VOT 74171

PRODUCTION OF ENZYMATIC GLUCOSE BIOSENSORS

DR. AZILA ABDUL AZIZ

PUSAT PENGURUSAN PENYELIDIKAN
UNIVERSITI TEKNOLOGI MALAYSIA

2006

ACKNOWLEDGEMENTS

I would like to express my appreciation to all those who have assisted me, whether directly or indirectly, in conducting this research. There are too many names to list down. However, special thanks goes to the research assistants, Wong Fui Ling and Norhana Mohd Jusoh for their dedications and tireless efforts in making this research work a success.

ABSTRACT

For this project, two types of glucose biosensors namely hydrogen peroxide-based glucose biosensor and mediated glucose biosensor have been developed. The performance of a glucose biosensor depends mostly on the immobilization method and support materials that are being used. For hydrogen peroxide-based glucose biosensor, selection of suitable materials for enzyme immobilization was done. Four types of immobilization materials, including chemically-linked PVA, TMOS sol-gel, alumina sol-gel, and freezed-thawed PVA cryogel, were used to immobilize glucose oxidase (GOD) to determine the most appropriate material for GOD immobilization. Generally the membranes had shown good sensitivity except for the chemically cross-linked PVA. However, the main differences with the enzyme immobilization methods were enzyme leakage and the values of K_m^{app}. Freeze-thawed PVA-GOD membranes, which showed satisfactory sensitivity and adequate value of K_m^{app} was chosen as the support material for immobilizing GOD. The enzyme leakage of this type of membrane was improved by reducing enzyme loading. Even though this type of sensor is very simple and easy to construct, it suffers from electrochemical interferences from common electroactive species present in blood such as acetaminophen. Thus, a selective inner layer based on permselectivity was studied. pHEMA, at a cross-linking ratio of 0.043 which resulted in a permselectivity of 10, successfully eliminated acetaminophen interference. Nafion membrane was used as the outer membrane to protect the biosensor.

For the mediated based glucose biosensor development, the scopes of work include the preparation of active layer, preparation of external layer and the fabrication of glucose biosensor. Three methods of tethering a mediator to an enzymatic membrane were studied to construct a non-leaking mediated glucose

biosensor. The methods were immobilization of glucose oxidase (GOD) and ferrocene redox polymer in cross-linked poly (vinyl alcohol) (CLPVA) with bovine serum albumin (BSA) as a protein stabilizer, immobilization of ferrocene carboxylic acid and glucose oxidase in a sol gel derived silica (SGS) matrix containing cross-linked poly (vinyl alcohol) (CLPVA) and nafion, and lastly multilayered construction of glucose oxidase and redox poly (allylamine) ferrocene utilizing layer-by-layer covalent attachment. After evaluating the biosonser response amperometrically at 0.363V, the first method, which was immobilization of glucose oxidase and ferrocene redox polymer in CLPVA with the addition of BSA was selected for the fabrication of disposable glucose biosensor since this type of sensor provided good responses over a wide range of concentration. Nafion was chosen as the external layer and the works on the fabrication of the glucose biosensor are ongoing.

ABSTRAK

Untuk projek ini, kajian telah dijalankan atas dua jenis biosensor glukosa, iaitu biosensor glukosa berdasarkan hidrogen peroksida dan biosensor glukosa berdasarkan pengantara. Prestasi sesuatu biosensor glukosa banyak bergantung kepada cara penyekatgerakan enzim dan jenis bahan sokongan yang digunakan. Bagi biosensor glukosa berdasarkan hidrogen peroksida, pemilihan bahan yang sesuai telah dilakukan. Empat jenis bahan penyekatgerak telah dikaji iaitu poli(vinil alkohol) (PVA) disambung-silang secara kimia, , sol-gel (tetrametoksi)silane (TMOS), sol-gel alumina, dan kryogel beku-cair PVA, untuk menentukan jenis bahan yang paling sesuai bagi penyekatgerakan glukosa oksida (GOD). Secara umum, membran-membran yang dihasilkan menunjukkan sensitiviti yang baik kecuali PVA disambung-silang secara kimia. Walau bagaimanapun, perbezaan utama antara cara-cara penyekatgerakan ialah kebocoran enzim dan nilai K_m^{app}. Kryogel beku-cair PVA, yang menunjukkan sensitiviti yang memuaskan dan nilai K_m^{app} yang memadai, telah dipilih sebagai bahan sokongan untuk menyekatgerak GOD. Kebocoran enzim bagi membran jenis ini telah diperbaiki dengan mengurangkan kuantiti enzim yang dimasukkan dalam proses penyekatgerakan. Walaupun sensor yang berasaskan hydrogen peroksida tidak kompleks dan mudah untuk dibina, ia menghadapi masalah gangguan elektrokimia daripada spesies elektroaktif yang biasanya wujud dalam darah seperti asetaminofen. Oleh sebab itu, suatu lapisan dalaman yang boleh menghalang gangguan elektrokimia berdasarkan ketertelapan selektif telah dikaji. Poli(hidroksietil metakrilat) (pHEMA), dengan nisbah sambung-silang 0.043 yang menunjukkan selektiviti 10, berjaya menyingkirkan gangguan asetaminofen. Membran Nafion telah digunakan sebagai lapisan luaran untuk melindungi biosensor tersebut.

