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Design of a Reagentless Enzymatic Amperometric Alcohol Biosensor: Yeast Alcohol Dehydrogenase and Nicotinamide Adenine Dinucleotide on Vertically Aligned Carbon Nanofibers

Martha Lee Weeks
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Martha Lee Weeks entitled "Design of a Reagentless Enzymatic Amperometric Alcohol Biosensor: Yeast Alcohol Dehydrogenase and Nicotinamide Adenine Dinucleotide on Vertically Aligned Carbon Nanofibers." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

Paul Frymier, Major Professor

We have read this thesis and recommend its acceptance:

Paul Bienkowski, Elizabeth Howell

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for the Masters of Science Degree

The University of Tennessee, Knoxville

Martha Lee Weeks

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DEDICATION

I would like to dedicate this thesis to my parents Olaf and Lourdes, my sister Ludmila, and to Wayne for their love and encouragement. You have all been an unwavering source of support and without it I would not be where I am today. I love you all.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Frymier who has given me much guidance throughout this process and for giving me the opportunity to work on such a rewarding project. I would also like to thank my committee members, Dr. Elizabeth Howell and Dr. Paul Bienkowski for all of their help with my project. My experience at the University of Tennessee is one that I will always remember fondly. I have learned so much and am thankful for all of the support I have received since I arrived here. Last but not least I would like to thank my family and friends who have always been there for me.

ABSTRACT

A reagentless amperometric enzymatic biosensor was constructed on a carbon substrate for the detection of ethanol, methanol, and isopropanol. Yeast alcohol dehydrogenase (YADH), an oxidoreductase enzyme, and its cofactor nicotinamide adenine dinuleotide (NAD^+) were immobilized by adsorption and covalent attachment to the carbon substrate. Carbon nanofibers grown by plasma enhanced chemical vapor deposition (PECVD) were chosen as the electrode material due to their excellent structural and electrical properties.

Electrochemical techniques were employed to test the function and performance of the constructed biosensor. Characterization of the electrode was performed using NADH. This allowed the function of the electrode to be examined as well as to determine the oxidation peak potential of NADH. Subsequently, amperometric measurements were conducted for the detection of ethanol, methanol, and isopropanol to determine the response in electrical current as a result of an increase in analyte concentration. The storage stability, reusability, and response time of the biosensor was also examined.

Carbon nanofibers were found to be an effective strategy for building a biosensor. At a working potential of 0.9 V, electrodes with covalently attached and adsorbed enzyme were found to provide a strong current response. The electrical current responses to methanol and isopropanol were found to be on

average one order of magnitude lower than that of the ethanol experiments. The response was stable, storage stability of the sensors was excellent, and the response time to analyte additions was quick.

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CHAPTER 1 INTRODUCTION

The desire to improve detection techniques used in chemical analysis has been the motivation for continuous research in biosensor technologies since their introduction in the early 1960's [2]. Biosensors detect the presence of a particular analyte, the substance to be detected, by producing a physical signal that is proportional to the concentration of the analyte present in the system. This technology seeks to develop methods which can determine concentrations with sensitivities that rival that of more laborious methods [28]. Biosensors have the potential to address problems of previously existing methods in terms of cost efficiency and real time evaluation. The prospect of real time monitoring as well as their ability for sensitive, stable, and reproducible results has made these sensors particularly useful to many applications such as the food industry for the detection of toxins, medical diagnostics for glucose monitoring, and chemical warfare for the detection of nerve agents [32]. Biosensors come in a variety of forms depending on their intended use, and each component of the sensor has great influence on the quality of data obtained. In order to ensure the success of the biosensor, each component of the design must be evaluated to develop a successful device.

There are many methods that have been used for chemical analysis; these include titration, gas chromatography, and ion spectroscopy. These methods have proven to be accurate in the detection of analyte concentrations, and under the correct conditions can be discriminatory providing detailed information for a specific analyte in a complex mixture. The disadvantage of these methods lies in that they are extremely laborious, complicated, and expensive procedures [17]. It is becoming increasingly desirable for much faster, more cost efficient solutions, that can accommodate real time detection; all of which are important attributes of a successful biosensor.

Progression into sensor technologies was not due to the existence of poorly developed methods; former methods had the advantage of being particularly precise. Instead, this technology has evolved in response to the shortcomings of older methods. Sensors can be of two kinds; they can either transduce physical or chemical signals. Physical sensors provide information about physical properties of a system and include sensors that measure temperature, pressure, and fluid flow [33]. Chemical sensing methods involve the transformation of chemical information into an analytically useful signal. Chemical sensors can sometimes display a lack of discriminatory sensing which led to the exploration of the idea of a device that could differentiate between chemical species, a device which could detect the presence of a particular analyte [9]. It was discovered that chemical

coatings or gel matrices could selectively bind to certain compounds and aid in the differentiation of analytes [28].

Chemical sensors are classified according to the method by which the chemical information of the sample is transformed into a useful analytical signal. Electrochemical sensors are devices that transform the effect of an electrochemical interaction between the chemical species and the electrode into a useful signal, from the production of an electrochemically active species. Expansion of the idea eventually brought to realization the possibility of using a biological element to selectively detect the presence of a particular compound. This in turn could potentially produce real-time readings with increased affinity for the analyte [38]. The resulting biosensor was a bio-molecular probe that allowed for the detection of molecules by converting the interaction of a biological component with another chemically reactive compound at the electrode surface [33]. This biochemical interaction could then be translated into a physical, detectable signal. Continuing research intends to advance the science to rival the sensitivity of other methods.

Materials that have been used for the construction of electrochemical sensors vary widely. Carbon electrodes have proven to be successful when used in enzyme biosensors. Among some of the most promising materials for a carbon electrode are carbon nanostructures, cylindrical or conical structures that have diameters varying from a few to hundreds of nanometers and lengths ranging from less than a micron to millimeters [6]. They have the potential to improve the

sensitivity as well as the cost efficiency of the sensor. Much work has been done in this area with carbon nanotubes and currently studies are emerging using carbon nanofibers. Carbon nanofibers have excellent conductive properties which make them exceptional candidates for an electrode material and their structural properties make them good candidates as an immobilization substrate [8].

The intent of this thesis is to document the development and characterization of a reagentless amperometric enzymatic biosensor that makes use of carbon nanofibers for the working electrode. Yeast alcohol dehydrogenase (YADH) and its coenzyme nicotinamide adenine dinucleotide (NAD^+) were chosen as the biological elements. The enzyme and coenzyme will be immobilized to the carbon nanofiber surface by two methods; adsorption and covalent attachment. YADH is selective most prominently with smaller, unbranched alcohols; however this enzyme will also react with larger, branched alcohols. Ethanol, methanol, and isopropanol will be used to test the effectiveness of the biosensor. The detection range, stability, reusability and response time of the sensor will be studied to evaluate its performance. Investigations by Holland reported current responses on the order of 1*10^-9A for a multi-walled carbon nanotube (MWNT) biosensor. Detection ranges obtained were 1.5M to 8.5M for YADH and NAD^+ adsorbed to the electrode and a range between 0.5M and 7.0M for YADH and NAD^+ covalently attached the electrode. The stability for the sensor developed by Holland provided excellent stability with a loss of less than

1% and the reusability for the sensor showed a 30% loss of current response after ten measurements [41]. This research hopes to improve upon the results obtained by Holland. In the following chapter, the considerations involved in the design of the biosensor will be discussed.

CHAPTER 2 BACKROUND

2.1 Considerations In Biosensor Design

There are many design considerations that go into the development of a biosensor. The purpose of a biosensor is to detect a compound by means of a biological element and convert the response into an electrical signal. The goal in developing new biosensors is to expand of the range of analytes that can be detected and increase the sensitivity of detection by the sensor [2]. Since their introduction, much progress has been made in advancing their capabilities. Careful studies should be made in order to ensure that each component employed will maximize the usefulness of the sensor. A method of detection should be established followed by the determination of the biological receptor. Finally the electrode material should be selected. The purpose for which a biosensor is intended will often dictate the materials and transduction methods that will be used [28]. Careful consideration of each component of a biosensor should be taken into account in order to ensure its success. All of these variables will work together to enhance the efficiency of the biosensor.

In order to be useful, a biosensor should possess certain desired features. The biological component should react very specifically and sensitively in its detection; it should include stable components allowing for storage, and should

allow for reuse over more than one experiment. It is preferable that conditions such as stirring, pH, and temperature have a minimal effect on the reaction [40]. This is not always possible as certain components are inherently sensitive to changes in these conditions. However, every effort should be made to keep these conditions under control. The current response should be sensitive and reproducible over a range of concentrations without dilution and should be kept free of electrical noise. Real time detection, analysis at the actual time the probe was put to use, is also an important attribute for the sensor to attain [4]. These characteristics are very important in determining the suitability of a biosensor regardless of its intended use.

2.2 General Biosensor

Biosensors can vary greatly in appearance and structure but there are some characteristics that are common to all no matter what their purpose. Every biosensor contains a biological element which reacts with the analyte of interest, and a transducing element which converts the signal giving it physical meaning [9]. Figure 2.1 illustrates materials that can be used as the biological element as well as methods that can be used to transduce the signal.

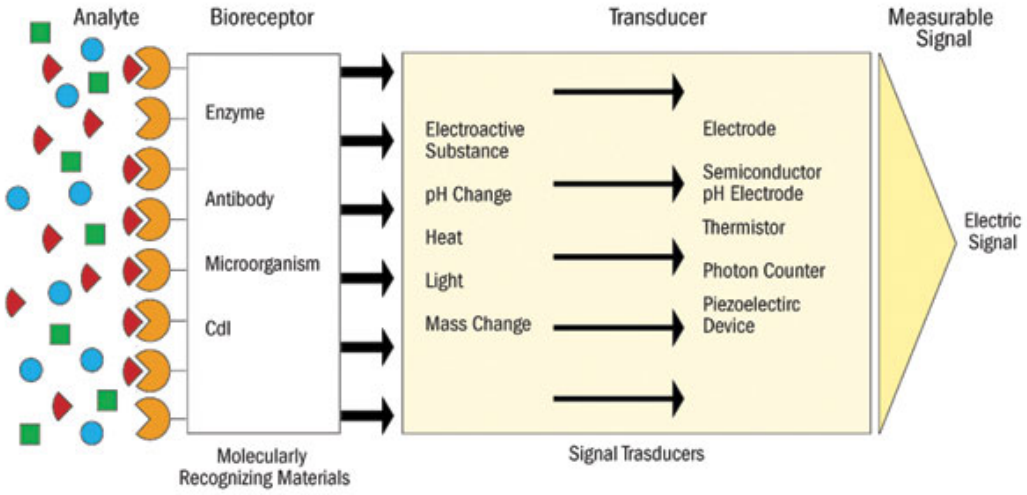


Figure 2. 1 Diagram of the signal path of a biosensor as well as elements that can be involved in the function of a biosensor [44].

The interaction between the target analyte and the bioreceptor causes a reaction which results in the production of an electrical signal. In order for the signal to be detected efficiently; the biological element should be in close contact with the electrode surface. In order that the sensor allow for reusability, all elements required for detection should be incorporated into the electrode [12]. For maximum simplicity and versatility, function of the biosensor should not require the introduction of additional reagents during use. The biological component should react in a very specific manner with the analyte in solution and should not be influenced by other analytes. The bioreceptor may be an enzyme, an antibody, microorganism, cell or tissue and may be chosen for a sensor because it reacts preferably with a certain molecule [28].

A biosensor requires the use of a transducer, to convert the information obtained from the reaction, and can be broken into two parts; detection and conversion to a physical signal. Once the analyte is detected and the chemical change is determined, the transducer must convert and transmit this information so that it may be analyzed [34]. There are several ways in which the signal might be measured. It is possible to measure a change in heat (calorimetric), light (optical), pH, mass (piezoelectric), or electroactivity (amperometric). The observed signal travels to the second part of the transducer which can be an electrode, semiconductor pH electrode, thermistor, photon counter, or piezoelectric device

[32]. Each component of the biosensor must be carefully considered as they all influence the resulting performance of the sensor.

2.3 Choice of Detection Method

The method of detection that is used should be chosen based on several factors. Data needs and the target molecule can be influential factors that should be incorporated into this decision. There are several different transduction methods that are available for consideration.

A sensor which requires a change in heat to create a signal is known as a thermometric biosensor. These sensors require a change in heat to create a signal and are based on the measurement of the heat effects of a specific chemical reaction or adsorption which involve the analyte. These as well as magnetic sensors, sensors based on the change of magnetic properties of a gas being analyzed, are very rarely implemented. Optical biosensors were introduced in the 1970's and consist of a fiber optic sensor with a biological element immobilized on it. These sensors detect the light output or the difference in absorbance between the reactants and products. This sensor is particularly good at measuring carbon dioxide and oxygen and is available for use *in vivo* [33].

Piezoelectric biosensors are yet another alternative for transduction of the signal. Essentially, this type of sensor measures the change in mass occurring as a

result of a reaction. This method makes use of crystals that vibrate upon exposure to an electric field; they experience a phase change when current is applied. An alternating AC current produces a standing wave in the crystal at a characteristic frequency. The frequency is dependant on the properties of the crystal so if a biological material is immobilized on it, the binding of the target element to the receptor will cause a change in the frequency, and induce a signal [33].

