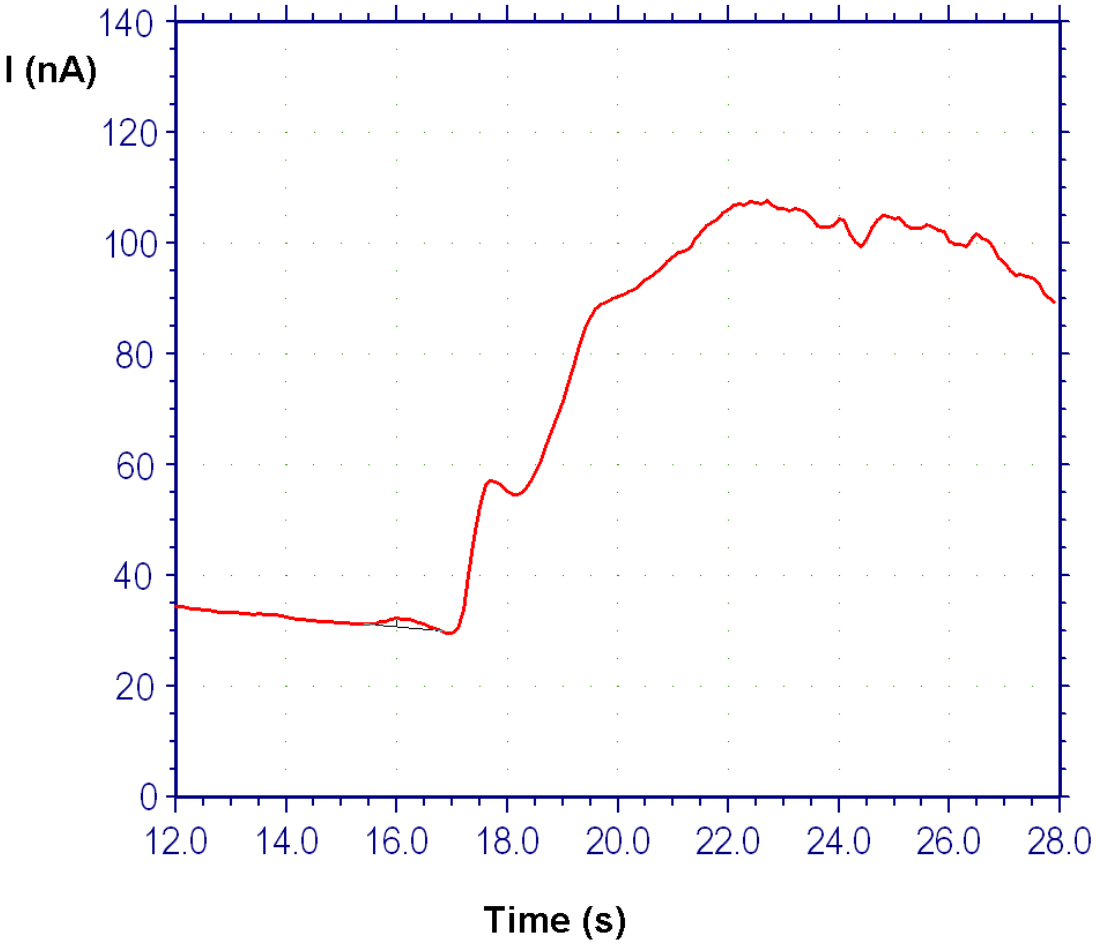


Figure 13: Typical current – time curve of 176µm non-conductive polymer based glucose sensor for flow cell analysis. Current increase is due to the additions of 250mM glucose. Sensors were kept at +0.6V constant potential (vs. Ag/AgCl).

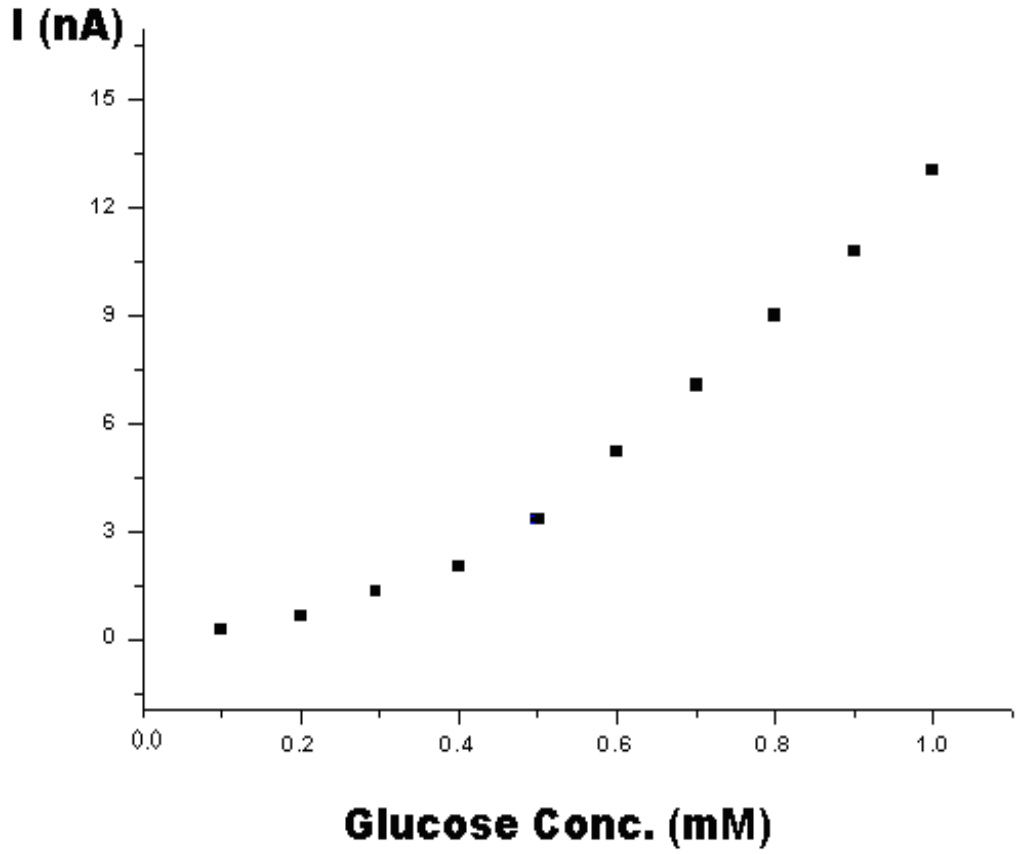


Figure 14: Response of 176μm glucose sensor against standard addition of glucose to pH 7.4 PBS solution. The final concentration of glucose in the cell after addition of glucose is from 0.1mM glucose to 1.0mM. Sensors were kept at +0.6 V (vs. Ag/AgCl) (n=2) (R^2 for 0.4-1.0mM is 0.9969)

Ascorbic acid and uric acid are present in biological samples and electrochemically active when a potential step is applied. Perm-selective coating on glucose sensor prevents these molecules reaching electrode surface. The current generated by oxidation of these species interfere with the signal produced by the analyte.

On the other hand, H_2O_2 produced by the GOx enzyme catalytic reaction is consumed by ascorbic acid and converts it into dehydroascorbic acid (equation 5) which does not have electroactive properties [88]. This also causes in reduction in the analyte signal.

$$H_2O_2 + \text{Ascorbic acid} \longrightarrow 2\,H_2O + \text{Dehyroascorbic acid} \quad (5)$$

Non-conductive polymer based glucose sensor exhibited significant discrimination over ascorbic and uric acids providing virtually no change in current on addition of both 0.1mM ascorbic acid and 0.1mM uric acid (Figure.15) However, this method was tested on 25μm Pt wire microelectrode. The microelectrodes coated only glucose oxidase enzyme, had current signals for glucose. However, the microelectrodes with enzyme and polymer coatings had very little, or no current increase for glucose. It can be assumed that the thickness of the three layers is large enough to result in low currents that arise from the oxidation of H_2O_2.