Bagi biosensor glukosa berdasarkan pengantara, skop kerja merangkumi penyediaan lapisan aktif, lapisan luaran, dan pembinaan biosensor glukosa. Tiga cara untuk mengikat pengantara ke membran yang mengandungi enzim telah dikaji untuk membentuk sebuah biosensor glukosa tanpa kebocoran pengantara. Cara-cara tersebut termasuk penyekatgerakan GOD dan polimer redoks ferrocene dalam PVA (CLPVA) tersambung-silang dengan menggunakan bovine serum albumin (BSA) sebagai agen penstabil protin, penyekatgerakan GOD dan asid karbosilik ferrocene dalam matriks sol gel silika (SGS) yang mengandungi CLPVA dan nafion, dan yang terakhirnya adalah multi-lapisan GOD dan redoks poli (allilamin) ferrocene menggunakan pelekatan kovalen lapisan demi lapisan. Selepas menilai gerak balas biosensor secara amperometrik pada 0.363V, cara pertama, iaitu penyekatgerakan GOD dan polimer redoks ferrocene dalam CLPVA bersama BSA telah dipilih untuk membentuk biosensor glukosa yang boleh dibuang selepas penggunaan memandangkan sensor jenis tersebut menunjukkan gerak balas yang baik dalam julat kepekatan yang luas. Nafion telah dipilih sebagai lapisan luaran dan kerja-kerja pembinaan biosensor glukosa yang lengkap sedang dijalankan.

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Research Background

Diabetes mellitus is a group of metabolic diseases characterized by high blood glucose levels, which result from defects in insulin secretion, or action, or both (Miller, 2003). In a person without diabetes, the body is able to regulate the amount of glucose in the blood between 3.5 to 6.5 mM with the help of the hormone insulin. Many people who are suffering from diabetes mellitus are not able to control their blood glucose level. In diabetes, the auto regulation of glucose fails and the blood glucose level of a diabetic sufferer may vary between 1 to 30 mM. The consequences of poor glucose regulation are at best, long term damage to organs from too much glucose (hyperglycemia), coma or death caused by too little glucose reaching the (hypoglycemia).

There are two major types of diabetes mellitus. Type 1 diabetes sometimes referred to as *juvenile diabetes* or *insulin dependent diabetes mellitus* (IDDM), usually strikes children and young adults. The insulin producing islet cells in the pancreas are destroyed by the diabetics' own immune system. These type 1 diabetics usually lose all insulin-producing capabilities and must inject themselves with insulin before each meal to allow their bodies to utilize glucose from the food. The type 2

diabetes or also referred as *non insulin dependent diabetes mellitus* (NIDDM) is very complex and usually strikes older people. Type 2 diabetics can usually increase their glucose regulation by losing weight and are initially treated with diet control and with drugs that help the body metabolizes glucose. Type 2 diabetics over time may need to start using insulin injections to maintain glucose regulation (Henning and Cunningham, 1998).

The findings of Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) clearly show that intensive control of elevated levels of blood glucose in patients with diabetes mellitus decreases the complications of nephropathy, neuropathy, retinopathy, and may reduce the occurrence and severity of large blood vessel diseases [Miller, 2003]. Tightly controlled blood glucose level means achieving fasting glucose level between 70-120 mg/dL, and glucose level of less than 180 mg/dL after meals. Studies in intensively treated type I patients have shown a decrease of diabetic eye disease by 76%, kidney disease by 54%, and nerve disease by 60%. In patients with type II diabetes mellitus, intensive blood glucose control shows similar beneficial effects on the eyes, kidneys, nerves and blood vessels (Miller, 2003).

Since the first publication on a glucose biosensor (Clark and Lyons, 1962), the detection of glucose has attracted a high degree of interest due to its biological importance (Liu *et al*., 2004). A biosensor is a sensor that is based on the use of biological material for its sensing function. The bio-component specifically reacts or interacts with the analyte of interest resulting in a detectable chemical or physical change. The amperometric glucose biosensor represents the most successful commercial biosensor development to date. Amperometric biosensors based on enzymes are interesting due to their high sensitivities, excellent selectivities, simplicity, low cost and rapid response.