Electrochemistry can also be used as a method to transduce and interpret the signal. These sensors are normally based on reactions that respond according to reduction-oxidation (redox) chemistry. Electrochemical biosensors generate electrons as the result of a chemical reaction between the analyte of interest and the biological receptor. There are two methods by which this can be accomplished; amperometric or potentiometric detection [13]. Amperometric and potentiometric detection will be discussed further in section 2.4. The signal is then transduced into a current response that can be read by a computer. Electrochemistry is most often used as a transduction method due to its versatility and relative ease of use [36].

2.4 Introduction to Electrochemistry

Electrochemistry is a subset of chemistry, a field that studies the electrical changes that occur as a result of chemical reactions occurring in the

electrochemical cell. An electrochemical cell can be described as a system consisting of an anode and a cathode which are electrically connected, are submersed in an electrolytic solution, and either use or generate an electrical current. Electrochemistry is a discipline that studies the reactions that take place at the interface of an electronic conductor and an ionic conductor [9]. Aqueous solutions are most often studied although other ionic conductors are becoming of increasing importance [5]. An electrochemical reaction can be caused by an external voltage or by a reaction that brings about a change in voltage or electrical current. Reduction-oxidation or redox reactions are those which drive the production of electrical current in an electrochemical cell. Redox chemistry occurs when a transfer of electrons passes from one species to another, one species must gain electrons and the other must lose electrons. The transfer of the electrons produces a current and the magnitude of the current can provide information regarding the substance being studied [29].

Most electrochemical biosensor research focuses on potentiometric and amperometric biosensors. Potentiometric sensors are based on the measurement of potential when no current is flowing between the electrodes. These sensors function by using ion-selective electrodes in order to transduce the signal between the receptor and the meter. These sensors are useful for the detection of ion concentrations in a solution and the potential produced is proportional to the concentration of ions present in solution. An example of the design of such a

sensor is a biological agent surrounded in a membrane at the tip of a pH probe. There are several drawbacks to this type of sensor including its inability to detect concentrations of analytes other than ions, its susceptibility to interference from ions that are not of interest, as well as susceptibility to electronic noise. These sensors are not as selective as the amperometric alternative and they require rapid electron kinetics for detection [32].

Amperometric sensors are different from potentiometric sensors in that a potential is applied between the electrodes resulting in a change in current. Response time, range, and sensitivity of amperometric sensors are similar but improved over potentiometric methods. These sensors are more versatile and have the advantage that they can detect the concentration of analytes other than ions. This method is particularly useful in the extraction of information of reduction-oxidation dependant species. The current that flows is a product of the electron exchange that occurs as a result of reduction-oxidation reactions [13]. Certain enzymes, specifically oxidoreductase enzymes, are ideal biological elements for this type of sensor. Oxidoreductases are most typically used with electrochemical detection because their turnover of electrons is most easily detected by amperometric electrochemistry [16].

Amperometric electrochemical sensors allow the concentration of analytes to be detected, and in general is the preferable detection and transduction method. Every electrochemical cell requires the use of at least two electrodes, a cathode

and an anode through which the electrons are allowed to flow. Reduction occurs at the cathode and oxidation occurs at the anode. In order that the circuit be complete either an ion junction or third electrode is required. The three electrode model consists of a working electrode which can be made from a multitude of materials, a silver/silver chloride (Ag/AgCl) reference electrode, and an auxiliary electrode made of a platinum wire [9]. It was thought for some time that only metallic substances could make up the working electrode but it was found that other materials have properties which allow them to conduct just as well [28]. Carbon electrodes have been found to work particularly well as they display excellent conductive and structural properties [10]. The working electrode is the interface at which the reaction between the biological component and the chemical analyte occurs; it is the site at which the analyte experiences a redox reaction. Chemical changes occurring at this site eventually give rise to the detectable current changes measured in the system. The working electrode is held a constant voltage potential relative to the Ag/AgCl reference electrode with a potentiostat. The platinum wire auxiliary electrode serves to complete the circuit [9]. As shown in Figure 2.2, the three electrodes are inserted into an electrochemical cell and the electrolytic solution of interest is subsequently added, allowing for experimentation to proceed.

Figure 2. 2 Electrochemical cell. Starting from the left; Ag/AgCl reference electrode (anode), platinum wire auxiliary electrode, and glassy carbon working electrode (cathode) in buffer solution.

2.5 Transport Considerations

Molecular transport should also be considered when constructing a biosensor. Transport factors include the mechanisms by which the particles move throughout the system. In an electrochemical cell, there are several modes of transport that can influence how quickly the molecules reach the electrode surface. Transport can occur due to migration, forced fluid movement, or diffusion. Migration occurs when molecules move through the solution but not as a result of an imposed gradient. Forced transport (ie. stirring) occurs when movement of the molecules is facilitated by physical movement of the solution. Diffusion occurs as the result of a concentration gradient; that is, the spontaneous spreading of molecules in response to an uneven concentration distribution present in the system. At a given point in time there may be a gradient of concentration of a particular molecule and the natural tendency is for the system to equilibrate, the molecules will spread out until the gradient is eliminated. As the solution in the vessel in question increases in size, the time during which diffusion acts on the system will also increase. Although all three methods of transport contribute to molecular movement to some degree, the most influential modes are induced movement by stirring and diffusion [9]. In the case of electrochemical biosensors these factors are important contributors to the response time.

Once the analyte molecules arrive at the reactive electrode surface, the biological element of the sensor must be available to react. Often, enzymes are

used in this capacity as they can differentiate between molecules and only react with a particular analyte. Enzymes possess reaction kinetic characteristics that make them ideal candidates for use as the biological element of a biosensor.

2.6 Enzymes

Enzymes are biological catalysts that speed up a chemical reaction without undergoing any permanent physical change in the reaction. Enzymes are an integral part of supporting chemical reactions in many living systems. They are important in living systems because they act to reduce the activation energy of a given reaction so that less energy is required to complete the reaction [31]. This knowledge has allowed many advances in clinical diagnosis and therapeutics. Enzymes are proteins and are high molecular weight compounds that are primarily made up of chains of amino acids linked together by peptide bonds. In their action, enzymes exhibit a high level of specificity for particular molecules. Enzymes are usually extremely discriminatory in the reactions they catalyze and analytes they use in these reactions [30]. Although they act as powerful catalysts, they are also extremely susceptible to denaturation. Denaturation is a change in the conformation of a protein which results in the loss of activity or function of the protein. Factors such as heat, pH, salts, and solvents can irreversibly denature proteins to the point of no activity. Enzymes typically have optimal activity in

narrow ranges of environmental conditions and once out of this range, the protein begins to denature and thus lose activity. All of this contributes to the fact that the tertiary and quaternary structure of the enzyme is extremely important to enzyme function [22].

Many enzymes require the presence of cofactors, molecules that bind to enzymes and are required for catalytic activity to take place and serve to facilitate the electron transfer from the reaction. These molecules may be organic or inorganic in nature. Cofactors of an organic nature are referred to as coenzymes. Coenzymes must bind to a special location of an enzyme known as the active site. Once the coenzyme is bound to the enzyme, a substrate may come into contact with this complex and a reaction occurs [25]. In most cases the forces that hold the enzyme and its substrate together are hydrogen, ionic or hydrophobic bonds, which are relatively weak non-covalent bonds. Successful binding of the enzyme and its substrates require that the two molecules be able to approach each other and must be in close proximity for binding at a specific catalytic active site. Not only must the coenzyme and substrate both be bound to the enzyme but they also must approach and bind to the enzyme in a specific orientation for a reaction to occur. This requirement for complementarity in the configuration of the substrate and the coenzyme is important in giving enzymes their unique specificity [15]. The temperature of the reaction system, the presence of inhibitors, and the pH of the system will affect the activity of the enzyme and in turn affect the velocity.

Enzymes are characterized by a constant called the Michaelis-Menten constant. This value is often taken as an indication of the affinity of an enzyme for a particular substrate, or the dissociation constant for the already formed enzyme-substrate complex [11]. The specificity of an enzyme comes from the complementarity they have for their substrates [18]. The velocity of the enzymatic reaction as well as the enzymes specificity for varying analytes can have a great influence on the response time of the biosensor. Due to these factors, careful consideration must be taken in choosing an appropriate enzyme.

2.7 Stabilization Methods

When enzymes are used as the biological element of a biosensor, stabilization of the enzyme is an important consideration. Enzymes are powerful catalytic proteins with immense potential in the biosensor technologies. Enzymes however can be relatively fragile and prone to fouling, and so special care often must be taken to protect their function [5]. Stabilization allows for the life of the enzyme to be preserved as well as to protect the enzyme from harmful agents in the solution. Enzymes operate within a small optimal zone of physical and chemical conditions. In order that the enzyme be less vulnerable to irreversible denaturation, which would occur in environments out of their optimal zone, stabilization methods are needed [41]. In attempts to effectively protect the

function of the enzyme and to allow the biosensor to be reused, it should be immobilized to a surface. Immobilization has the potential to cause a decrease in the catalytic activity of the enzyme as it can affect the orientation and flexibility of the enzyme [1]. As the enzyme will be unable to migrate once it is immobilized, it must be placed at a site that will allow it to contribute to the function of the biosensor. The most appropriate location in which to immobilization an enzyme is the working electrode surface, as this is the site at which the reaction and electron transfer will occur [17].

The final function of the working electrode depends a great deal on the method of immobilization that is used. The method chosen will often depend on the surface on which the enzyme is to be immobilized. The most commonly employed methods of immobilization of an enzyme to an electrode surface are entrapment, adsorption, and covalent attachment [5].

Entrapment generally involves the incorporation of the enzyme molecules into a gel matrix or by restricting diffusion of the enzyme from the sensor by barricading it behind a membrane. These methods are less imposing on the structure of the enzyme and also aid in protecting the enzyme from harmful agents in the solution. Even with the physical barrier, the activity of the enzyme is not completely protected and still experiences continuous deactivation. Negative aspects of this type of immobilization also include large barriers to the diffusion of

molecules which results in delayed reaction at the electrode surface and long response times [36].

Adsorption is another method of immobilization that rarely requires the use of reagents for attachment [41]. Depending on the orientation of the enzyme approaching the surface, Van der Waals forces, dipole bonding, and hydrogen bonding may be used to bridge the enzyme with the electrode surface. The bonds involved in adsorption are very weak and it is possible that binding may be reversible. Because of these weak forces the enzyme will be prone to damage from the environment and desorption. Despite these shortcomings, this method of bonding is less restrictive on the conformation of the attached enzyme, is relatively easy to employ, and is applicable to many types of molecules [38].

Covalent immobilization makes use of nucleophilic amino acid groups on the enzyme surface. Immobilization by covalent attachment of the enzyme to the electrode surface must occur in such a way as to not interfere with bonds essential to the biological functions of the enzyme [1]. In order for bonding of the enzyme to be effective, the surface of the electrode must first be modified to provide a reactive surface. The nature of the electrode surface will determine the exact reagents to be used, however all covalent immobilization procedures involve the attachment of the enzyme to the reactive electrode surface. This method is typically used on metallic and carbon surfaces [28]. Covalent immobilization can change the conformation of the enzyme causing some loss of activity however it is

potentially the most irreversible method providing increased stability for the enzyme [1].

2.8 Working Electrode Materials

Immobilization methods must be applied to the working electrode so it is important to consider the materials to be used in the construction of the working electrode. It is important that the material used display strong structural as well as electrically conductive properties. The working electrode is the site at which the electrochemical reaction occurs. The electrode must be made from materials that will promote electron transfer once the reaction occurs [9]. Since the focus for the electrode is electrochemical methods, materials must be considered that will promote the flow of current. Metals such as platinum, gold, silver and stainless steel have long been used for electrochemical electrodes due to their excellent electrical and mechanical properties. It was thought for some time that metal materials were best suited for use as the electrode material over others [2]. Carbon based materials such as graphite, and carbon black have been found to work remarkably well in this capacity, not only due to their conductivity and structure but also their relative cost. Carbon has great potential in electrode construction as it is a versatile material with the ability to make use of large potentials and exhibit low resistances. They are commonly used as materials for electrochemical

transducers. The surfaces of these structures allow the possibility of immobilization by various methods providing an opportunity for reproducible electrode surfaces [35].