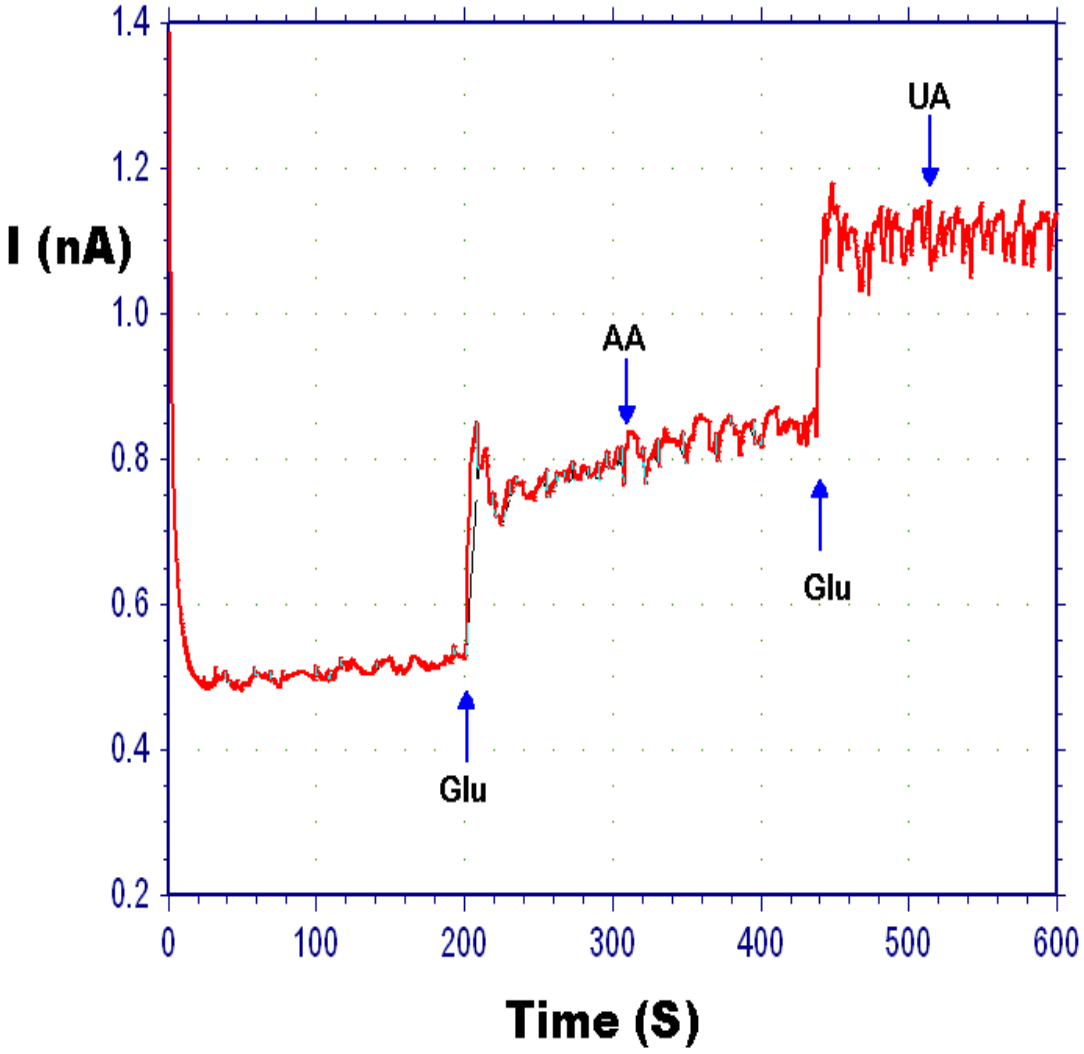


Figure 15: Current-time curve of 176μm glucose sensor for successive injection of 5.0mM glucose 0.1mM ascorbic acid and 0.1mM uric acid in pH 7.4 PBS solution. Sensors were kept at +0.6V (vs. Ag/AgCl)

Stability test for sensor response to glucose showed increased signals for the first 10 days. According to the statistical analysis, there was a significant increase in current compared to the day 1 and day12 ($p > 0.05$). Then, the sensitivity for glucose decreased gradually and significantly low current signals were observed by the day 50. The glucose sensor had about a life time of 50 days. These findings are compatible with previous studies that employed a similar approach [49]. The reason for longer stability of sensor is that the non-conductive polymer films are long lasting and does not deteriorate easily. The electrodeposited GOx enzyme has a thin coat of polyphenol. Polyphenol is a hydroxyl compound that has an ability to attract water molecules [49; 69]. Having an aqueous microenvironment on polyphenol, enzyme tends to maintain the catalytic activity for over 50 days. Even though the sensors were refrigerated dry after each test, the enzymes co-deposited with polyphenol retained the activity. Hence, the sensor was stable over time. The application of 3-ATS layer to make cross-links between phenols and silanes has demonstrated increased stability of phenol film [49].

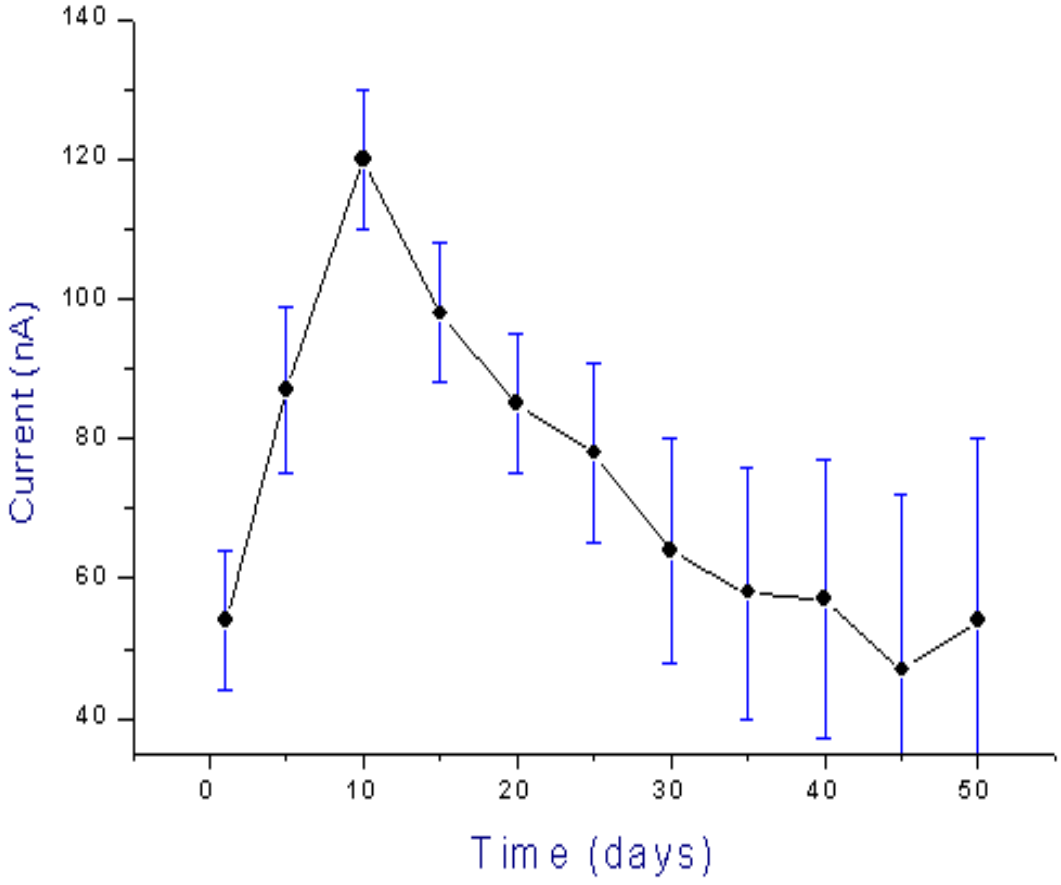


Figure 16: Long term stability of 176 µm glucose sensors with response to 5mM glucose in PBS solution (pH7.4). Working potential: +0.6V (vs. Ag/AgCl) (n=3)

3.2 Pt-Fused silica conductive polymer based glucose sensor

Conductive polypyrrole polymer based 25μm diameter Pt/PPy/GOx sensors were constructed and tested for glucose. The first test was to find the best method to polymerize pyrrole on the platinum surface along with GOx enzyme.

After performing chemical and electrochemical polymerization of pyrrole, chemical polymerization catalyzed by K_2PtCl_6 was chosen (Figure: 17). The response to glucose for chemically polymerized Pt/PPy/GOx sensors and electrochemically polymerized Pt/PPy/GOx sensors had no apparent difference in current response except that the signals obtained 24 hours after dip-coating electrodes appeared to be greater using chemically polymerized electrodes compared to $LiClO_4$ electropolymerized electrodes. One potential cause for this difference may be denaturation of enzyme. The other potential cause is that the enzyme and electrochemically polymerized pyrrole may be leaching [64] .Therefore, chemical polymerization was chosen for further studies.