The most frequently used enzymatic methods for glucose determination employ glucose oxidase (GOD), due to its high selectivity towards ß-D-glucose. GOD happens to be easy and cheap to obtain; secondly, it is one of the most robust enzymes around (it withstands greater extremes of pH, ionic strength, temperature than many other enzymes), thus allowing less stringent conditions during the

manufacturing process and also relatively care-free storage and use by the home-user of the biosensor) and thirdly, the concentration range of glucose with which GOD reacts optimally happens to coincide with the range of concentrations encountered in human blood. The other, less coincidental factor is that the glucose-test market has always been and looks set to remain the largest single market for home-diagnostics and biosensors.

Three general strategies are used for the electrochemical sensing of glucose, all of which use immobilized glucose oxidase, an enzyme that catalyzes the oxidation of glucose to gluconic acid with the production of hydrogen peroxide. The first detection scheme measures oxygen consumption; the second measures the hydrogen peroxide produced by the enzyme reaction; and a third uses a diffusable or immobilized mediator to transfer the electrons from the glucose oxidase to the electrode.

Among the amperometric biosensors, the peroxide based glucose biosensor is the simplest. There are three membrane layers in a peroxide based amperometric glucose biosensor which are the outer layer, the active layer, and the inner layer. The electro oxidation of hydrogen peroxide requires high potential that results in oxidation of easily oxidable substances in blood simultaneously, thus adding to the electrical signal and giving a non-accurate reading of measured glucose concentration. An interfering molecule is a species that is electroactive at the operating potential of the amperometric sensor. This includes ascorbic acid, urate, and acetaminophen.

In the efforts to minimize the interference effects, a selective layer is often placed between the enzymatic active layer and the electrode to filter out and interfering species. A permselective membrane can be used as the inner membrane of the sensor. A permselective membrane restricts the passage of larger molecular weight species based on MWCO (molecular weight cut off) (Kermis *et al*., 2003). On the other hand, the permselective membranes may lead to the diffusional constraints to analyte, while excluding the interference species (Poyard *et al*.,1998). Therefore, studying the characteristics of the permselective layer is required to optimize the function of this selective layer.

However, the use of an artificial electron acceptor or mediator to replace the natural acceptor oxygen in the oxidation of glucose by glucose oxidase is also a preferable approach that has been explored to overcome tissue oxygen dependence. In addition, the oxidation of the reduced mediator occurs at low potential thus reducing the sensitivity of the sensor to interfering substances. Claremont *et al*. 1986 were the first who reported an implantable amperometric ferrocene-modified glucose sensor. However, the initial promise exhibited by mediator based glucose sensors for in vivo applications, has failed to materialize.

The main problem remains the limited long-time-use stability of mediated glucose sensors, which has been attributed to the leaching of the mediator. In addition, the loss of mediator is a particularly important issue for implantable sensors because of the inherent toxic effect of the mediators used. Therefore, in order to develop a stable implantable mediated glucose sensor, a suitable immobilization method should be investigated to avoid the leaking of mediator as well as the enzyme. However, for disposable mediated glucose biosensor for home monitoring, the issues of stability and leakage are not as crucial. What is more important is an immobilization method that results in high sensitivity of the sensor and adequate kinetics to extend the detection limit of the sensor.

In this work, for both hydrogen peroxide based glucose and mediated glucose biosensor, various immobilization methods were investigated to determine which one was the most stable and could able to retain enzyme with good responses over a wide range of concentration. Besides, for hydrogen peroxide-based glucose sensor, the characteristic of the permselective layer was studied in order to develop an interference-free hydrogen peroxide-based glucose biosensor. Furthermore, for mediated glucose sensor, selection of the appropriate method to tether the mediator to the enzymatic membrane was important in order to develop a non leaking mediator based biosensor. The methods involved in this research part were immobilization of GOD and ferrocene redox polymer in cross-linked poly (vinyl alcohol) (CLPVA) with bovine serum albumin (BSA) as a protein stabilizer, immobilization of ferrocene carboxylic acid and GOD in a sol gel derived silica (SGS) matrix containing cross-linked poly (vinyl alcohol) (CLPVA) and nafion, and

lastly multilayered construction of GOD and redox poly(allylamine) ferrocene utilizing layer-by-layer covalent attachment.