Carbon nanostructures have received much attention due to their excellent conductive and structural properties. These features make them very attractive for use in working electrodes for biosensor applications. They have been found to possess the ability to greatly improve electrical current detection capabilities for biosensors. Much of the research that has been done with carbon nanostructures as a working electrode material has concentrated on carbon nanotubes. This attention has been due to their smooth, defect free walls and strong mechanical properties [8]. Experimentation in the past with carbon nanofibers had not been as successful as that with carbon nanotubes due to the lack of ability to control their features during growth. Methods have since been developed that allow for the ability of a controlled synthesis process to grow the fibers on a silicon support. Carbon nanofibers are cylindrical or conical structures that have diameters of several hundred nanometers, and lengths that range between micrometers to millimeters. Carbon nanofibers are made up of different arrangements of graphene sheets; each layer is made of hexagonal sheets of covalently bonded carbon atoms. Figure 2.3a shows a schematic of the graphene layer. Each graphene layer folds producing a single three dimensional stacks of conical

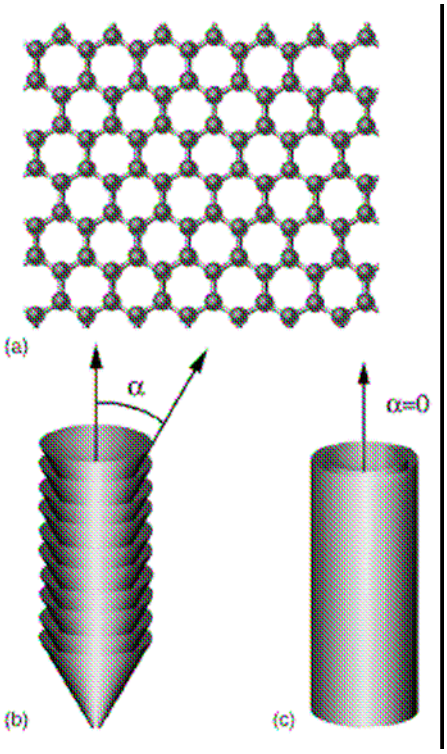


Figure 2. 3 a. Carbon nanofiber graphene sheets made up of hexagonal arrangements of covalently bonded carbon atoms. b. Carbon nanofibers made up of stacks of conical graphene sheets. α refers to the angle between the fiber axis and the graphene sheet near the sidewall surface, for a carbon nanofiber this value will be greater than 0. c. Carbon nanotubes made up of a single graphene sheet. α for carbon nanotubes will equal 0 [6].

structures made of the sheets as can be seen in Figure 2.3b. Carbon nanotubes differ in that they are made of a cylindrical structure of the graphene sheets, see Figure 2.3c. These structures are made of short, poorly connected sheets which span only a portion of the fiber resulting in defect sites. The defect sites are composed of unsaturated bonds and as a result are more reactive than carbon nanotubes. Exposed defect sites on the surface of the carbon nanofibers provide many points at which the immobilization of biological elements can take place [6].

2.9 Fabrication of the Carbon Nanofiber Forest

Carbon nanofibers can be synthesized in a controlled manner by a reliable process known as plasma enhanced chemical vapor deposition or PECVD. Use of the PECVD process allows for control in the size, location, chemical composition, as well as the internal structure of the carbon nanofibers. The nanofibers created by this process are grown perpendicularly to the substrate on which they are grown. These vertically aligned carbon nanofibers can be grown as individual freestanding structures or many fibers can be grown in close proximity to each other creating a forest of fibers. Growth of a forest of fibers can be controlled to a greater degree than the growth of freestanding carbon nanofiber structures. When grown in a forest, the carbon nanofibers are kept aligned by Van der Waals forces between the fibers themselves [6].

The location of the vertically aligned carbon nanofibers or VACNF's are determined by placement of a catalyst material which is applied using a photo- or electron-beam lithography procedure to a silicon substrate. The length of time during which the growth process is allowed to take place as well as the diameter and width of the catalyst particle determines the length of the fiber which is typically between 5nm and 500nm. A setup of the PECVD process can be seen in Figure 2.4. To synthesize VACNF's, a silicon oxide substrate is placed on an electrically conductive heated cathode substrate. On top of the silicon oxide substrate a titanium layer is added in locations where carbon nanofibers are to be grown. This metal layer provides the substrate with electrical conductivity and ensures that silicon is not incorporated into the growing fibers. The nickel catalyst dots of approximately 100nm in diameter and 40nm thick are deposited to the surface of the titanium layer which sits on top of the silicon oxide substrate. From under each catalyst dot will emerge one carbon nanofiber so that the final fiber will have the catalyst on its tip. The vacuum chamber in which this process occurs is controlled at a temperature of 700°C and at a pressure of approximately 1*10^-5 torr. This process makes use of direct current discharge plasma, activating molecules by electron impact, which serves to reduce the activation energy of the process. Subsequently, ammonia is uniformly introduced

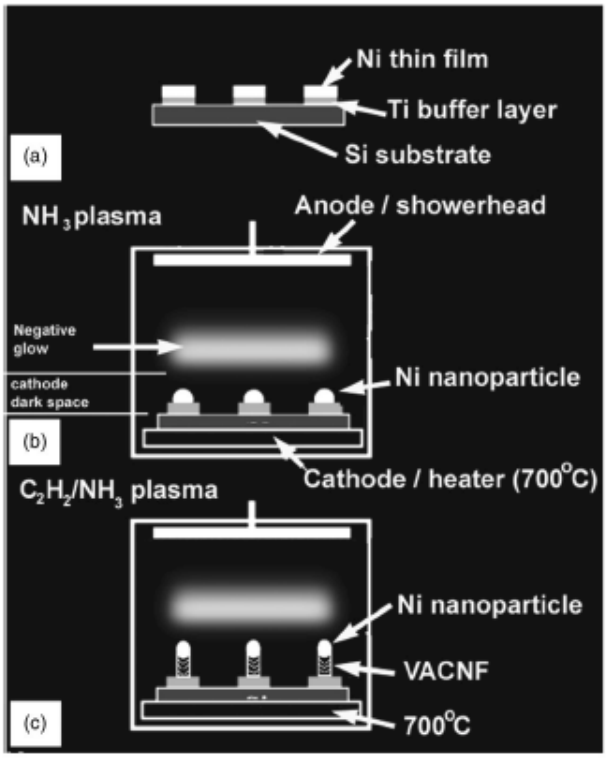


Figure 2. 4 Schematic of the plasma enhanced chemical vapor deposition process for the growth of vertically aligned carbon nanofibers. a. catalyst deposition, b. nanoparticle formation, c. growth of carbon nanofibers [6].

into the vacuum chamber through a gas showerhead which also serves as the anode. Acetylene (C_2H_2) is then introduced into the chamber while maintaining the plasma energy, which in turn begins growth of the carbon nanofibers [6]. Scanning electron micrographs of the resulting forest of carbon nanofibers can be seen in Figure 2.5.

2.10 Electrochemical Methods for the Evaluation of a Biosensor

In the evaluation of a biosensor there are several experiments that can be performed in order to determine its success. Cyclic voltammetry is a common electrochemical procedure used in the evaluation of biosensors. These experiments serve to study the electroactivity of compounds, study electrode surfaces, as well as to determine the rates of oxidation and reduction of a reaction [9]. Cyclic voltammetry experiments are conducted in an unstirred environment between a range of voltage potentials that are applied between the working electrode and the reference electrode [13]. An example of a cyclic voltammogram can be seen in Figure 2.6. In cyclic voltammetry analysis, the potential of the electrode is linearly cycled from a starting potential to a final potential and then back to the original potential. The redox reaction is cycled as the potential is cycled, and the electrical current is measured as a result [5]. The initial state of the

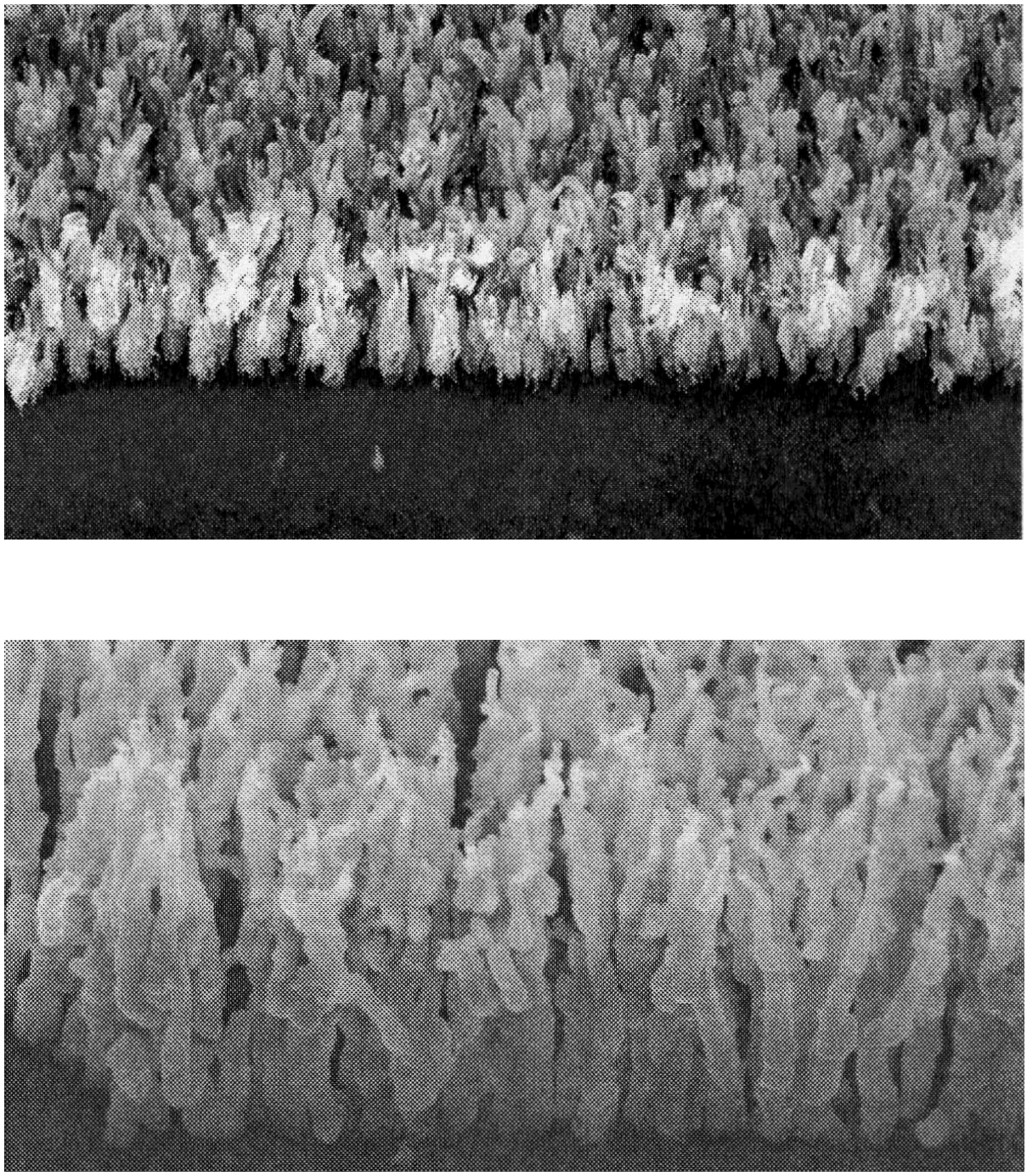


Figure 2. 5 Scanning electron micrographs of carbon nanofibers.

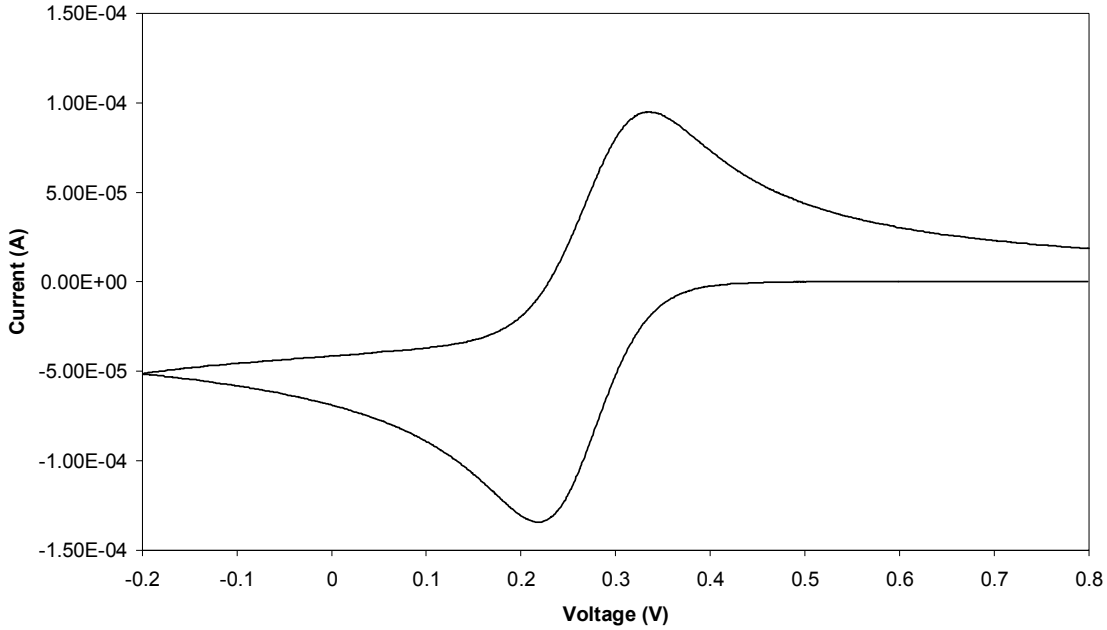


Figure 2. 6 Cyclic voltammogram of NADH with glassy carbon electrode. NADH oxidation starts at a potential of 0.8V, is cycled to a potential of 0.2V generating NAD^+, and then returned to its original potential.

species will determine whether it is oxidized or reduced during the forward reaction. In the case of NADH, a critical point is reached during the forward reaction at which it will begin to be oxidized to NAD^+. A cathodic current peak occurs when most of the species has been oxidized. The current then declines until the final potential is reached, it is at this point that the reverse scan begins. During the reverse scan the potential is cycled back to the starting point and the electroactive species is reduced back to NADH. An anodic peak appears once most of the species has been reduced and the current decays at this point and the potential is returned to its original voltage [34].

The next step in evaluating a biosensor is to study its effectiveness as a detection device. In order to do this different variables including the stability, reusability, and sensitivity of the biosensor must be studied. As enzymes are inherently prone to denaturation it should be stabilized in order to slow deactivation. Stabilization of the enzyme should increase the reusability of the sensor which is an indication of the current loss after repeated uses. Reusability is also important as the biosensor should be available to return consistent results over many uses [17]. The sensitivity is important to evaluate so that the detection range of the biosensor may be determined [37].