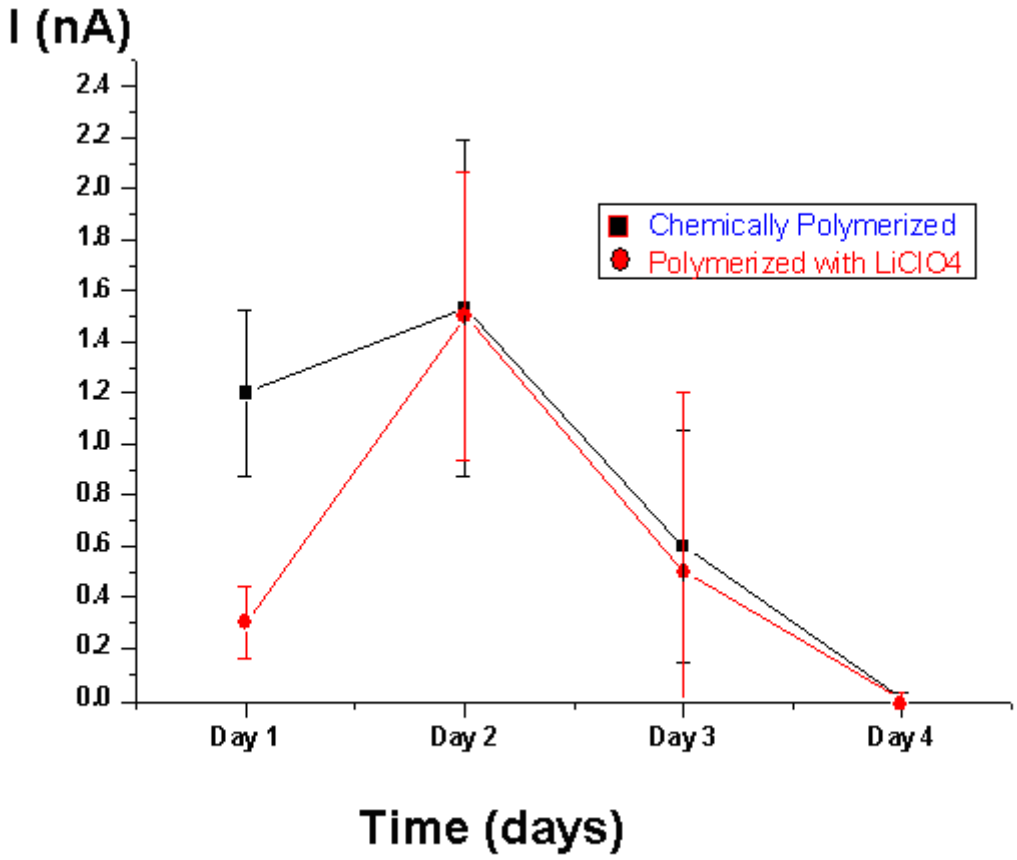


Figure 17: Stability comparison of 25µm Pt/PPy/GOx sensors polymerized electrochemically with $LiClO_4$ and 25µm Pt/PPy/GOx sensors polymerized chemically/ naturally without $LiClO_4$ by addition of 5mM glucose in PBS (pH 7.0, 0.1M). Sensors were kept in +0.6 V (vs. Ag/AgCl) (n=3)

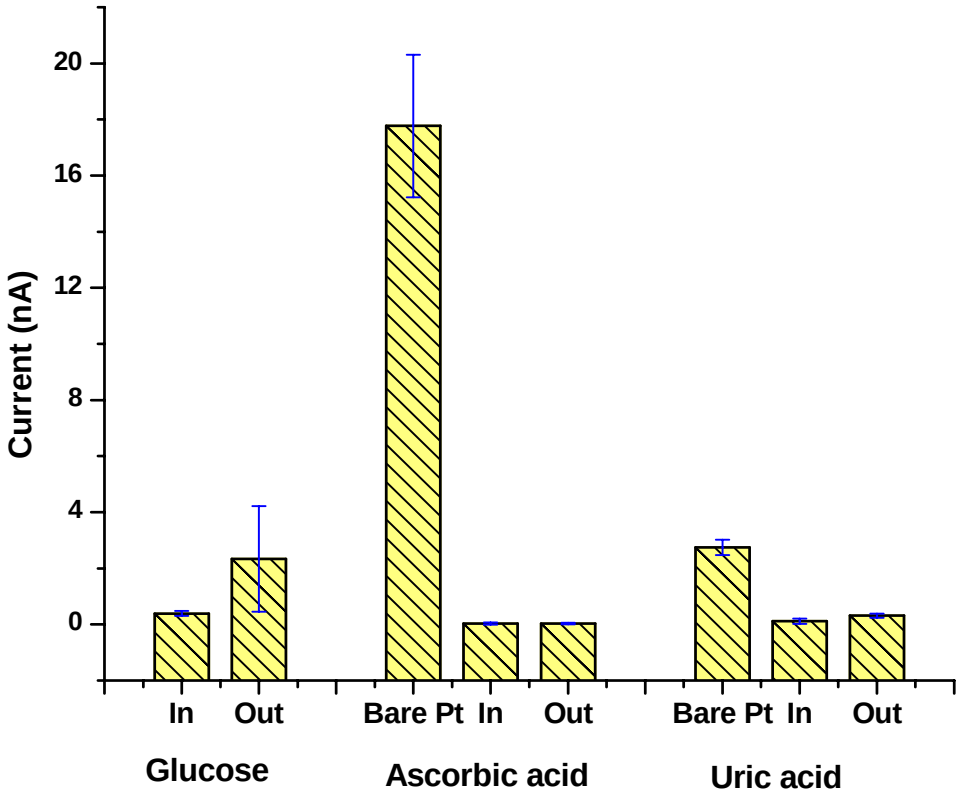


Figure 18: Response of 5% Nafion directly coated (In) 25µm electrodes and Nafion coated after the enzyme/pyrrole layer (Out) and 25µm bare Pt electrodes against 0.1mM glucose, 0.1mM ascorbic acid, and 0.1mM uric acid additions. Working potential is +0.6V against Ag/AgCl reference. (N=4-6 sensors)

In the mammalian brain, ascorbic acid is present at extracellular concentrations of 0.5-5 mM depending on the brain region [70]. Ascorbic acid may interfere with the glucose signal by consuming the H_2O_2 produced by the enzymatic reaction, resulting in a lower glucose-initiated current. Therefore, it is essential to block ascorbic acid entry to the electrode surface.

The experiments carried out with the sensors coated with Nafion® directly on the electrode surface and the sensors coated with Nafion® outside of the enzyme Pt/PPy/GOx layer did not have a considerable difference for ascorbic acid and uric acid (Figure 18). But the glucose test on sensors coated with Nafion® on the outside showed higher currents compared to the sensors coated with Nafion® directly on electrode surface. After performing interference rejection and glucose response tests of the inner and outer Nafion® coated electrodes, outer Nafion® coated electrodes were chosen as the best way to modify protective Nafion® film on electrodes. Coating Nafion® outside of the enzyme layer is consistent with most of the reports that have been published on Nafion® coated electrodes [7; 57; 59; 71].

A concentration of 5% Nafion® has proven effective in blocking ascorbic acid and uric acid (Figure: 19). A concern is that such a high Nafion® concentration impedes glucose from reaching the electrode surface.