1.2 Objective

The objectives of this work are as follows:

(i) To develop an interference-free hydrogen peroxide-based glucoe biosensor
(ii) To develop a practical and stable mediated amperometric glucose sensor

1.3 Scopes

To achieve objective (i), the following specific areas were investigated:

(i) Selection of the appropriate methods of immobilizing GOD. The enzymatic membranes developed were chemically cross-linked poly(vinyl alcohol) (PVA)-glucose oxidase (GOD) membranes, freeze-thawed poly(vinyl alcohol) (PVA)-glucose oxidase (GOD) membranes, tetramethoxysilane (TMOS) sol-gel -glucose oxidase (GOD) membranes , and alumina sol-gel -glucose oxidase (GOD) membranes.
(ii) Determination of the optimum cross-linking density of poly(2-hydroxyethyl methacrylate) (pHEMA) membrane for it to successfully perform as a selective inner membrane.
(v) Testing of a complete lab-scale sized peroxide-based glucose biosensor.

To achieve objective (ii), the following specific areas were investigated:

(i) Selection of the appropriate methods of immobilizing GOD to the polymer to construct a non-leaking mediated glucose sensor. Three methods were studied:

a) Immobilization of glucose oxidase and ferrocene redox polymer in cross-linked poly (vinyl alcohol) with bovine serum albumin as protein stabilizer

b) Immobilization of glucose oxidase/ferrocene carbozylic acid in composite silica sol gel (SGS) /cross-linked poly (vinyl alcohol) (CLPVA)/nafion membrane

c) Multilayered construction of glucose oxidase and poly(allylamine) ferrocene

(ii) Preparation of a nafion protective membrane

(iii) Fabrication of the mediator-based glucose sensor

CHAPTER 2

LITERATURE REVIEW

2.1 Historical Overview of Biosensor Technology

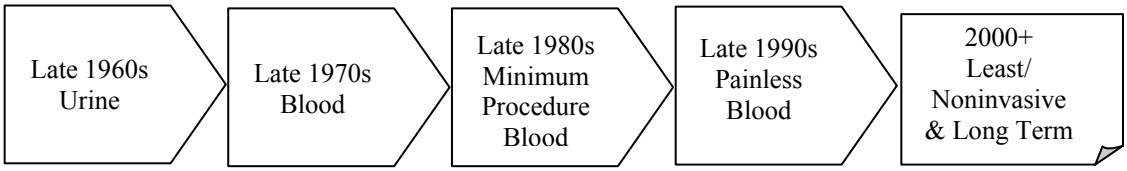


Figure 2.1 Evolution of home blood glucose monitoring technology

The first prototype biosensor was an enzyme electrode reported in 1962, which utilized immobilized GOD on a Clark pO_2 electrode for measuring the concentration of glucose in solution. This prototype enzyme electrode later served as the basis for the development of the first commercialized enzyme electrode and glucose analyzer (Taylor, 1991]. The first step towards commercial exploitation was that taken by the Yellow Springs Instrument Company in the seventies. YSI- in close collaboration with Clark- developed a series of laboratory-scale glucose sensors. Much work was invested in finding suitable membranes that rendered the GOD-platinum electrode technique reproducible and accurate.

The key research that lead to the next generation of home-testing glucose sensors was performed in the early 80's by H.A.O.Hill and I.J. Higgins and their respective colleagues at the University of Oxford and the Cranfield Institute of Technology (Newmann, 2005). The oxidized form of the mediator reacted with reduced GOD instead of oxygen and thus reduced mediator is formed instead of hydrogen peroxide. The reduced mediator is then re-oxidized at the electrode, giving a current signal and regenerating the oxidized form of the mediator. This eliminates the problem with variable oxygen concentrations in the sample and partially eliminates electrochemical interference. The commercial reality of the mediated sensor came with the foundation of Genetics International (later to change name to Medisense) and the launch of the pen-sized Exactech glucose sensors in 1987. The system consists of small, disposable, single-use glucose-sensitive electrodes (based on a mixture of GOD and mediator in a conductive carbon-paste binder) and the corresponding pen-sized (later pocket-calculator-sized) meter containing the electronics and an LCD display (Newmann, 2005).

2.2 Principle of Glucose Biosensor

Biosensors are a class of extremely sensitive and selective sensors that convert a biological action into an electrical signal to detect or quantitatively determine a specific compound. This technology is the creative synergistic combination of biotechnology, biochemistry, membrane technology and microelectronics. Biosensors are analytical devices that incorporating a biological or a biomimic material, such as tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids etc., which recognizes the analyte, and is intimately associated with or integrated within a physicochemical transducer or transducing microsystem, that translates the recognition event into a signal. The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes (Eggins, 1996). In a glucose biosensor, glucose oxidase enzymes are employed as

the biological components of the sensors for molecular recognition. In the context of glucose biosensor, the analyte involved is glucose and it is available in the blood. Figure 2.2 shows the schematic representation of possible glucose biosensor construction.