Amperometric measurements, or measurements of the electrical current response at a constant applied potential, are used for evaluation purposes of the effectiveness of the biosensor. The potential at which amperometric

measurements are conducted can be determined by studying the cyclic voltammograms of the electroactive redox species. The potential at which the cathodic or oxidation peaks occur is the potential at which amperometric experiments should be conducted.

2.11 YADH Biosensor for the Detection of Alcohol

The goal of this project is to construct an amperometric enzymatic reagentless biosensor. The choice of transduction method, biological element and working electrode material will be presented here. Subsequent discussion will focus on the considerations that were involved in the building such a sensor. This biosensor will be based on an electrochemical method of detection and transduction as it is the preferable method for detection of concentrations of analytes for biosensors.

Yeast alcohol dehydrogenase or YADH, was chosen to be the biological element that would be incorporated into the biosensor as it is a redox enzyme which displays strong catalytic activity. YADH is an oxidoreductase enzyme, one which catalyzes redox reactions and is isolated from *Saccharomyces cerevisiae,* also known as Bakers yeast. The molecular weight of this enzyme is approximately 141,000 Daltons. YADH is a tetramer consisting of four equal subunits weighing approximately 35,000 Daltons each [3]. Each subunit contains two zinc molecules, one which serves a structural role and the other which serves

a catalytic role at the active site [21]. The structural zinc atom serves to stabilize the ternary structure of the enzyme. Most proteins are very sensitive to heat and operate within a small temperature range. Removal of the structural zinc atom would increase the unfolding, inactivation and increase the heat sensitivity of the enzyme [23]. The second zinc atom serves a catalytic role and the activity of YADH is dependant on the proper functioning of this zinc atom [20]. YADH is a tetramer and each monomer has a firmly bound catalytic zinc atom, a histidine, and a cysteine residue all of which are essential to catalytic activity [7]. Each YADH molecule has four active sites and will bind with four NAD^+ coenzyme molecules which are required for reaction. Each catalytic zinc atom operates independently of the others and serves as a locus of reversible attachment for a molecule of the coenzyme. Should one of the catalytic zinc atoms be inhibited in any way, the coenzyme will no longer bind at that active site and no reaction will occur [21]. At each active site of YADH there are two domains, a catalytic domain and a narrow coenzyme binding domain buried in the protein. The two parts are unequal in size and there is a physical cleft dividing the two regions [3].

The redox reaction catalyzed by YADH is:

$$\text{ethanol} + NAD^+ \leftrightarrow \text{acetaldehyde} + NADH$$

The kinetic mechanism by which this enzyme reacts has been shown to be a steady state random mechanism. This indicates that the order in which substrate and coenzyme are bound to the enzyme is not important; the only requirement is that

they both be bound for reaction to occur [25]. YADH will react with primary and secondary alcohols although the specificity with which it reacts will decrease as the size of the alcohol increases and as the branches extending off the alcohol increase. YADH is most reactive with ethanol as it is a small unbranched alcohol [26]. The Michaelis-Menten constant, K_m, has value of 2.1mM for ethanol. K_m values for larger, branched alcohols such as methanol and isopropanol are 130mM and 140mM respectively [44].

Nicotinamide adenine dinucleotide, is the coenzyme used by YADH and it is required that it be bound to the active site of the enzyme for a reaction to occur. In the active site of the coenzyme binding pocket, NAD^+ will bind with several amino acid residues including threonine, leucine, methionine, and cysteine [27]. Electron transfer by the coenzyme occurs via a direct hydride transfer mechanism [25]. Once both NAD^+ and the alcohol are bound, the alcohol group is oxidized by the removal of a proton from the hydroxyl group. Transfer of a hydride ion from the adjacent carbon atom to NAD then occurs [3]. The overall oxidation of alcohol to aldehyde involves the net release of one proton from the alcohol. The release of a proton from the bound alcohol occurs in the hydrophobic center of the enzyme molecule and the proton is transferred by the enzyme and is released [22].

Both YADH and NAD were to be immobilized to the electrode surface so methods were required that would not hinder the function of the enzyme and coenzyme. Two methods of immobilization were used in the evaluation of this

biosensor; adsorption and covalent immobilization (see Section 2.7). The specific method of covalent attachment used for this biosensor was diimide-activated amidation. This method of attachment is among the most widely used for covalent attachment. Enzymes are covalently coupled to the exposed carboxylic acid sites on the surface of the carbon nanofibers [14]. Both methods were used to test whether one resulted in a better performing biosensor.

Carbon nanofibers were chosen as the working electrode material to be used in the construction of this biosensor as carbon materials have been found to perform excellently in electrochemical analysis. Defect sites on the surface of the carbon nanofibers provide many sites at which immobilization of the enzyme can occur. The increased surface area of these fibers has the potential to allow for increased current output as a result. The process by which these fibers were made was plasma enhanced chemical vapor deposition (see section 2.9).

2.12 Proposal for Biosensor

An amperometric enzymatic alcohol biosensor will be constructed and its function will be evaluated. The enzyme used will be YADH which will also require the use of NAD^+. Adsorption and covalent attachment immobilization methods will be used to determine if one provides improved results over the other. The working electrode will be made from carbon nanofiber arrays that have been

grown onto a silicon oxide substrate. The response of the biosensor will be tested with three alcohols, ethanol, methanol, and isopropanol. The detection range, stability, reusability and response time of this sensor will be examined as well as the response time and the effect of carbon nanofiber pretreatment on the function of the biosensor.

CHAPTER 3 MATERIALS AND METHODS

3.1 Reagents and Materials

Yeast alcohol dehydrogenase (YADH, EC 1.1.1.1, 451 U/mg, 90% protein, Sigma-Aldrich product no. A3263) and both the oxidized and reduced form of nicotinamide adenine dinucleotide (NAD^+, NADH) were obtained from Sigma Aldrich (Sigma Aldrich Corp., St. Louis, MO) and used as they were received with no further purification. N-hydroxylsuccinimide (NHS), 1-ethyl-3-(3-dimethlyaminopropyl) carbodiimide (EDAC), and 2-(N-morpholino) ethanesulfonic acid (MES) were also obtained from Sigma Aldrich Corp. and were used as received. Ethanol (200 Proof) was obtained from Fisher Scientific (Fisher Scientific, Hampton, NH). Carbon nanofibers grown onto silicon wafers were obtained from Oak Ridge National Laboratory (Oak Ridge, TN). The silicon wafers were produced by Kate Klein and the Molecular-Scale Engineering and Nanoscale Technologies Research Group (MENT). Each working area (the area of the silicon oxide wafer with one 0.5mm x 0.5mm carbon nanofiber array grown onto it) was approximately 2mm x 2mm. The carbon nanofibers were approximately 100nm in diameter and 4μm in length. Sodium phosphate (monobasic and dibasic), sodium pyrophosphate, phosphoric acid, sulfuric acid,

and sodium hydroxide were all of analytical grade and were used without further purification.

The following solutions were prepared in the laboratory. Sodium phosphate buffer solution (PBS) at pH 7.4 was prepared by adding 0.1M monobasic sodium phosphate to 0.1M dibasic sodium phosphate until a pH of 7.4 was reached. Sodium pyrophosphate buffer solution was prepared in the following manner: an 8% (v/v) solution of phosphoric acid was added to a 50mM sodium pyrophosphate solution until a pH of 8.8 was reached. MES (2-(N-morpholino) ethanesulfonic acid) buffer solution was prepared by adding a 1mM solution of NaOH to 50mL of MES buffer until pH 6.0 was reached. All solutions were prepared with deionized water.

3.2 Apparatus

Amperometric measurements for this project were performed using a computer controlled CHI660A potentiostat (CHI Company), as seen in Figure 3.1. Electrochemical experiments were conducted in a three electrode electrochemical cell with a working volume of 8mL. The reference electrode was an Ag/AgCl electrode (BAS, model RE-5B) and the auxiliary electrode was a platinum wire (BAS, model MW-4130). A working electrode was constructed using carbon nanofibers grown onto a silicon wafer (see Figure 3.2). Fabrication of the working

Figure 3. 1 CHI660A Potential. Picture of the potentiostat used in analysis of this biosensor.

Figure 3. 2 A carbon nanofiber array (shown as the dark area in the middle of the field) grown on a silicon oxide substrate.
2mm x 2mm piece of silicon oxide with 0.5mm x 0.5mm areas of carbon nanofibers grown to the surface. Shown here are two, titanium leads extending from the left and right sides of the carbon nanofiber array area; the leads are approximately 2mm in length.

electrode is described in the following section. The working electrode was operated both by sweeping through a range of potentials and by holding the electrode at constant specific potentials in order to measure the resulting electrical current. All electrochemical experiments were conducted at room temperature, approximately 24°C. A 1.3cm long and 0.32cm wide magnetic stir bar, was obtained from Fisher Scientific (Fisher Scientific, Hampton, NH) and was placed in the electrochemical cell in order to ensure adequate mixing of the solution (at a speed of 400rpm) and facilitate convective transport in the cell for experiments in which the potential was held at a constant value.

3.3 Fabrication of the Working Electrode

Silicon oxide wafers with vertically aligned carbon nanofibers were grown by a vapor deposition procedure (see Section 2.9). The wafers were then altered to assume the role of a working electrode for electrochemical measurements. There were nine separate carbon nanofiber areas grown onto a single circular silicon oxide wafer with a diameter of 15.24cm. The wafer was cut into 2mm x 2mm pieces using a diamond scribe; each piece contained one area of carbon nanofibers, see Figure 3.2. Each area of carbon nanofibers was approximately 0.5mm x 0.5mm in area and the diameter and length of each fiber was approximately 100nm and 4μm respectively. An example of a single vertically

aligned carbon nanofiber can be seen in Figure 3.3. Extending from each carbon nanofiber area was at least one small, thin titanium wire lead ranging from 2mm to 4mm in length, see Figure 3.2. Scanning electron micrograph images of the carbon nanofibers can be seen in Figure 2.6. On top of each titanium lead were randomly grown carbon nanofibers. This occurred as a result of the vapor deposition process used to create the silicon wafer with the carbon nanofiber areas as described in section 2.9. To facilitate a better electrical contact, the fibers were gently brushed off the top of the titanium lead by a dry cotton swab.

The 2mm x 2mm silicon oxide wafer with the carbon nanofibers grown to them were sonicated in a 1M sulfuric acid wash for 5 minutes [6]. This was done in order to ensure that the fibers were as clean as possible and to reduce the chance of contaminants attaching to the fibers by adsorption or other mechanisms. The wafer was then rinsed gently with distilled water. These precautions were carefully adhered to in order to ensure minimization of contamination of the working electrode surface. Once these procedures were accomplished they could not be applied after modification of the electrode was complete due to the fragile nature of the immobilized species (the enzyme and cofactor).

An insulated 30 gauge wire was cut to 4 inches in length, and 1mm of each end of the wire was stripped. To one end, a drop of solder was deposited and this soldered end was then glued to a corner of the wafer and positioned so that it did not lie on top of the carbon nanofibers or the metal lead, but instead on top of

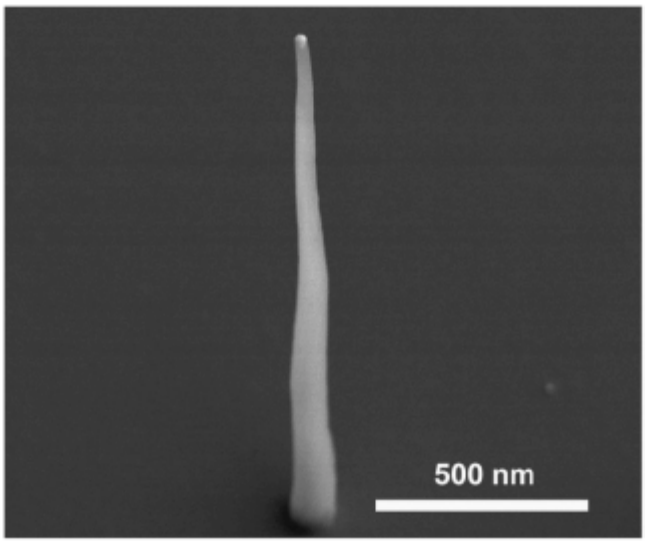


Figure 3. 3 Scanning Electron Micrograph of vertically-aligned carbon nanofiber (VACNF) grown via the PECVD process (described in the text). The fiber pictured is at a 30° tilt angle, so the apparent height is approximately two times smaller than the actual height of the fiber [6].

silicon oxide. The glue used was Loctite®Epoxy Gel which is both waterproof and allows adhesion to glass surfaces. In order for the glue to fully set, it was allowed to cure for 24 hours before the working electrode was used. It was discovered that attempts to solder the 30 gauge wire directly to the metal lead were not very successful. The solder did not adhere well to the silicon oxide surface. By gluing the soldered wire to the silicon, it was assured that the wire would stay attached to the wafer. The resistance through the metal lead was lower than the silicon oxide portion of the wafer so it was important that a connection be established through the titanium lead. Using a Circuit Writer™ silver pen (Caig Labs, Inc.), a line was drawn connecting the metal lead and the solder so that current, when applied, would run through the carbon nanofibers to the wire and eventually to the potentiostat. The final electrode can be seen in Figure 3.4. The electrical resistance through the titanium wire lead is much lower than the resistance through the silicon oxide portion. It was important that a good electrical connection be established from the wire lead to the titanium lead in order to ensure minimal resistance. The resistance of this electrode was tested and found to be approximately 1000 ohms. Carbon electrodes typically have low resistances on the order of several hundred ohms. The resistances associated with this experimental set up are unique to this procedure, other methods of connection might provide lower resistances.