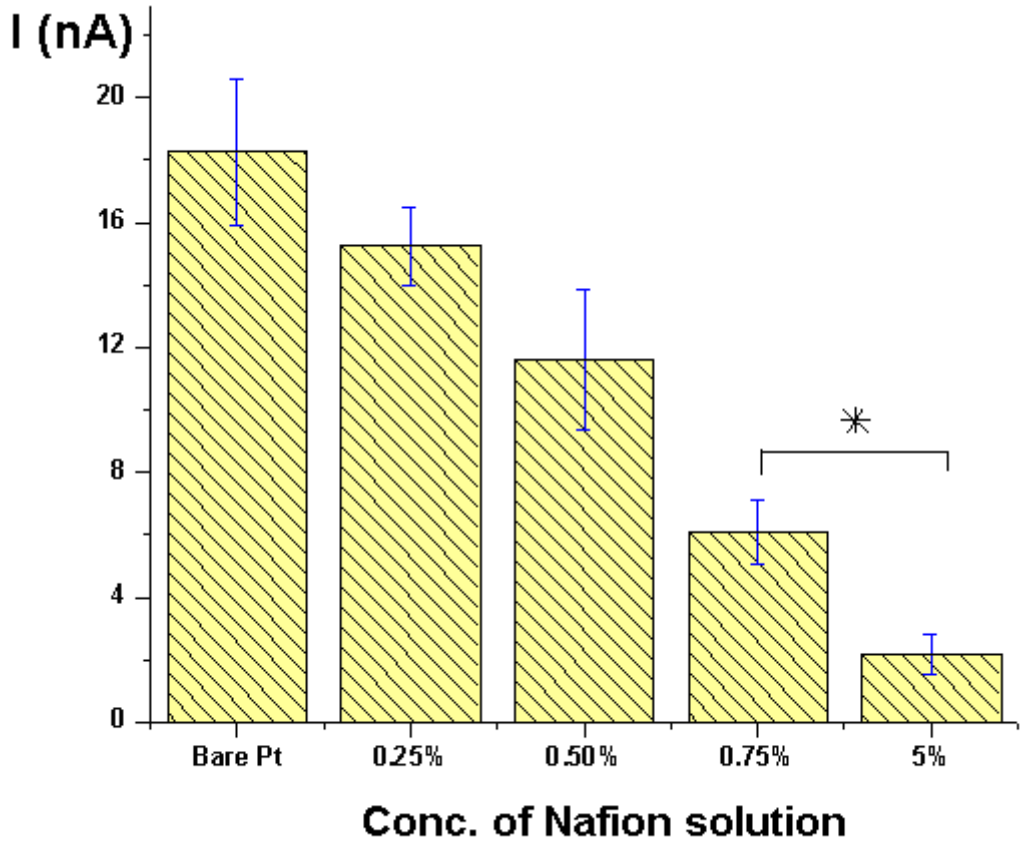


Figure 19: Current generated by oxidation of 0.1mM H_2O_2 against 25µm microelectrodes coated different concentrations (weight %) of Nafion®. (* P> 0.05) (n=4-6)

Having a higher concentration of Nafion® of electrodes not only prevented interfering species from diffusing towards the electrode, but it also decreased the permeability of analyte. Therefore, electrodes were tested with H_2O_2 and we compared the currents with currents generated by oxidation of H_2O_2 at bare Pt electrodes. During the enzyme catalytic decomposition of glucose, H_2O_2 is released. During a potential step H_2O_2 undergoes a two electron oxidation to form H_2O and O_2 while generating a current at the electrode. Therefore, current generated by the decomposition of H_2O_2 is proportional to the concentration of glucose in the sample. In this study, for determine the best Nafion® concentration, H_2O_2 was chosen as the analyte instead of glucose in order to eliminate the loss of signal due to enzyme denaturation or other enzyme-glucose related problems. Thus, the current comparison will be only based on diffusion control of the Nafion® layer. The selected Nafion® layer has to be thick enough to control interference species and thin enough to allow glucose / H_2O_2 molecules to pass through without decreasing current significantly.

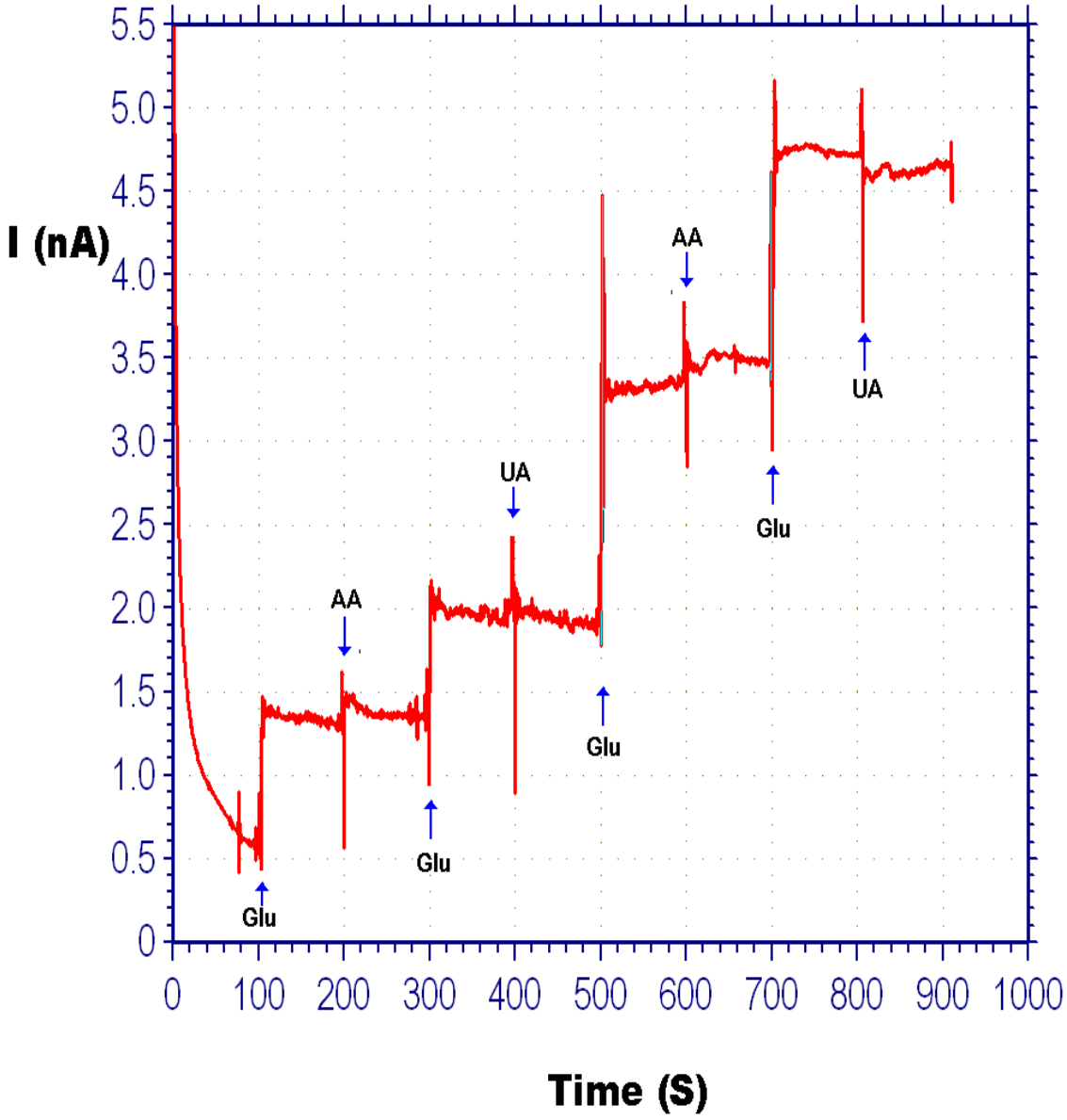


Figure 20: Typical current-time curve for 0.75% Nafion® coated Pt/PPy/Gox 25µm microelectrode upon sequential addition of 5.0mM D-glucose (Glu), 0.1 mM ascorbic acid (AA) and 0.1 mM uric acid (UA). Working electrode potential: +0.6V (vs. Ag/AgCl)

Nafion® with a concentration of 0.75% was chosen as the optimal concentration as the sensors coated with 0.75% Nafion® had 16% current signal for ascorbic acid and 8% current signal for uric acid compared with the glucose current signal and the sensor response to glucose did not decrease significantly (Figure 20). Additionally, the response time was calculated using the results obtained by the flow cell analysis. The average time for the glucose sensor to go from 10% to 90% of peak amperometric signal was 1.4 ± 0.5 seconds. (n=5)

Glucose sensors with a 0.75% Nafion® coat were tested *in vitro* for a range of glucose concentrations starting from 5mM to 45 mM in PBS as the electrolyte solution in the cell (Figure 22). The dynamic range of the sensor where it showed a linear response to the analyte was determined. The linear dynamic range of Pt/PPy/GOx sensor was 5mM to15 mM of glucose concentrations.