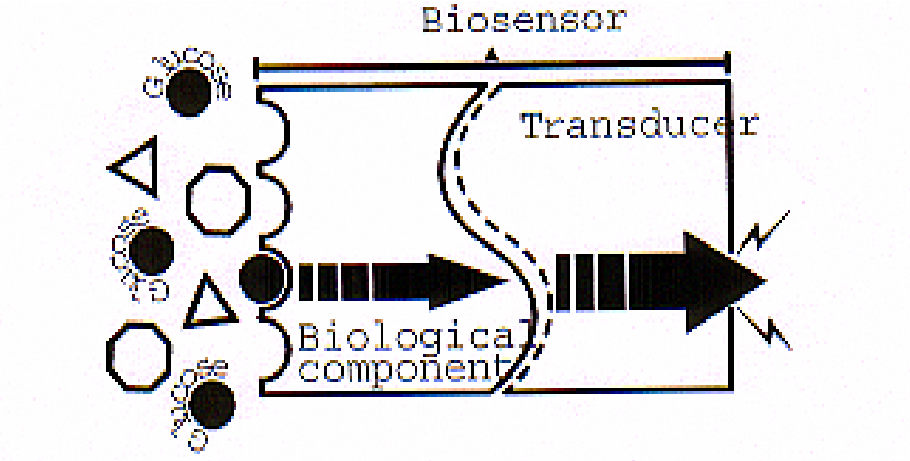


Figure 2.2 Schematic representation of possible glucose biosensor construction

Glucose biosensors are based on the fact that the enzyme glucose oxidase catalyses the oxidation of glucose to gluconic acid. Glucose and oxygen would diffuse into the enzyme layer from the sample site and the consequent depletion of oxygen would provide a measurement of the glucose concentration. The most common strategies for glucose detection can be partitioned into the following groups: those employing glucose oxidase; those using a dehydrogenase enzyme or those relying on an inorganic catalyst for oxidation of glucose or fluorescence due to the combination of fluorescein and glucose. The first article describing an immobilized enzyme electrode was due to Updike & Hicks in 1967. They immobilized the enzyme glucose oxidase in a polyacrylamide gel at an oxygen electrode.

Since three decades, the search for an ideal glucose biosensor continues to be one of the main motivations in this research field. The refinement of electrochemical approaches for glucose sensitivity has occupied many research groups. Every year there are lots of papers in glucose biosensor published. Most papers found by the literature search appear to use glucose oxidase to oxidize one of the anomers of glucose (Niu and Lee, 2002; Zhang *et al*., 2004; Yoon *et al*., 2000; Hodak *et al*., 1997; Koide and Yokoyama, 1999). Today, glucose sensor research is a relative

mature and well-worked research field. The majority of sensors is based on electrochemical principles and employ enzymes as biological component for molecular recognition. A successful biosensor must possess at least some of the following beneficial features:

1. The biocatalyst must be highly specific for the purpose of the analyses, be stable under normal storage conditions and show good stability over a large number of assays (i.e. much greater than 100).
2. The reaction should be as independent of such physical parameters as stirring, pH and temperature as is manageable.
3. The response should be accurate, precise, reproducible and linear over the useful analytical range, without dilution or concentration. It should also be free from electrical noise.
4. If the biosensor is to be used for invasive monitoring in clinical situations, the probe must be tiny and biocompatible, having no toxic or antigenic effects
5. The complete biosensor should be cheap, small, portable and capable of being used by semi-skilled operators.
6. There should be a market for the biosensor

2.3 Three Generations of Glucose Biosensor

Sometimes these three modes of oxidation are referred to as first, second and third generation biosensors. First generation is oxygen electrode based sensors and second generation is mediator-based sensor. Meanwhile, the third generation is directly coupled enzyme electrode. However there is some evidence that the mode of action of conducting salt electrodes is really the same as that of a mediator, so that the third generation description may not be strictly accurate (Eggins, 1996). The advantages of the mediated sensor are numerous. The reaction of GOD with mediator is much better defined because of non-dependence on ambient oxygen. Therefore, there is no need to worry about variable oxygen concentrations in blood.

Secondly, mediators can be re-oxidized at an electrode at less extreme potentials than are necessary for hydrogen peroxide. This partially eliminates electrochemical interference that always occurred when H_2O_2 detection method is used. Working at such high potentials will increase the risk of interference from easily oxidizable compounds. The hydrogen peroxide method is very sensitive to many common interfering species present in the blood such as uric acid, vitamin C and paracetamol. These substances will break down electrochemically and thus give interfering signals. Besides mediated biosensors offer other advantages of increased linear response and perhaps an extended biosensor lifetime, because hydrogen peroxide is not being generated, which can contribute to the deactivation of the enzyme (Reynolds et. al., 1992).