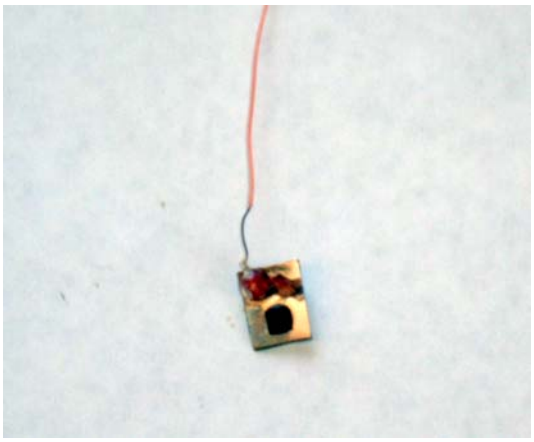


Figure 3. 4 Constructed working electrode**.**
The electrode shown has an area of 4mm x 3mm. The carbon nanofiber area grown to the silicon oxide wafer piece is 1.5mm x 1.5mm. A 30 gauge wire with solder deposited on one end was glued to one edge of the silicon oxide wafer. Using a silver pen, a line was drawn connecting the solder and the titanium lead.

3.4 Immobilization of YADH and NAD

Two techniques were used to immobilize the enzyme and coenzyme to the carbon nanofiber electrode. Each technique required that different types of chemical bonds be used and each technique had their own unique properties which could affect the outcome of the electrochemical measurements. The intent was to determine if a different immobilization technique would affect the outcome. The first technique was immobilization by adsorption to the surface. First a 6000U/mL (0.05mg in 10mL of buffer solution) solution of YADH in pH 7.4 sodium phosphate buffer was prepared. A clean carbon nanofiber electrode was immersed in this solution for two hours. A magnetic stir bar was present to allow stirring at a speed of 400rpm during this period. After two hours the electrode was removed from the solution and the excess enzyme was gently washed off with distilled water and the electrode was allowed to dry. A 5mM solution of NAD^+ in pH 7.4 sodium phosphate buffer was prepared. The electrode was immersed into this solution for two hours as the solution was stirred at 400rpm. After two hours the electrode was removed from the solution and the excess coenzyme was gently removed by washing it with distilled water making the electrode ready for use [42].

Immobilization by covalent bonding involves several treatment steps before the enzyme and coenzyme can be immobilized. The particular technique employed was diimide-activated amidation. Treatment with EDAC forms a

reactive intermediate at the carboxylic acid site on the carbon nanofiber that can couple with available amines to form amides and NHS works to stabilize the reaction [6]. Concentrations of 26mM of EDAC and 43mM of NHS were added to 50mL of pH 6.0 MES buffer and the electrode was allowed to sit in this solution for 2 hours as the solution is stirred. Then a 6000U/mL (0.05mg in 10mL of buffer solution) solution YADH in pH 7.4 sodium phosphate buffer was prepared and then the electrode was transferred to this solution. The electrode was allowed to sit in this solution for 2 hours with stirring. After two hours the electrode was rinsed with deionized water to remove excess and loosely bound enzyme and then allowed to dry. A solution of 5mM NAD^+ was then added to a pH 7.4 sodium phosphate buffer and the electrode was added to this solution. The electrode was allowed to sit for another two hours with stirring at 400rpm. At the end of this final treatment the electrode was washed with deionized water to remove excess and loosely bound coenzyme and then allowed to dry. The electrode preparation was complete and the electrode was ready for use [43].

The finished electrodes were stored in a dry state to minimize coenzyme leakage and in a desiccator so as to minimize adsorption of contaminants. The electrodes were stored at 4^o C and between measurements no additional modifications or preparations were made to the electrodes (See section 3.3).

CHAPTER 4 RESULTS AND DISCUSSION

The goal of this project was to construct and evaluate the function of an enzymatic biosensor that could quantify the amount of ethanol, methanol and isopropanol in a solution. Experiments were first conducted with an unmodified electrode, an electrode to which enzyme and coenzyme are not immobilized. This allowed the function of the electrode itself to be checked to determine if it was working properly before immobilization of the enzyme and coenzyme. This electrode was characterized by NADH and electrochemical trials were then conducted of ethanol, methanol, and isopropanol. The modified electrode, the electrode to which enzyme and coenzyme were immobilized, was then used to evaluate the biosensor in the detection of ethanol, methanol, isopropanol.

4.1 Electrochemical Analysis Of NADH

Electrochemical analysis of aqueous phase NADH with the unmodified electrode allowed for the electrode to be tested for function and to determine the potential at which later experiments would be conducted. In order to better understand the nature of the newly constructed electrode, experiments were first conducted with the unmodified electrode, one without YADH or NAD^+

immobilized to the carbon nanofiber surface. This study would allow for any flaws in the electrode design to be more easily identified and corrected. This permitted elimination the of electrode design as a possible source of error in later experiments. The characterization of the unmodified electrode with a solution of NADH is a relatively straightforward experiment compared to the complexities involved in using YADH and NAD^+ that would arise in the later experiments. This characterization reduced the possibility for random error and thus allowed for more control over experimental variables.

The first step in characterizing the electrode with NADH was to make observations of the electron transfer kinetics between the working electrode surface and NADH as the electrochemically active species. The characterization of the electrode with NADH is essential in determination of the voltage potential at which future experiments would be conducted. The oxidation peak potential determined from these experiments indicated an approximate potential at which future amperometric experiments should be performed. The electrode used for amperometric detection of NADH was constructed as explained in Chapter 3; however no enzyme was immobilized on the surface. A 1mM solution of NADH in pH 7.4 sodium phosphate buffer was prepared and 6mL of the solution was added to a 10mL electrochemical cell. The reference, auxiliary, and working electrodes were then introduced into the electrochemical cell. Cyclic voltammetry measurements were conducted without stirring. The potential was cycled between

-0.4V and 0.6V. In the forward scan, the potential started at -0.4V and ended at 0.6V, oxidizing NADH in the process. In the reverse scan, the potential was cycled back to its starting potential of -0.4V reducing NAD^+ back to NADH. From the cyclic voltammogram of NADH pictured in Figure 4.1, two extrema can be seen at 0.75V and at -0.24V, the first corresponding to the oxidation peak and the second to the reduction peak of the species. Other studies have obtained oxidation peaks of 0.82V with a glassy carbon electrode [34], and 0.55V with a multi-walled carbon nanotube electrode [35]. The difference between these experimental results and other published data is most likely due to the difference in chemical composition of each electrode surface which in turn affects their electron transfer kinetics characteristics.

The next step in the characterization was to perform amperometric measurements on NADH with the unmodified electrode. The reference, auxiliary, carbon nanofiber electrodes and the stir bar were introduced into a 10mL electrochemical cell. Then, 6mL of pH 7.4 sodium phosphate buffer was added to the cell. Experiments were performed with stirring at 400 rpm. The peak oxidation for cyclic voltammetry experiments occurred at a potential of 0.75V, this potential was initially applied between the working and reference electrodes for the amperometric measurements. In later trials (results not shown), the current

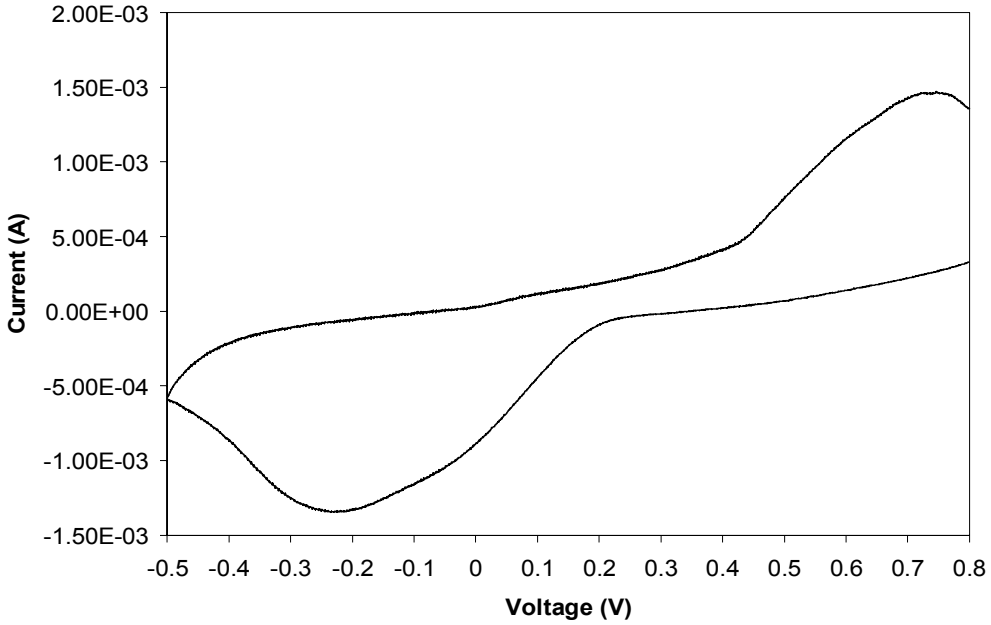
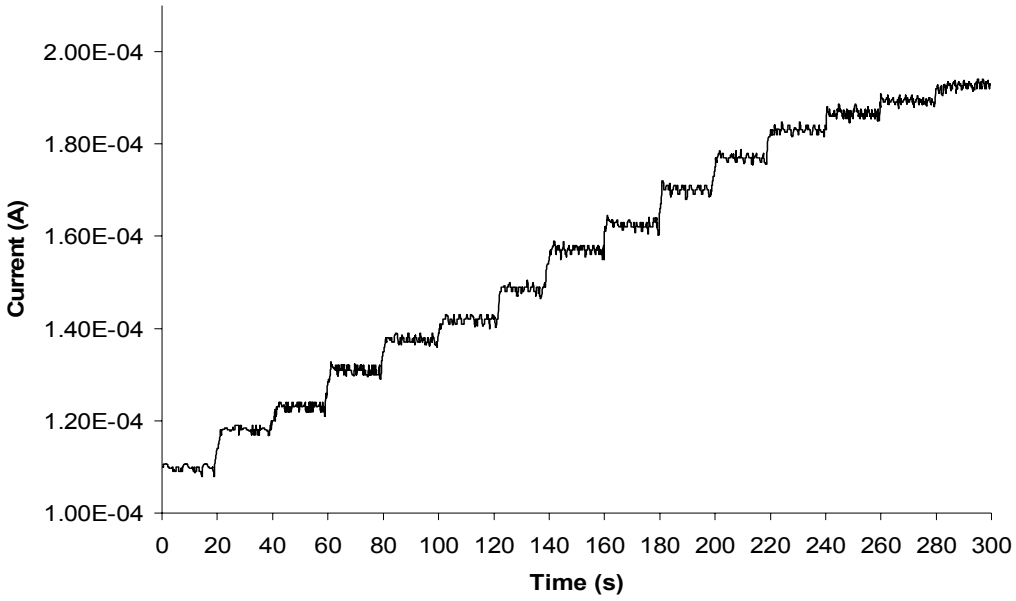


Figure 4. 1 Cyclic voltammetry of 1mM NADH with the unmodified carbon nanofiber electrode.

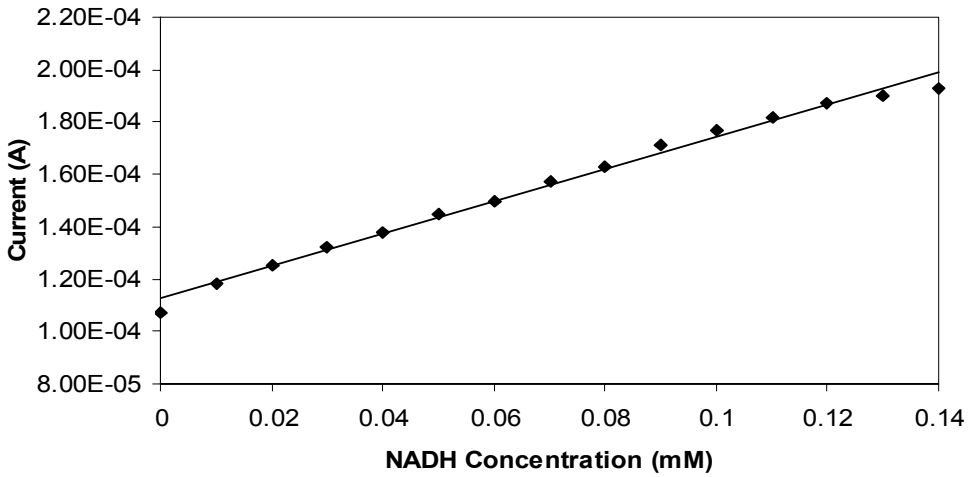
response from an applied potential of 0.9V was found to provide greatly improved results over the oxidation peak potential indicated from the cyclic voltammetry experiments of NADH. Additions of 0.1mL of 1mM NADH were pipetted into the electrochemical cell every 20 seconds. Addition of NADH into the cell generated a current that was then measured by the potentiostat. The resulting amperometric measurements can be seen in Figure 4.2. The response time of the unmodified electrode is shown to be quick and after each addition steady state electrical current was achieved in approximately three seconds.