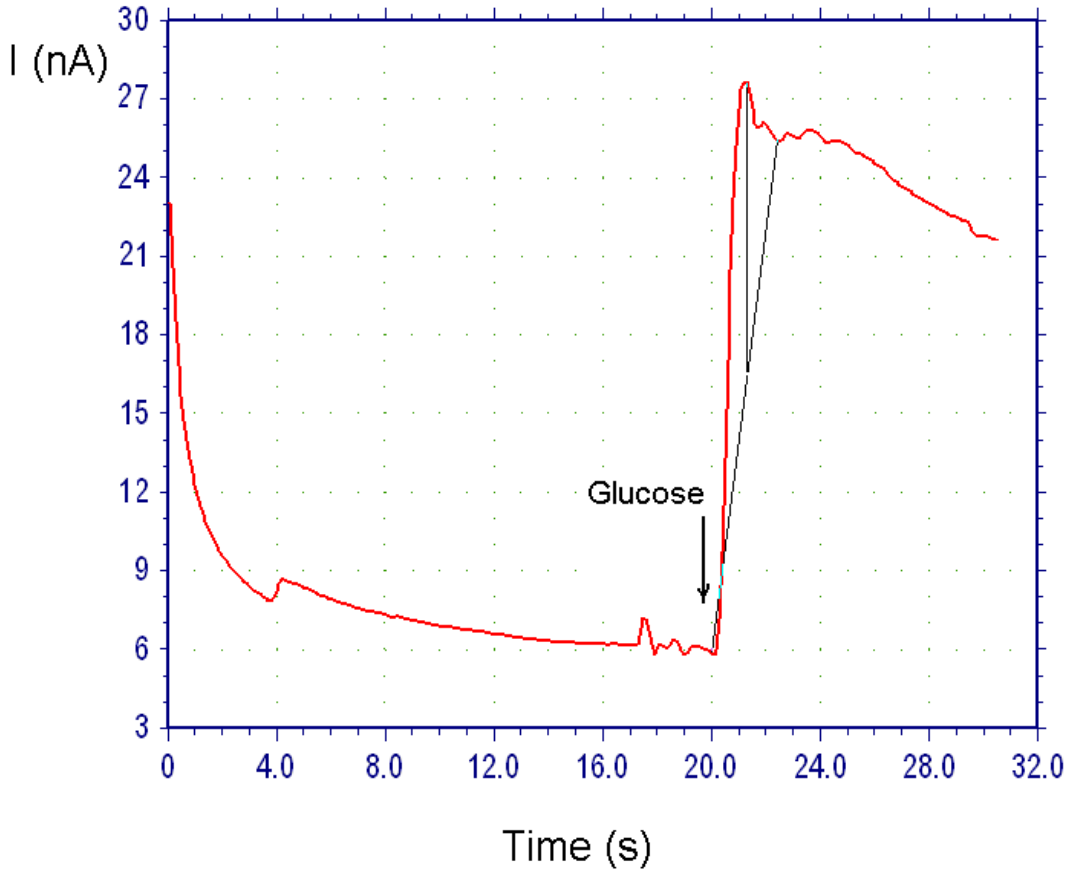


Figure 21: Typical current – time curve of 25µm Pt/PPy/GOx glucose sensor for flow cell analysis. Current increase is due to the additions of 250mM glucose at 20.0s. Sensors were kept at +0.6V constant potential (vs. Ag/AgCl). (n =6) The average time for the glucose sensor to go from 10% to 90% of peak amperometric signal was 1.4 ± 0.5 seconds.

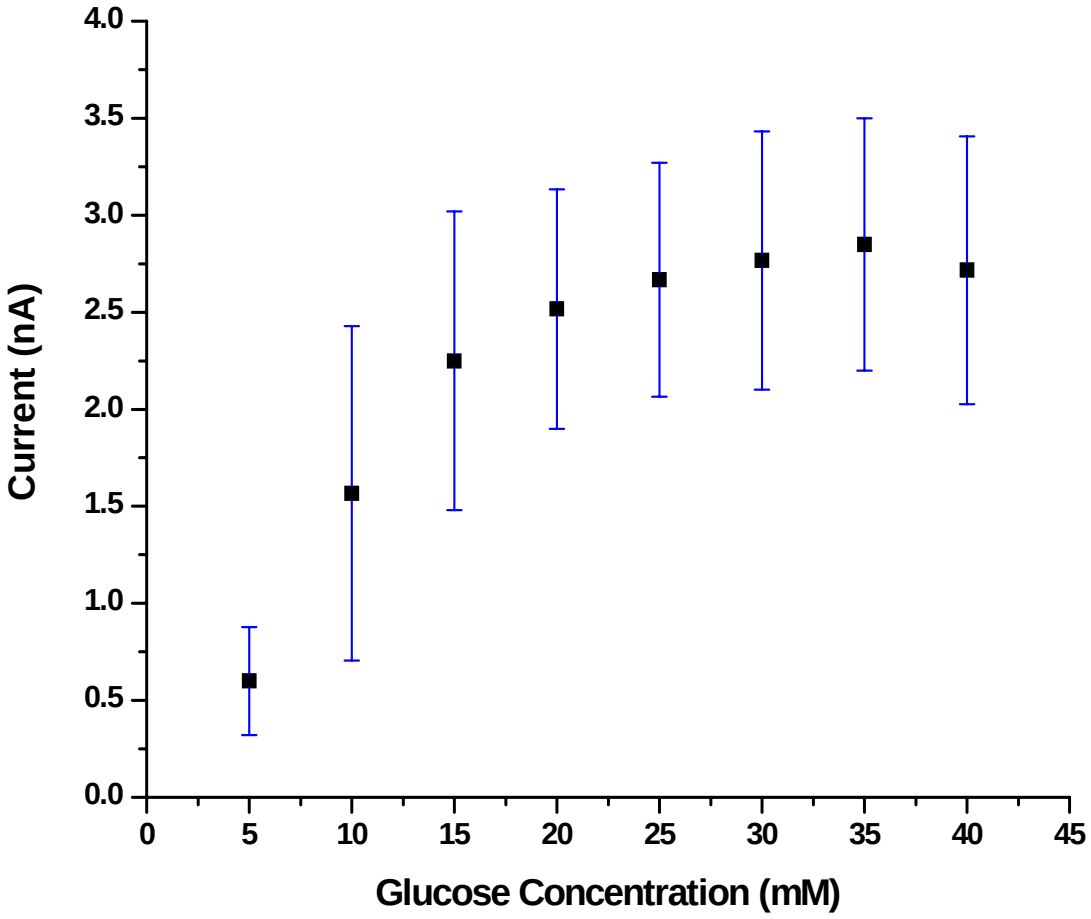


Figure 22: Calibration plot of 0.75% Nafion coated 25µm Pt/PPy/GOx biosensor against standard additions of glucose concentrations from 5mM up to 45mM in PBS (pH 7.0, 0.1M) (n=3) The sensors had linear response to glucose from 5mM to 15mM ($R^2 = 0.9999$).

Finally, glucose biosensors were tested to determine if Pt/PPy/GOx based 25μm diameter Pt biosensors can detect extracellular glucose concentrations *in vivo*. Biosensors were implanted in the cortex (figure 23) and 5μL volume of 50mM solution of glucose was injected from a microsyringe located about 200μm from the sensor. A current jump, likely due to the increased glucose concentration in the brain was observed (Figure 24). Although, fluctuations in current were present, a current increase of about +0.6nA was observed.

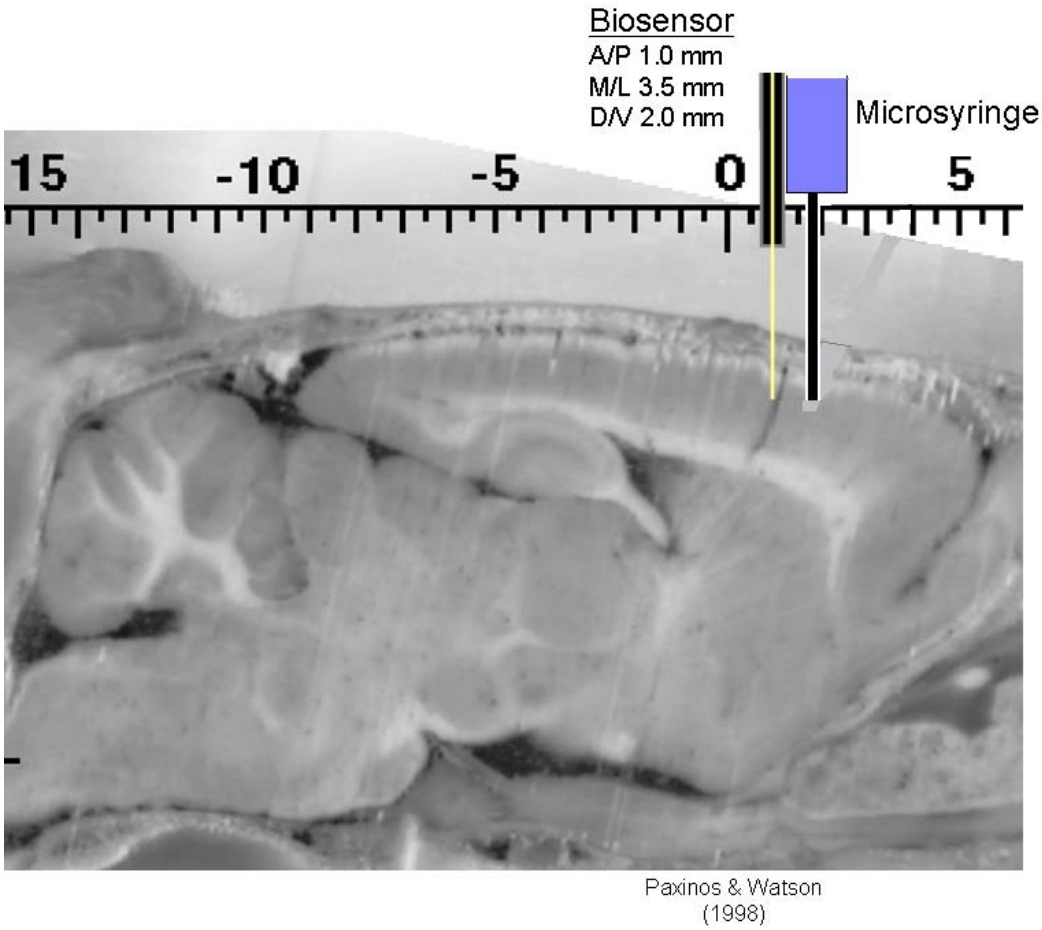


Figure 23: Diagram of 25µm Pt/PPy/GOx sensor and microsyringe implantation on a rat cortex.