2.3.1 First Generation Glucose Biosensor

Glucose biosensors are generally based on the enzyme glucose oxidase. This enzyme catalyzes the oxidation of β -D-glucose by molecular oxygen producing gluconolactone and hydrogen peroxide. The detail reactions involved are as shown below:

$$\beta\text{-D-glucose} + \text{GOD(FAD)} \rightarrow \text{Glucono-}\delta\text{-lactone} + \text{GOD}(FADH_2)$$

$$\text{GOD}(FADH_2) + O_2 \rightarrow \text{GOD(FAD)} + H_2O_2$$

$$\text{Glucono-}\delta\text{-lactone} + H_2O_2 \rightarrow \text{Gluconic acid}$$

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2$$

The very simple first generation glucose sensor, which generates hydrogen peroxide in the presence of oxygen and glucose are the most widely used. The signal is due to the oxidation of the hydrogen peroxide at a catalytic (usually platinum) anode.

$$H_2O_2 \xrightarrow{\text{700 mV vs Ag/AgCl}} O_2 + 2H^+ + 2e^-$$

The most important advantage of the hydrogen peroxide electrode based sensors is their ease of fabrication and the possibility of constructing them in small sizes even

when simple technology is used (Wilkins and Atanasov, 1996). Figure 2.3 shows the different between oxygen electrode based sensors and mediator based sensor.

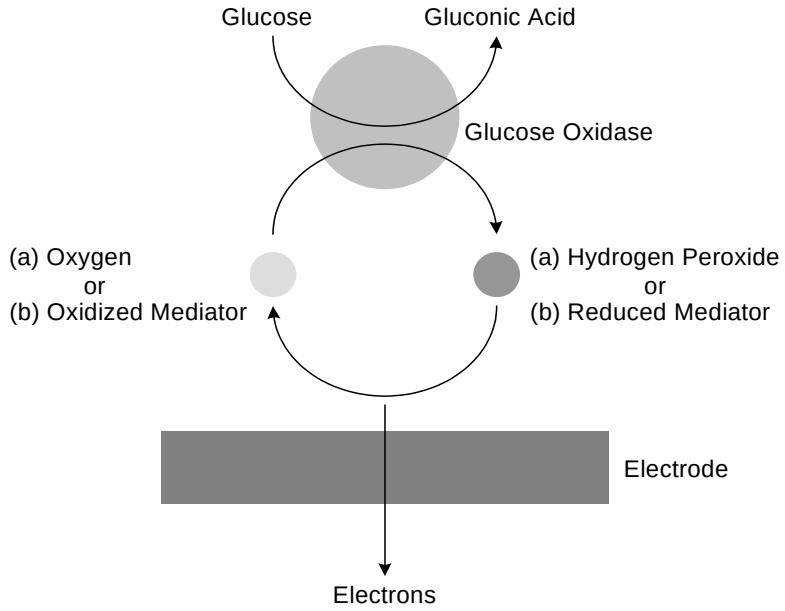


Figure 2.3 The differences between oxygen electrode based sensors and mediator based sensor (Clark and Lyons, 1962)

2.3.2 Second Generation Glucose Biosensor

The problems that occur in hydrogen peroxide-based sensor can be overcome using mediated glucose sensor, which is second generation glucose sensor. The oxidized form of the mediator reacted with reduced GOD instead of oxygen and thus reduced mediator is formed instead of hydrogen peroxide. The reduced mediator is then deoxidized at the electrode, giving a current signal and regenerating the oxidized form of the mediator. This eliminates the problem with variable oxygen concentrations in the sample and partially eliminates electrochemical interference. Figure 2.4 shows the electron transfer between the mediator and the enzyme.

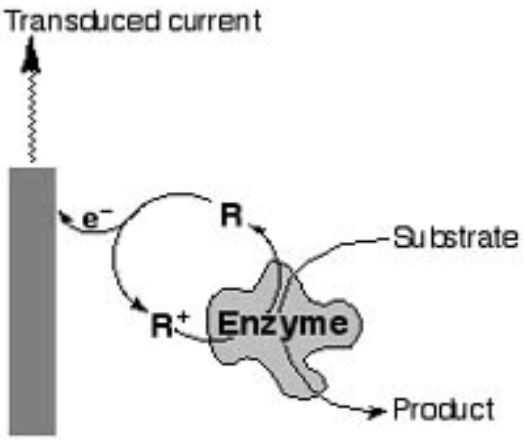


Figure 2.4 The electrical 'wiring' of an oxidative redox-enzyme via a diffusional electron-transfer mediator shuttling between the enzyme reaction centre and the electrode**.** R: reduced mediator, R^+: oxidized mediator (Degani and Heller, 1987)