4.2 Electrochemical Analysis of Ethanol Using Unmodified Electrode With Enzyme and Coenzyme in Solution

Before the modified electrode was tested, amperometric measurements were conducted using the unmodified electrode to detect the presence of ethanol in the solution. This required that the enzyme and coenzyme be present in the solution. Into the electrochemical cell, 1mL of 6000U/mL (0.05mg in 10mL of buffer solution) solution of pH 7.4 YADH in sodium phosphate buffer was added to the electrochemical cell along with 5mM of NAD and 50mM of pH 8.8 sodium pyrophosphate (final pH 8.1, within the enzymes optimal pH range of 7.0 to 9.0). The reference, auxiliary, unmodified carbon nanofiber electrodes and the stir bar were introduced into the cell. The solution was stirred at a speed of 400rpm.



(a)



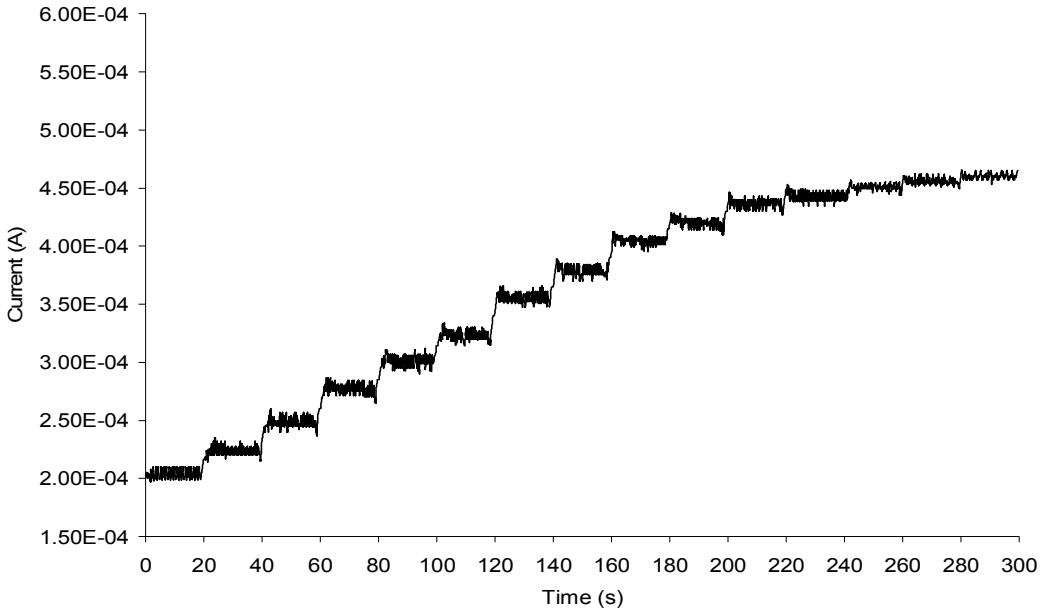
(b)

Figure 4. 2 (a) Amperometric measurements of 1mM NADH with unmodified carbon nanofiber electrode. (b) Current versus NADH concentration curve for the amperometric measurements of NADH.

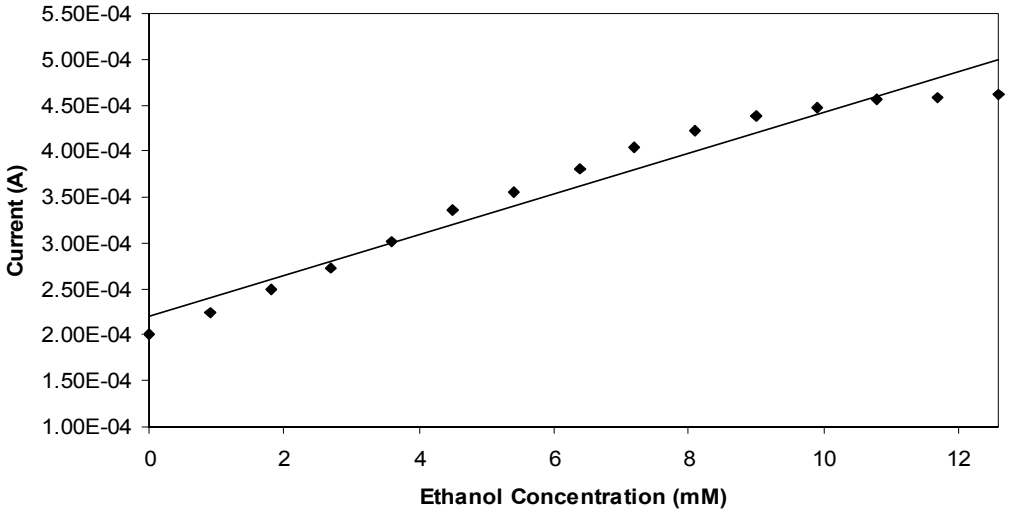
Additions of 0.1mL of 2M ethanol were pipetted into the solution every 20 seconds. A voltage potential of 0.9V was applied to the system instead of the oxidation potential of NADH as described in section 4.1. The amperometric measurements obtained can be seen in Figure 4.3. Steady state electrical current for this system was reached within approximately five seconds.

4.3 Electrochemical Analysis of Ethanol Using Modified Working Electrode With Immobilized Enzyme and Coenzyme

After the amperometric measurements of ethanol with the unmodified electrode, all subsequent experiments made use of the modified electrode with YADH and NAD^+ immobilized to the carbon nanofiber surface. Amperometric measurements were conducted with the modified electrode for the detection of ethanol. Electrode surfaces were used that were modified with YADH and NAD by adsorption and covalent attachment methods. Into a 10mL electrochemical cell, 3mL of pH 7.4 PBS and 3mL of pH 8.8 sodium pyrophosphate were added. The reference, auxiliary, modified carbon nanofiber electrodes and the stir bar were introduced in the cell. The solution was stirred at speed of 400 rpm. Additions of 0.1mL of 2M ethanol were introduced into the solution every 20 seconds. Characterization of the unmodified electrode in NADH was described in section 4.1, so the potential at which amperometric measurements would be



(a)



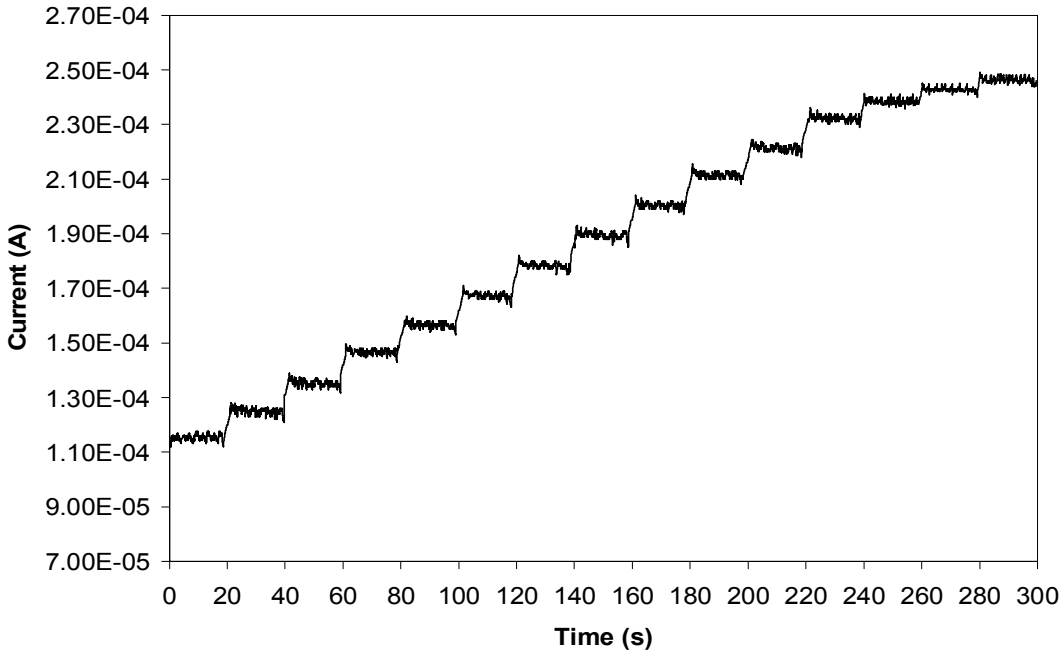
(b)

Figure 4. 3 (a)Amperometric measurements of ethanol with unmodified carbon nanofiber electrode. (b) Current versus ethanol concentration curve for the amperometric detection of ethanol.

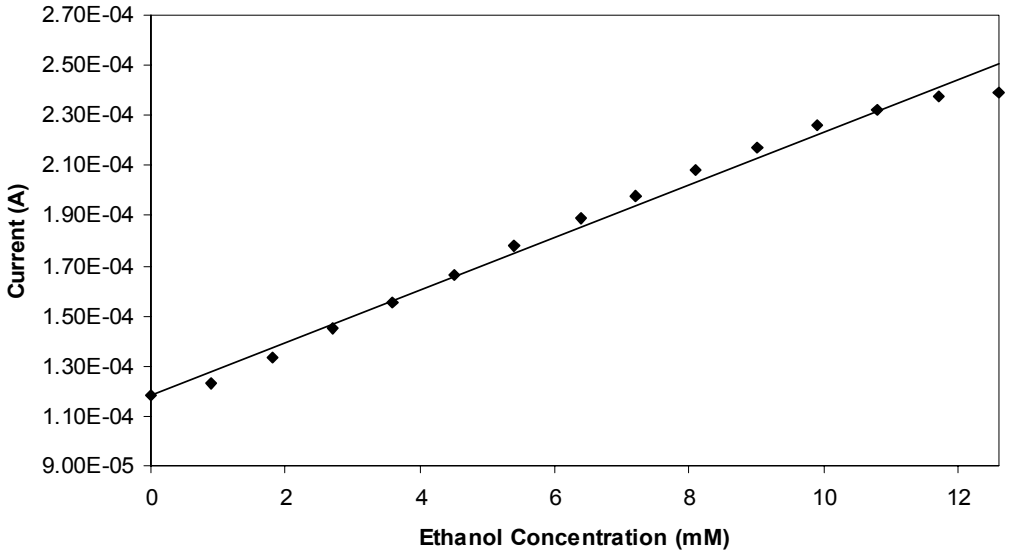
conducted had already been determined. The oxidation voltage potential was set at 0.75V as determined by the cyclic voltammograms of NADH. This potential provided poor results but a slightly higher potential of 0.9V was found to provide improved results. Figure 4.4 and 4.5 illustrate the amperometric measurements obtained from electrodes on which the enzyme was adsorbed and covalently attached, respectively. Steady state was achieved in approximately six seconds for both biosensors.

4.4 Detection Limit

The ethanol concentration range for which this detection electrode functions is important to consider when evaluating a biosensor. Different biosensors will have varying concentration ranges for which they are effective. The detection limit for a biosensor is the smallest concentration that can be measured by the device and for which it will respond to changes in concentration [32]. The modified electrode with enzyme adsorbed to the surface displayed a detection limit of 0.1mM. The modified electrode with enzyme covalently attached to the surface displayed a detection limit of 0.1mM (data not shown). Below a concentration of 0.1mM ethanol, the biosensor displayed a poor response with no clear distinction between changes in concentration of ethanol. Although the biosensor will detect concentrations equal to or larger than the

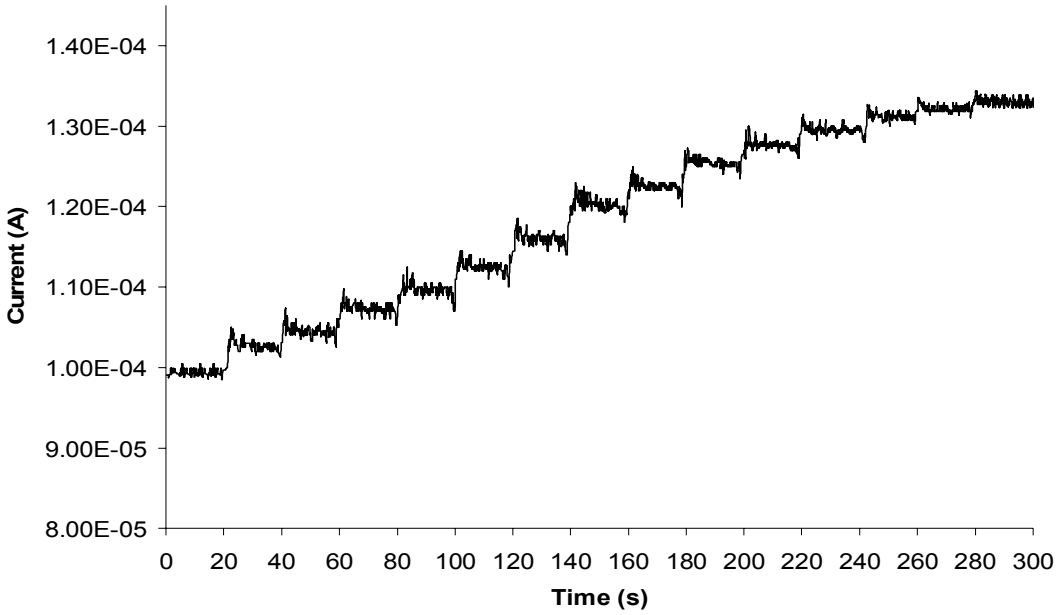


(a)