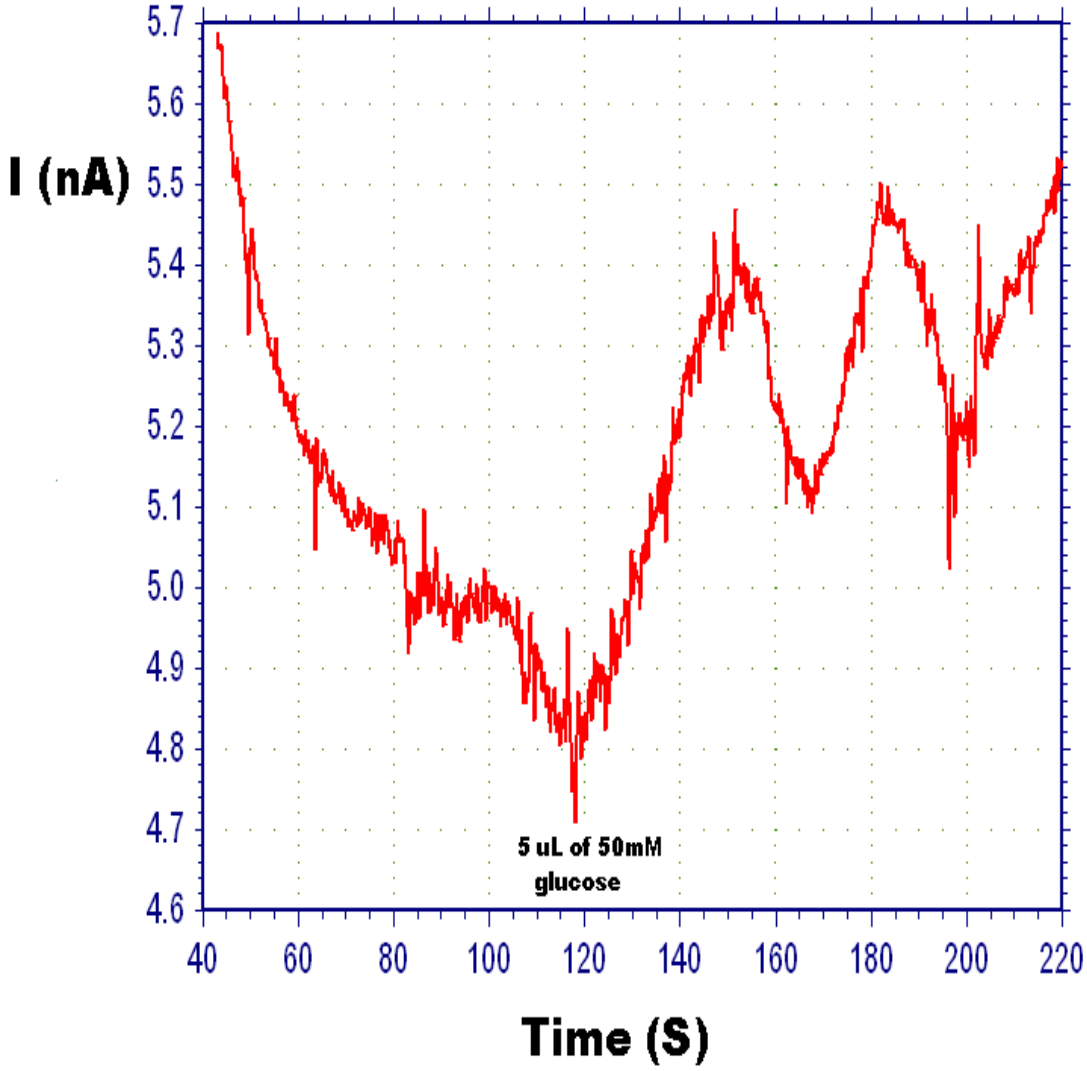


Figure 24: *In-vivo* Current Response of 25µm Pt/PPy/GOx sensor after injecting 0.25 mM glucose. (5µL of 50mM solution of glucose) Initially, the sensor had a decreasing background current and after the glucose was injected the current signal was increased. Then, the current signal was fluctuated between 5.5 -5.1nA.

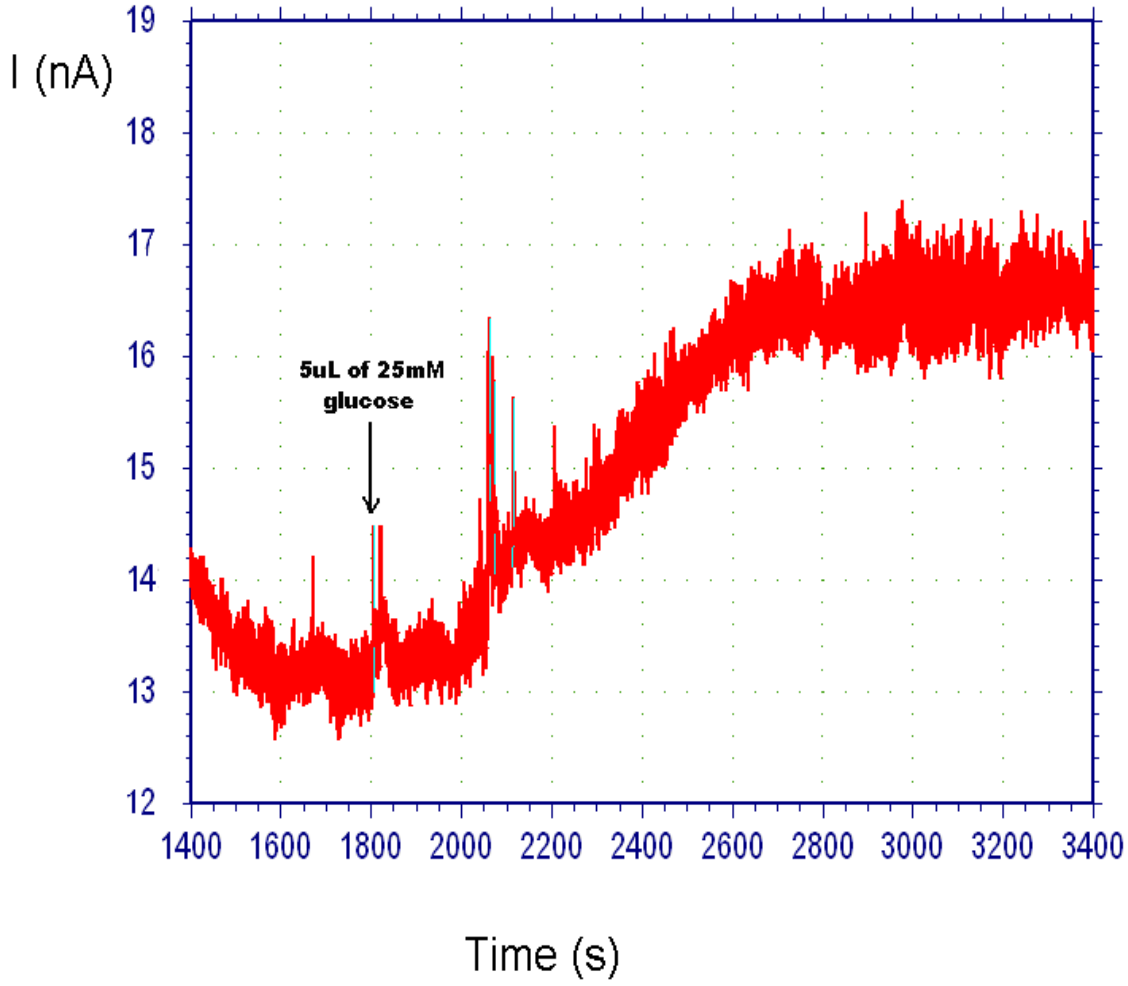


Figure 25: *In-vivo* Current Response of 176µm Pt/PPy/GOx sensor after injecting 0.125 mM of glucose. (5µL of 25mM solution of glucose)

For comparison, a 176μm glucose sensor was made following the same procedure used for the 25μm sensor and tested in the rat brain cortex. The glucose sensors were constructed with 176μm diameter Pt wire immobilized with Pt/PPy/GOx solution and dip coated with 0.75% Nafion®. A current increase of 4mA was observed following micro-injection of 5μL of 25mM glucose (figure 25). Thus, the 25μm sensor was able to detect increasing glucose levels in the brain, similar to the 176μm sensor. However, *in-vivo* experiments and optimization of analytical performance of the sensor's detection of physiological glucose levels in normal and disease stage animal models still needs to be accomplished.