There are three main steps in the reaction of the mediators with glucose oxidase. The first one is the diffusion of the substrate from the bulk solution to the surface of the enzyme. And the second one is the transfer of the electron from the reaction centre of the enzyme to the mediator. Finally is the transport of the electron from the mediators to the electrode. The distance between the electrode and the reaction centre of enzyme will influence the membrane response time. Ferrocene derivatives, organic dyes, ferricyanide, Ru-complexes and other electrochemically active substrates have been employed as diffusional mediator and for the electrical activation of soluble redox-enzymes lacking direct electrical contact with the conductive support (Bartlett *et al*, 1991).

A ferrocene is a typical mediator for enzyme biosensor (Kase and Muguruma, 2004). Ferrocene fit all criteria of a good mediator such as no reaction with oxygen, stable in both the oxidized and reduced forms, independent of pH, show reversible electron transfer kinetics and react rapidly with the enzyme (Eggins, 1996). Besides, in order to successfully mediate an enzyme reaction a potential mediator must posses the following attributes such as low redox potential, reversible electrochemistry, fast electron transfer kinetics and good stability. There are many ways in which mediators can be incorporated into biosensors. In a biosensor, both the enzyme and the ferrocene must be immobilized on the electrode. For glucose, the operation of mediator is as follows:

$$\text{Glucose} + GOD_{OX} \longleftrightarrow \text{Gluconolactone} + GOD_{RED} + 2H^{+}$$

$$GOD_{RED} + 2Fc^{+} \longleftrightarrow GOD_{OX} + 2Fc$$

$$2Fc - 2e^{-} \longleftrightarrow 2Fc^{+}$$

The actual oxidation of the glucose is carried out by the FAD component of the glucose oxidase, which is converted into $FADH_2$. The $FADH_2$ is reoxidized to the FAD by the mediator, Fc^+ . Then, the Fc is reoxidized to Fc^+ directly at an electrode. The current flowing through the electrode is an amperometric measure of the glucose concentration. This is better shown in the cyclic diagram in figure 2.5.

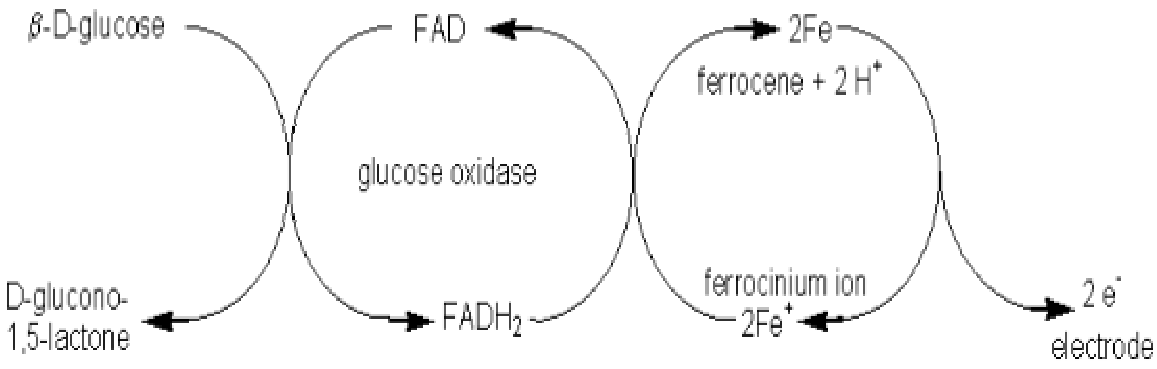


Figure 2.5 A ferrocene mediated biosensor for glucose

2.3.3 Third Generation Glucose Biosensor

The third generation is directly coupled enzyme electrode. It may be strange that a mediator is needed to couple an enzyme to an electrode. It is not possible to reduce an enzyme directly on the electrode because the proteins tend to be denatured on electrode surfaces. A better solution was developed by Albery and Cranston (1987) and Bartlett (1987) using organic conducting salt electrodes. Tetrathiafulvane (TTF) is reversibly oxidized, and tetracyanoquinodimethane (TCNQ) is similarly reversibly reduced. These conducting salts can be built into electrodes in three ways which are as single crystals as pressed pellet or a paste with graphite powder (Albery and Cranston, 1987; Bartlett, 1987). Recently, immobilization techniques have been developed to wire an enzyme directly to an electrode, facilitating rapid electron

transfer and hence high current densities. In general they involve an in situ polymerization process using redox polymer.