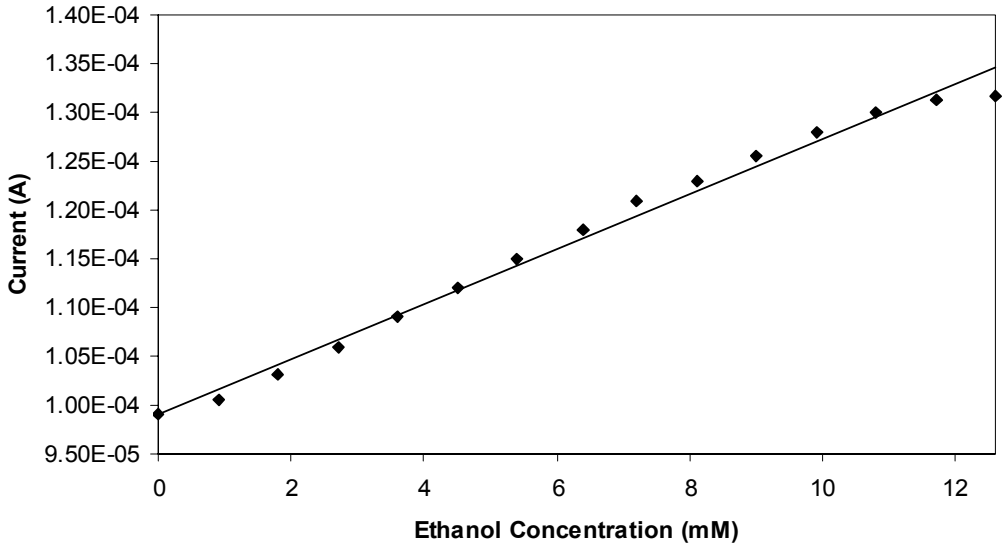


(b)

Figure 4. 4 (a) Amperometric measurements of ethanol using modified carbon nanofiber electrode (covalent attachment). (b) Current versus ethanol concentration of amperometric measurements of ethanol using modified electrode**.**



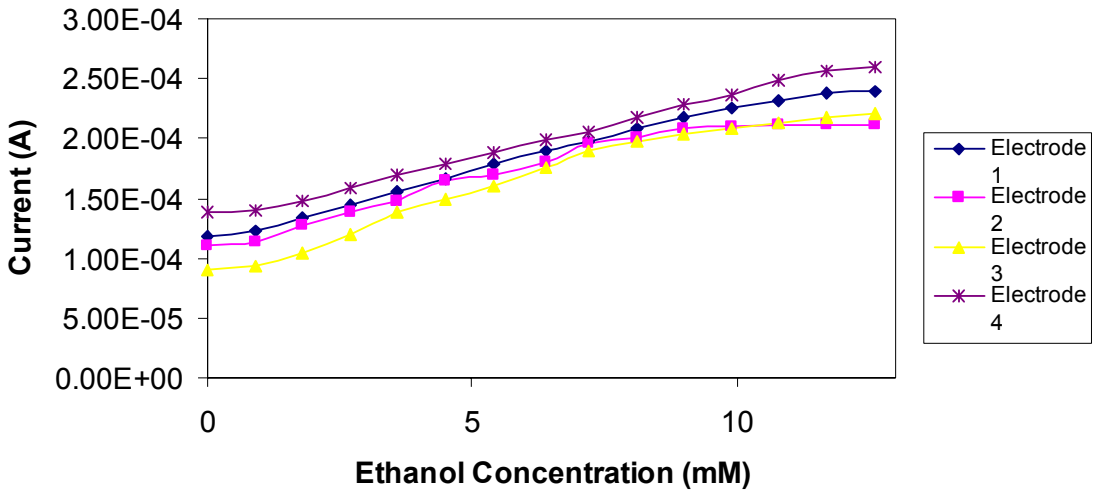
(a)



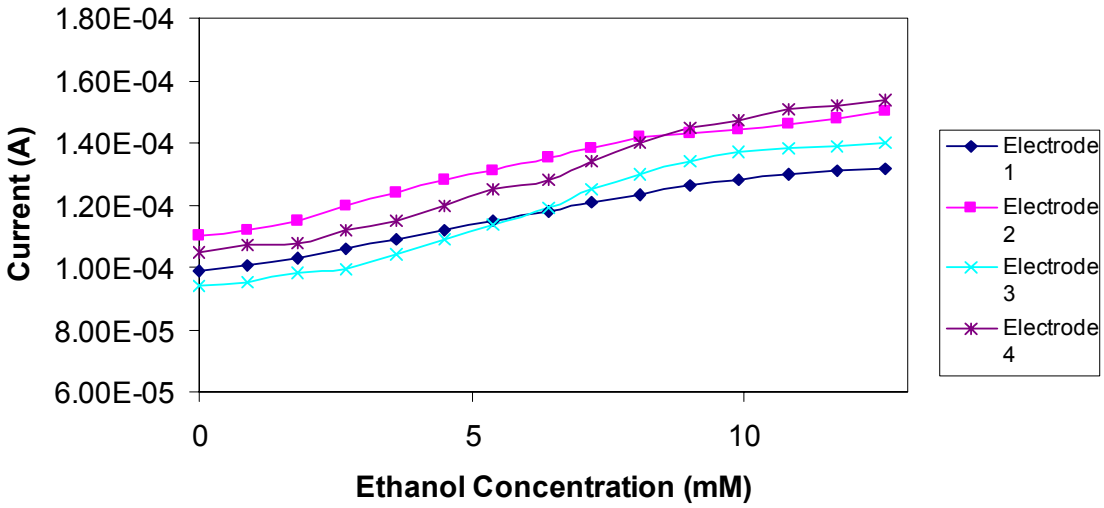
(b)

Figure 4. 5 (a) Amperometric measurements of ethanol with modified carbon nanofiber electrode (adsorption). (b) Current versus ethanol concentration for amperometric measurements with modified electrode.

detection limit, the largest linear concentration range is generally an indication of the strongest change in current per change in analyte concentration. Biosensors are often evaluated by comparing their linear concentration range with those of previously developed sensors. By measuring the slopes between the data points along the current versus concentration curve, the largest linear concentration range was determined for the modified electrodes. Results from eight different trials (four for the electrode with adsorbed enzyme and four for the electrode with covalently attached enzyme) were compared to determine the linear concentration range, see Figures 4.6a and 4.6b. The linear concentration range for the modified electrode used in these experiments with the enzyme adsorbed to the surface is approximately 1.75mM to 6mM (see Figure 4.5). The linear concentration range for the modified electrode was determined by measuring the slopes along the length of the curve. For the electrode with the enzyme covalently attached to the surface the linear concentration range was found between 2mM to 8mM (see Figure 4.4). Studies of other alcohol biosensors have displayed concentration ranges of 45μM-4mM for a carbon paste electrode [37] and 1.5M to 8.5M for a multi-walled carbon nanotube electrode [41].



(a)

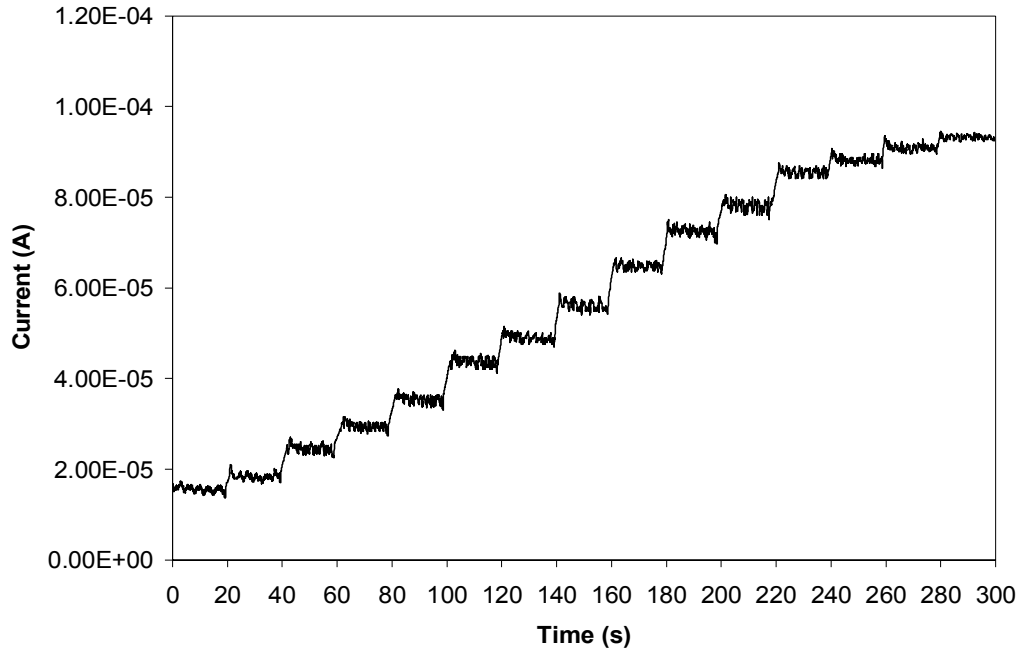


(b)

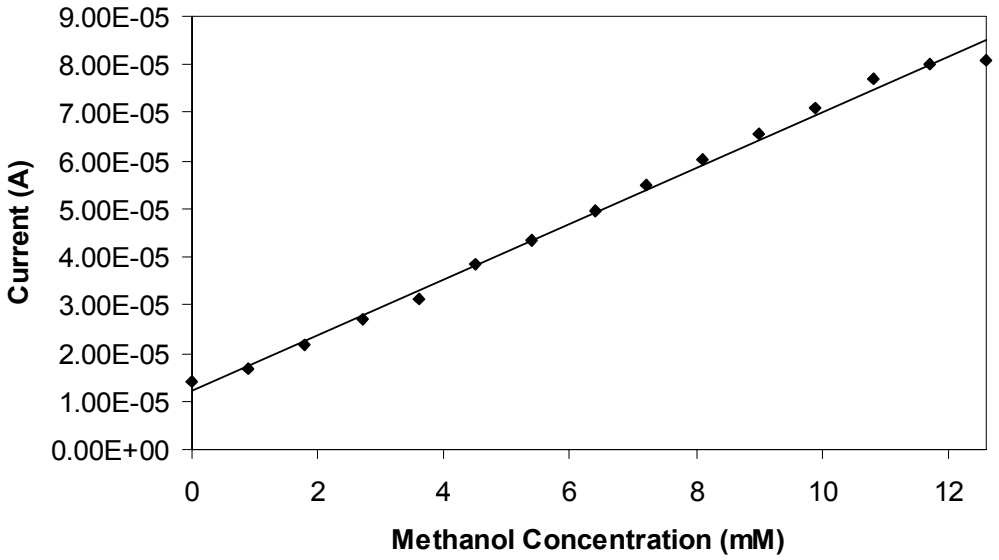
Figure 4. 6 (a) Amperometric data for four different modified electrodes with covalently attached enzyme (b) amperometric data for four different modified electrodes with adsorbed attached enzyme.

4.5 Amperometric Measurements of Methanol and Isopropanol

YADH is most reactive with small unbranched alcohols like ethanol but has the capacity to react with other, larger and branched alcohols. Methanol and isopropanol are two such alcohols with which YADH will react. The sensor with covalently attached enzyme was used for these experiments due to the fact that this sensor was found to operate over a larger concentration range and had similar sensitivity to that of the electrode with adsorbed enzyme. Amperometric measurements were conducted with this electrode to detect methanol and isopropanol. 3mL of pH 7.4 PBS and 3mL of pH 8.8 sodium pyrophosphate were added to a 10mL electrochemical cell. The reference, auxiliary, modified carbon nanofiber electrode and stir bar were introduced in the cell as before. The solution was stirred at a speed of 400rpm. Additions of 0.1mL of 1.5M methanol and 1.5M isopropanol were added, in different trials, to the solution every 20 seconds. A working potential of 0.9V was applied for amperometric characterization. The results from these trials can be seen for methanol in Figure 4.7 and for isopropanol in Figure 4.8. The electrical current response from methanol and isopropanol was much weaker than that of ethanol. At a concentration of 4.5mM ethanol the current response was 1.66*10^-4A for a modified electrode with covalently attached enzyme. At the same concentration of 4.5mM methanol and 4.5mM isopropanol, the current response was 3.86*10^-5A and 1.83*10^-5A, respectively. The linear concentration ranges of methanol and

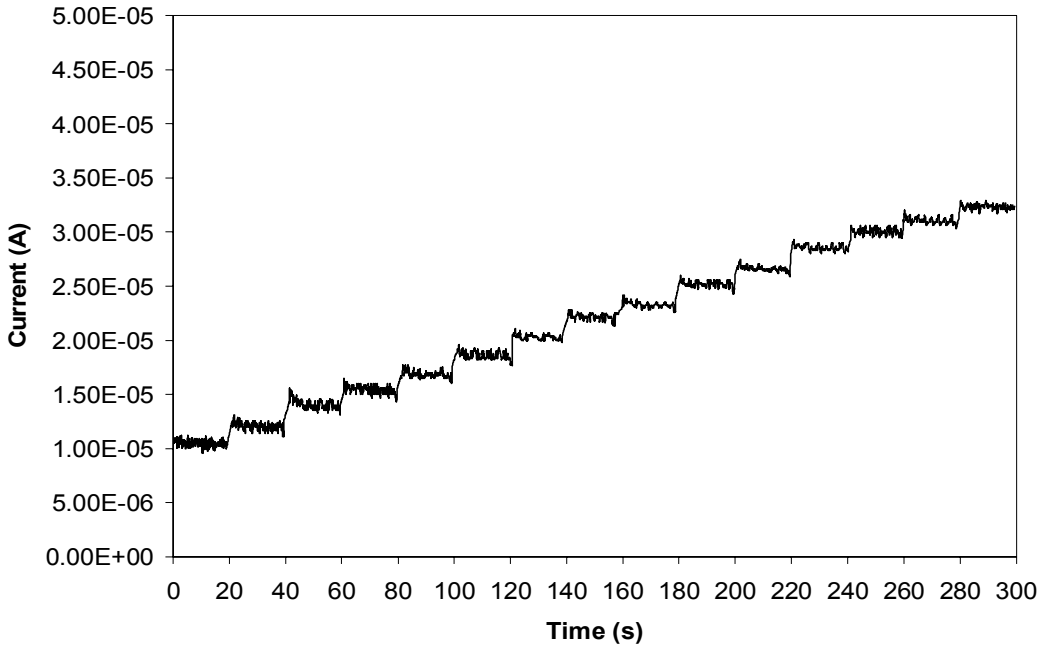


(a)