Conclusion:

Non-conductive polymer based glucose sensor was fabricated and *in-vitro* experiments were carried out to evaluate the sensor performance. The glucose sensor was able to use for over 50 days for *in-vitro* glucose measurements. There was negligible interference to the glucose signal from ascorbic and uric acid.

Polypyrrole conductive polymer based glucose sensors were prepared by co-deposition of enzyme and pyrrole. Pyrrole was polymerized by K_2PtCl_6 catalyzed chemical polymerization and electrochemical polymerization. However, there was not a significant difference with the stability of sensor or the response to glucose from the polymerization method. Nafion® coated glucose sensors were showed 16% current signal from ascorbic acid and 8% current signal from uric acid compared to the glucose signal. *In-vivo* experimental results of glucose sensor show good promise for applications in brain glucose monitoring.

Future Directions of the Research

The techniques illustrated in this study can be applied after a few modifications to construct a glutamate sensor. As future directions of this project, the coupling enzyme with conductive polymers can be applied to develop biosensors that can be used to detect glutamate, GABA, or other neurotransmitters present in the brain.

Nafion® can be applied to electrodes electrochemically instead of dip coating. It would be interesting to know how the electrodeposition is different from dip-coating for the stability of Nafion® layer over time.

The thickness of polypyrrole polymer film prepared by both polymerization methods should be investigated further using scanning electron microscopy to obtain a clear picture of surface properties. Rigid fibers of polymer could cover the active sites of enzyme, thereby impeding access to analyte [64]. These problems need to be addressed to enhance the performance of glucose sensor.

References

[1] E. Tamiya,T. Takeuchi, M. Suzuki, I. Karube, A. Akiyama, Ultra micro glutamate sensor using platinized carbon-fiber electrode and integrated counter electrode. Sensors and Actuators B-Chemical 10 (1993) 179-184.

[2] R.M. Wightman, D.O. Wipf, Voltammetry at ultramicroelectrodes. Electroanalytical Chemistry 15 (1989) 267-353.

[3] O.M. Schuvailo, S. Gasper, A.P. Soldatkin, E.Csoregi, Ultramicrobiosensor for the selective detection of glutamate. Electroanalysis 19 (2007) 71-78.

[4] R.S. Kelly, R.M. Wightman, Beveled carbon-fiber ultramicroelectrodes. Analytica Chimica Acta 187 (1986) 79-87.

[5] M.K. Justin, R.M. Wightman, Microelectrodes for studying neurobiology. Current Opinion in Chemical Biology 12 (2008) 491-496.

[6] W.Dimpfel, Pharmacological modulation of dopaminergic brain activity and its reflection in spectral frequencies of the rat electropharmacogram. Neuropsychobiology 58 (2008) 178-86.

[7] N.V Kulagina, L. Shankar, A.C. Michael, Monitoring glutamate and ascorbate in the extracellular space of brain tissue with electrochemical microsensors. Analytical Chemistry 71 (1999).

[8] M.I. Montenegro. M. Queiros, J.L Daschbach, Microelectrodes:Theory and applications, Vol. 197. 1990: Kluwer Academic Publishers. 3-15.

[9] P.M. Kovach, M.R.Deakin, R.M. Wightman, Electrochemistry at partially blocked carbon-fiber microcylinder electrodes. Journal of Physical Chemistry 90 (1986) 4612-4617.

[10] C.G. Zoski, M.V. Mirkin, Steady-state limiting currents at finite conical microelectrodes. Analytical Chemistry 74 (2002) 1986-92.

[11] P.T. Kissinger, W.R. Heineman, Laboratory techniques in electroanalytical chemistry, (1996) 403-420.

[12] P.J. Welford, J. Freeman, S.J.Wilkins, J.D. Wadhawan, C.E. Hahn, R.G. Compton, Laminated microelectrodes: A simple approach to the construction of inexpensive microelectrodes with a variety of geometries. Analytical Chemistry 73 (2001) 6088-92.

[13] P.M. Kovach, W.L. Caudill, D.G. Peters, R.M. Wightman, Faradaic electrochemistry at microcylinder, band, and tubular band electrodes. Journal of Electroanalytical Chemistry 185 (1985) 285-295.

[14] J. Guo, E. Linder, Cyclic voltammograms at coplanar and shallow recessed microdisk electrode arrays: Guidelines for design and experiment. Analytical Chemistry 81 (2009) 130-8.

[15] J.J. Burmeister, G.A. Gerhardt, Ceramic-based multisite microelectrode arrays for in vivo electrochemical recordings of glutamate and other neurochemicals. Trac-trends in Analytical Chemistry 22 (2003) 498-502.

[16] J.J. Burmeister, F.Pomerleau, P. Huettl, C.R. Gash, C.E. Wemer, J.P. Bruno, G.A. Gerhardt, Ceramic-based multisite microelectrode arrays for

simultaneous measures of choline and acetylcholine in cns. Biosensors and Bioelectronics 23 (2008) 1382-1389.

[17] G.S. Wilson, P.G. Yu, An independently addressable microbiosensor array: What are the limits of sensing element density? Faraday Discussions 116 (2000) 305-317.

[18] Y. Lee, B.K. Oh, M.E. Meyerhoff, Improved planar amperometric nitric oxide sensor based on platinized platinum anode. 1. Experimental results and theory when applied for monitoring no release from diazeniumdiolate-doped polymeric films. Analytical Chemistry 76 (2004).

[19] R.M. Wightman, W.G. Kuhr, A.G. Ewing, Voltammetric detection of dopamine release in the rat corpus striatum. Annals of the New York Academy of Sciences 473 (1986) 92-105.

[20] H. Tang, J.H. Chen, S.Z. Yao, L.H. Nie, G.H. Deng, Y.F. Kuang , Amperometric glucose biosensor based on adsorption of glucose oxidase at platinum nanoparticle-modified carbon nanotube electrode. Analytical Biochemistry 331 (2004) 89-97.

[21] L. Yu, Y.T. Wang, Z.Q. Zhu, J. Zhang, J.Z. Zhu, C. H. Fan, Improved enzyme immobilization for enhanced bioelectrocatalytic activity of glucose sensor. Sensors and Actuators B-Chemical 136 (2009) 332-337.

[22] E.R. Reynolds, A.M. Yacynych, Direct sensing platinum ultramicrobiosensors for glucose. Biosensors and Bioelectronics 9 (1994) 283-293.

[23] V. Yegnaraman, A.M. Yacynych, Enzyme immobllization on ultramicroelectrodes through electropolymerization: Effect of polymeric film thickness on the amperometric response of the electrode. Bulletin of Electrochemistry 16 (2000) 21-24.

[24] J.G. White, J.W. Jorgenson, Improvements in scanning voltammetric detection for open-tubular liquid-chromatography. Analytical Chemistry 58 (1986) 2992-2995.

[25] R.A. Wallingford, A.G. Ewing, Capillary zone electrophoresis with electrochemical detection. Analytical Chemistry 59 (1987) 1762-1766.

[26] W.G. Kuhr, R.M. Wightman, Real-time measurement of dopamine release in rat-brain. Brain Research 381 (1986) 168-171.

[27] Y.B. Hu, G.S. Wilson, Rapid changes in local extracellular rat brain glucose observed with an in vivo glucose sensor. Journal of Neurochemistry 68 (1997) 1745-1752.

[28] J. Kehr, Prof. Ralph n. Adams (1924-2002) - in memoriam. Journal of Neuroscience Methods 140 (2004) 3-4.

[29] N. Matsumoto, X. H. Chen, G.S. Wilson, Fundamental studies of glucose oxidase deposition on a pt electrode. Analytical Chemistry 74 (2002) 362-367.

[30] K.W. Johnson, D.J. Allen, J.J. Mastrototaro, R.J. Morff, R.S.Nevin, Reproducible electrodeposition technique for immobilizing glucose-oxidase and a differentially permeable outer-membrane material for use on a miniature

implantable glucose sensor. Diagnostic Biosensor Polymers 556 (1994) 84-95.

[31] D.R. Thevenot, K. Toth, R.A. Durst, and G.S. Wilson, Electrochemical biosensors: Recommended definitions and classification. Analytical Letters 34 (2001) 635-659.

[32] D.R. Thevenot, K.Toth, R.A. Durst, and G.S. Wilson, Electrochemical biosensors: Recommended definitions and classification - (technical report). Pure and Applied Chemistry 71 (1999) 2333-2348.

[33] A.P.F. Turner, I. Karube, G.S. Wilson, Biosensors: Fundamentals and applications, 1987.

[34] American Diabetes Association: Standards of medical care in diabetes – 2008 (Position Statement), Diabetes care 31 (Suppl 1): S12-S54, 2008.

[35] D.L. Loriaux, Diabetes and the ebers papyrus. Endocrinologist 16 (2006) 55-56

[36] N.S. Oliver, C. Toumazou, A. E. G. Cass, D.G. Johnston, Glucose sensors: A review of current and emerging technology. Diabetic Medicine 26 (2009) 197-210.

[37] O.M. Schuvailo, O.O. Soldatkin, A. Lefebvre, R. Cespuglio, A.P. Soldatkin, Highly selective microbiosensor for in vivo measurement of glucose, lactate and glutamate Analytica Chimica Acta 573-574 (2006) 110-116.

[38] W.R. Vieth, S.S. Wang, R. Saini, Immobilization of whole cells in a membraneous form. Biotechnology and Bioengineering 15 (1973) 565-569.

[39] X.H. Chen, Y. B. Hu, G.S. Wilson, Glucose microbiosensor based on alumina sol-gel matrix/electropolymerized composite membrane. Biosensors and Bioelectronics 17 (2002) 1005-1013.

[40] K.W.Johnson, D.J. Allen, J.J. Mastrototaro, R.J. Morff, R.S. Nevin, Reproducible electrodeposition technique for immobilizing glucose-oxidase and a differentially permeable outer-membrane material for use on a miniature implantable glucose sensor. Diagnostic Agnostic Biosensor Polymers 556 (1994) 84-95.

[41] R. G. Nuzzo, L.H. Dubois, Synthesis, structure, and properties of model organic-surfaces. Annual Review of Physical Chemistry 43 (1992) 437-463.

[42] R.W. Murray, J. Facci, Silanization and non-aqueous electrochemistry of 2 oxide states on platinum-electrodes. Journal of Electroanalytical Chemistry 112 (1980) 221-229.

[43] D. Olea, O. Viratelle, C. Faure, Polypyrrole-glucose oxidase biosensor - Effect of enzyme encapsulation in multilamellar vesicles on analytical properties. Biosensors and Bioelectronics 23 (2008) 788-794.

[44] D. Olea, P. Morean, C. Faure, Polypyrrole-glucose oxidase biosensor; Effect of enzyme encapsulation in multilamellar vesicles on film growth and morphology. Journal of Electroanalytical Chemistry 605 (2007) 125-135.

[45] R. Pauliukaite, M. Schoenleber, P. Vadgama, C.M.A.Brett, Development of electrochemical biosensors based on sol-gel enzyme encapsulation and protective polymer membranes. Analytical and Bioanalytical Chemistry 390 (2008) 1121-1131.

[46] I. Rohm, W. Kunnecke, U. Bilitewski, UV-polymerizable screen-printed enzyme pastes. Analytical Chemistry 67 (1995) 2304-2307.

[47] D. Zigah, M. Pellissier, B. Fabre, F. Barriere, P. Hapiot, Covalent immobilization and SECM analysis in feedback mode of glucose oxidase on a modified oxidized silicon surface. Journal of Electroanalytical 628 (2009) 144-147.

[48] S.K. Jung, G.S. Wilson, Polymeric mercaptosilane-modified platinum electrodes for elimination of interferants in glucose biosensors. Analytical Chemistry 68 (1996) 591-596.

[49] X.H. Chen, N. Matsumoto, Y.B. Hu, G.S. Wilson, Electrochemically mediated electrodeposition/electropolymerization to yield a glucose microbiosensor with improved characteristics. Analytical Chemistry 74 (2002) 368-372.

[50] A. Gill, G. Lillie, G. Farace, P. Vadgama, Biocompatible interfaces for biosensors. International Journal of Environmental Analytical Chemistry 85 (2005) 699-725.

[51] Q.L. Yang, P. Atanosov, E. Wilkins, A novel amperometric transducer design for needle-type implantable biosensor applications. Electroanalysis 9 (1997) 1252-1256.

[52] G.F.Khan, Layer-layer assembly for extremely stable glucose sensors. Electroanalysis 9 (1997) 533-536.

[53] O. Takei, S. Toyama, M. Someya, T. Kurokawa, R. Usami, K. Horikosi, Y. Ikariyama, Glucose sensor based on Au-Pt black electrode - preparation of functionally different sites on electrode surface. Electrochemistry 69 (2001) 956-958.

[54] J.M. Cooper. D.J. Pritchard, Biomolecular sensors for neurotransmiter determination:Electrochemical immobilization of glutamate oxidase at microelectrodes in a poly(o-phenylenediamine) film. Journal of materials science:materials in electronics 5 (1994) 111-116.

[55] Rajesh, T. Ahuja, D. Kumar, Recent progress in the development of nano-structured conducting polymers/nanocomposites for sensor applications. Sensors and Actuators B-Chemical 136 (2009) 275-286.

[56] D.J. Harrison, R.F.B. Turner, H.P. Baltes, Characterization of perfluorosulfonic acid polymer coated enzyme electrodes and a miniaturized integrated potentiostat for glucose analysis in whole-blood. Analytical Chemistry 60 (1988) 2002-2007.

[57] S. Daniele, C. Bragato, M.A. Baldo, Preparation and characterization of nafion (r) coated sphere-cap mercury microelectrodes. Journal of Electroanalytical Chemistry 471 (1999) 48-54.

[58] N. Suraj, Bhadani, K. Madhuri, K. Sumanta, G. Sen, C. Guru, Sahu, Preparation of conducting fibers via the electrochemical polymerization of pyrrole. Journal of Applied Polymer Science 64 (1997) 1073-1076.

[59] M.A. Baldo, S. Daniele, M. Corbetta, G.A. Mazzocchin, Performance of platinum-based spherical mercury microelectrodes in cyclic voltammetry and stripping analysis. Electroanalysis 7 (1995) 980-986.

[60] D.L. Nelson, M.M. Cox, Lehninger principles of biochemistry.

[61] U. Tamer, C. Kanbes, N. Ertas, Branched fibers of conducting polypyrrole: Synthesis and characterization. International Journal of Polymer Analysis and Characterization, 14 (2009) 259-270.

[62] Y.S. Yang, M.X. Wan, Microtubules of polypyrrole synthesized by an electrochemical template-free method. Journal of Materials Chemistry, 11 (2001) 2022-2027.

[63] F. Ullah, T.T. Dang, J. Heinicke, A. Villinger, P. Langer, Regioselective sonogashira reactions of n-methyltetrabromopyrrole: First synthesis of tri- and tetra(1-alkynyl)pyrroles. Synlett 5 (2009) 838-842.

[64] W. Aifang, X. Ye, P. He, Y. Fang, A new technique for chemical deposition of pt nanoparticles and its applications on biosensor design. Electroanalysis 19 (2007) 1603-1608.

[65] D. Dwyer, Glucose metabolism in the brain, 2002.

[66] L. Penicaud, C. Leloup, X. Fioramonti, A. Lorsignol, A.Benani, Brain glucose sensing: A subtle mechanism. Current Opinion in Clinical Nutrition and Metabolic Care 9 (2006) 458-462.

[67] A.J. Bard, L.R.Faulkner, Electrochemical methods: Fundamentals and applications Wiley; (December 18, 2000), 2000.

[68] G. Wohlfahrt, S. Witt, J. Hendle, D. Schomburg, H.M. Kalisz, H.J. Hecht, 1.8 and 1.9 a resolution structures of the penicillium amagasakiense and aspergillus niger glucose oxidases as a basis for modelling substrate complexes. Acta Crystallogr D Biol Crystallogr 55 (1999) 969-77.

[69] M. Koudelkahep, A. Vandenberg, N.F. Derooij, Polymeric membranes for miniature biosensors and chemical sensors. ACS Symposium series 487 (1992) 252-260.

[70] E.R. Margaret, Ascorbate regulation and its neuroprotective role in the brain, Trends in Neurosciences. 23 (2000) 209-216.

[71] C. Bala, G.L. Radu, D.E. Gheorghe, V. Magearu, Determination of cholesterol with a nafion-polypyrrole-modified biosensor. Journal of Medicine and Biochemistry 1 (1997) 47-54.