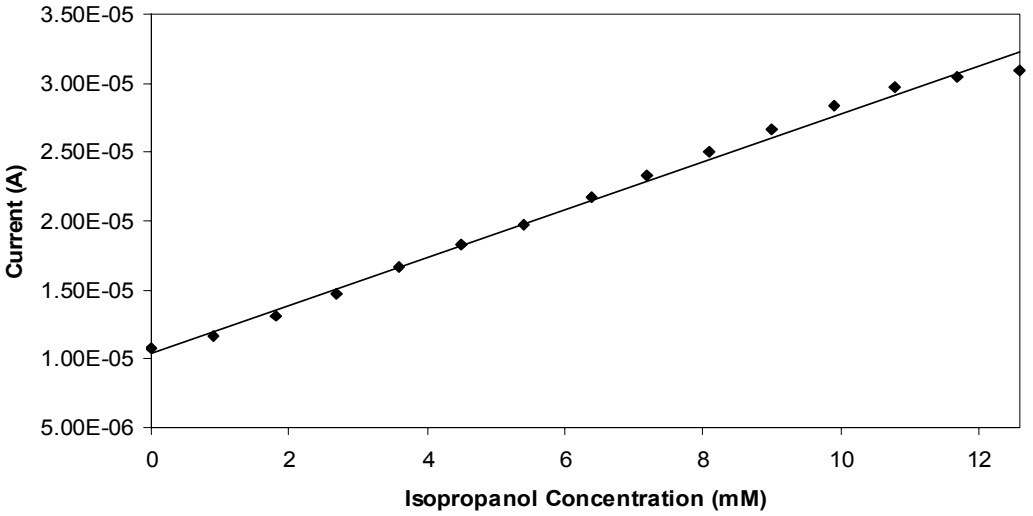


(b)

Figure 4. 7 (a) Amperometric measurements of methanol with modified carbon nanofiber electrode. (b) Current versus methanol concentration of amperometric measurements with modified electrode.



(a)



(b)

Figure 4. 8 (a) Amperometric measurements of isopropanol with modified carbon nanofiber electrode. (b) Current versus isopropanol concentration of amperometric measurements using modified electrode.

isopropanol for which the biosensor was effective were similar to that for ethanol.

4.6 Immobilized Enzyme on Electrode

Determination of the concentration and the activity of YADH on the carbon nanofiber electrode were not tested. In order to ensure that sufficient enzyme be exhausted too quickly, an excess concentration of enzyme solution was used for the immobilization step. An excess in enzyme concentration, as compared to the concentration of enzyme used in the standard assay for enzyme activity as specified by Sigma-Aldrich, was available in solution in the event that a small fraction of the bulk concentration actually immobilized onto the carbon nanofiber surface. Immobilization can have a profound effect on the conformation and function of the enzyme that is able to bind to the electrode surface [28]. These procedures can severely limit the activity of the successfully immobilized enzyme.

4.7 Storage Stability

A biosensor should be able to function and provide a strong current response even after periods of storage. The stability of the carbon nanofiber electrode with YADH and NAD^+ immobilized to the surface was tested. These tests were conducted for both the adsorbed and covalently attached enzyme. The electrodes were stored in a dry state to minimize coenzyme leakage from the

electrode surface. Amperometric measurements were made on the first day, the day in which the electrode was constructed and modified by both immobilization methods. The electrodes were stored at 4°C and the stability was tested after three, five, and seven days. For each test the electrode was used to measure the current resulting from 0.1mL additions of 2M ethanol. All three electrodes were introduced into the electrochemical cell and a working potential of 0.9V was applied. The resulting current was compared to measurements from the first day. Both electrodes proved to have very little loss in stability after a week of storage. After seven days there was approximately a 1.5% loss in electrical current response for both electrodes (results not shown). This was determined by comparing the final current obtained, after 300 seconds, on each day. These results indicate excellent storage stability for this biosensor.

4.8 Reusability

Reusability of a biosensor is an important criterion to consider in its evaluation. A biosensor should be able to withstand multiple uses and still retain the ability provide an accurate and reproducible current. Both the electrodes with adsorbed and covalently attached enzyme were tested. The reusability of the electrode was tested by conducting 20 amperometric measurements and observing the decline in current response with the repeated uses. These tests were conducted

under the same conditions as the amperometric measurements of ethanol with the modified carbon nanofiber electrode. Between each trial, the electrode was rinsed with deionized water. Both the modified electrodes with adsorbed and covalently attached enzyme were evaluated. The change in the response upon repeated use was determined by comparing the difference in current response at three difference concentrations and taking the average. After 10 measurements the average current as defined above was 76% of the original current and after 20 measurements was approximately 60% of the original current. This was true of modified electrodes with adsorbed and covalently attached enzyme. Additions of enzyme were applied and did not have a visible effect on the current response. It appears that enzyme detachment is not a contributing factor to the decline in current response, however this cannot be proven as the amount of enzyme initially immobilized to the carbon nanofiber surface is unknown. Additions of coenzyme were applied before use of the sensor and during amperometric measurements. The electrodes showed an increase in current response suggesting that NAD^+ will dissociate from the active site of the enzyme or become inactive over repeated uses. These results indicate that coenzyme detachment may play a role in the decline in current response.

4.9 Response Time

Real time detection is an important quality in determining the success of a biosensor. Response time can determine the suitability of a sensor for *in vivo* detection, for applications in which real time response is useful or necessary. It is important that a biosensor exhibit a relatively short response time. Response time was approximated by taking determining the average length of time taken to reach a current response that deviated within a certain range after each addition of analyte, this range differed depending on the analyte or electrode used. Amperometric detection of NADH exhibited a response time of approximately three seconds. For the trials in which YADH and NAD^+ were in free solution and were used to detect ethanol, the response was approximately five seconds. In the case of the electrode with YADH and NAD^+ immobilized to the surface, the response was determined to be approximately six seconds, as it was for methanol and isopropanol.

4.10 Importance of Carbon Nanofiber Pretreatment

Before construction of the electrode commenced, an acid wash was applied to rid the surface of contaminants. The effect of this acid wash was studied to determine how it affected the detection capabilities of the final biosensor. Amperometric measurements were compared between an electrode to which an

acid wash was applied and electrode to which no acid wash was applied, only a wash with deionized water. There was no observed effect on cyclic voltammetry results however there was an observed difference on the amperometric measurements. Untreated electrodes were on average about 30% less responsive (the current produced in response to a particular ethanol concentration) than treated electrodes. This was tested by evaluating the difference in current response at three different concentrations (3.6mM, 6.4mM, and 8.1mM) and then taking the average. This not only would affect a single test, but it could potentially affect the reusability and stability of the electrode.

CHAPTER 5 CONCLUSION

5.1 Summary

The objective of this project was to construct a biosensor based on carbon nanofibers. The biosensor was to be reagentless, amperometric and enzymatic in its function. After construction, investigations were conducted to evaluate the efficiency of the sensor. The performance of this biosensor was to be compared to results obtained by Holland. Holland made use of multi-walled carbon nanotube (MWNT) electrodes to evaluate the electrical current response to ethanol. The cyclic voltammetry and amperometric procedures used by Holland were used to evaluate the sensors described in this study. When compared to experiments conducted by Holland with a multi-walled carbon nanotube (MWNT) electrode, results obtained in terms of current response and linear concentration range, were improved by methods used in this study and will be addressed later in this chapter [41]. Different immobilization strategies were studied as was the current response to several alcohols. Finally, investigations were performed to test the stability, reusability and range of concentrations that could be used with the sensor.

Carbon nanofibers were found to be an effective strategy for building a biosensor. Their structure is conducive to immobilization because carbon nanofibers have many defect sites which have exposed carboxyl groups that allow successful immobilization of the biological element. The abundance of defect

sites is not only good for immobilization but also for strong electrical current response. Two immobilization strategies were tested; adsorption and covalent attachment. At a working potential of 0.9V, both electrodes were found to provide a considerably strong current response.

Characterization of the electrode was first conducted with aqueous phase NADH. This served to test the function of the electrode as well as to determine the oxidation peak of NADH with the electrode. This information was important for amperometric measurements that would subsequently be conducted. Amperometric experiments were then conducted with ethanol. Before testing the modified electrode, experiments were conducted with the unmodified electrode to detect the presence of ethanol. A potential of 0.9V was found to provide maximum current response. For a 300 second trial the current ranged between 2.02*10^-4A and 4.65*10^-4A, and steady state was achieved within approximately five seconds. Amperometric measurements were then conducted with the modified electrodes; electrodes to which enzyme was immobilized by adsorption and covalent attachment. A potential of 0.9V was used and the current response from the electrode modified with adsorbed enzyme provided a current response ranging between 9.98*10^-5A and 1.34*10^-4A. The current response from the electrode modified with covalently attached enzyme provided a current response ranging from 1.12*10^-4A to 2.47*10^-4A. Both trials were conducted for 300 seconds and steady state was achieved in approximately six seconds for

both electrodes. Results obtained by Holland for 300 seconds indicated current responses that ranged from approximately 2*10^-9 A to 100*10^-9 A for an electrode with adsorbed enzyme on a MWNT electrode, and a range of approximately 20*10^-9 A to 75*10^-9 A for an electrode with covalently attached enzyme to a MWNT electrode.

Furthermore, of interest were the linear concentration range, reusability, and storage stability of the amperometric enzymatic biosensor. The linear concentration range of the electrode with adsorbed enzyme was 1.75mM to 6mM ethanol. The linear concentration range of the electrode with covalently immobilized enzyme was 2mm to 8mM ethanol. Results obtained by Holland reported a linear concentration range of 1.5M to 8.5M for a MWNT electrode with adsorbed enzyme. A linear concentration range of 0.5M to 7.0M was reported for Holland's MWNT electrode with covalently attached enzyme [41]. Both sensors were found to still be effective after repeated uses. The loss in activity over 10 and 20 uses was approximately 25% and 40% respectively. Loss in activity for Holland's MWNT electrode was approximately 30% after 10 uses [41]. As for the storage stability of the sensors, both stood up extremely well after three, five, and seven days of storage with a loss in activity of approximately 1.5%, compared to a loss in activity of less than 1% obtained from studies by Holland [41].

The behavior of the biosensor was also tested with methanol, and isopropanol. The electrode with enzyme covalently attached was used to test the

performance of the biosensor with these alcohols. The response to methanol and isopropanol proved to be substantial for this biosensor. The current response was not as strong as for ethanol; current values were on average one order of magnitude lower than that of the ethanol experiments. The loss in activity, and reusability were the same as for the ethanol experiments.

The carbon nanofibers were exposed an acid wash pretreatment before electrode construction and enzyme immobilization, to observe the effect of the acid wash on the performance of the biosensor. The silicon wafer piece (2mm x 2mm) with carbon nanofibers grown to the surface was sonicated in a 1M sulfuric acid wash for five minutes. The electrode was then constructed and the enzyme and coenzyme were immobilized to the surface. The results of this experiment seem to indicate that the acid wash has a great influence on the current response of the biosensor. The purpose of applying the acid wash was to attempt to remove contaminants from the carbon nanofiber surface. In this study the result was a 30% difference in current response, with the electrode to which the acid wash was applied displaying a much stronger current response.

5.2 Future Work

Future work that would further the development of the research presented in this thesis would include the determination of the concentration of active

enzyme that was successfully immobilized on the surface of the carbon nanofibers. It would be useful to determine the effect on current response if any, due to irreversible oxidation that occurs to the carbon nanofibers, and to determine the effectiveness of the acid washes. With the setup used in this research there was much resistance that is believed to be largely attributed to the manner in which the wire was connected. Other methods such as wire bonding could help decrease the resistance. It would be interesting to see how much of an effect entrapment as an immobilization method might have on the sensor response. Entrapment might also increase the sensitivity and stability of the biosensor by slowing down the deactivation of the enzyme immobilized within it and may also restrict the enzyme which could decrease leakage from the surface. Considering the relatively few studies that have been done with carbon nanofibers for an enzymatic biosensor, more studies in this field will naturally help in the understanding of these structures and how they can contribute to biosensor development.

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VITA

Martha Weeks was born in Skokie, Illinois on April 3, 1981. She graduated from Glenbard South High School in May of 1998. In August 1998 she entered the University of Illinois at Urbana-Champaign and earned a Bachelor of Science degree in Biology and a Bachelor of Arts degree in Spanish. In August of 2004 she entered the University of Tennessee at Knoxville to earn a Masters degree in Chemical Engineering which she completed in August of 2006.