2.4 Enzyme Immobilization

Enzymes are biocatalytically active entities upon which the metabolisms of all living organisms are based. They speed up biochemical reactions by lowering the energy of activation, without themselves appearing in the reaction products. The catalytic action of enzymes involves their ability to alter the distribution of charges on the compound to be converted, thus bringing about a lowering of the energy of activation. Furthermore, they are highly specific, thus side reactions can be avoided by employing enzymatic breakdown. A biocatalyst is termed “immobilized” if its mobility has been restricted by chemical or physical means. This limitation of mobility may be achieved by widely differing methods, such as trapping in the network of a polymer matrix or by membrane confinement.

Immobilization of an enzyme results in a considerable change in the microenvironment of the enzyme and may affect the properties of the enzyme, as well as changes in the physical and kinetic properties. These changes may affect their usefulness in biochemical analysis. With immobilized enzymes the measured reaction rate depends not only on the substrate concentration and the kinetic constants K_M and V_{max}, but also on immobilization effects. These effects are due to the following alterations of the enzyme by the immobilization process. A variety of immobilization methods have been used in the development of successful biosensors.

2.4.1 Adsorption

Adsorption of enzymes onto insoluble supports is a very simple method of wide applicability and capable of high enzyme loading, which is about one gram per gram of matrix. Simply mixing the enzyme with a suitable adsorbent under appropriate conditions of pH and ionic strength, followed by a sufficient incubation period, and finally washing off weakly bound or unbound enzyme will produce the immobilized enzyme in directly usable form. The driving force causing this binding is usually due to a combination of hydrophobic effects and the formation of several salt links per enzyme molecule. The particular choice of adsorbent depends principally upon minimizing leakage of the enzyme during use. Although the physical links between the enzyme molecules and the support are often very strong, they may be reduced by many factors including the introduction of the substrate. Binding forces should not be weakened during use by inappropriate changes in pH or ionic strength (Chaplin, 1990).

2.4.2 Entrapment

By matrix entrapment the enzymes are embedded in natural or synthetic polymers, mostly of a gel-like structure. In order for the entrapped enzyme to fulfill its catalytic function it is essential that the substrates and products of the reaction are able to traverse the matrix. At the same time, the pores of the matrix should not be so large that the enzyme itself can escape. Entrapment is a convenient method for use in processes involving low molecular weight substrates and products. Amounts in excess of 1g of enzyme per gram of gel or fiber may be entrapped. The advantage of entrapping method is that enzymes are not subjected to serious modification, and immobilization eliminates the effect of proteases and enzyme inhibitors of high molecular weight. However, the difficulty which large molecules have in approaching the catalytic sites of entrapped enzymes precludes the use of entrapped enzymes with high molecular weight substrates. The entrapment process may be a purely physical caging or involve covalent binding (Chaplin, 1990).

The natural polymers that are used for entrapment of enzyme usually lead to relatively soft products. Subsequent hardening procedures, such as treatment with glutaraldehyde are required. Synthetic polymers produced by polycondensation or polymerization are frequently used for entrapping enzymes. The network of the polymer can be made dense enough to retain the enzyme molecules. One of the deficiencies of this method of immobilization is that the enzyme may slowly leak out of gel matrix. This leakage is more pronounced with gels that have high water content. Further linking procedures which provides additional cross-linking are needed for enzyme because the mesh diameter is too large to retain single enzyme molecules (Chaplin, 1990).

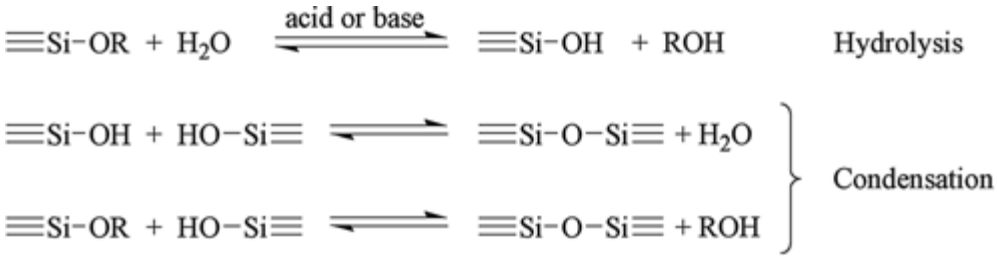
2.4.2.1 Sol-gel

An interesting recent entrapment procedure used is the sol gel method. Sol gels are chemically inert, can resist swelling, are processed at low-temperatures, and have tuneable porosity. Over 80% of GOD remained active in sol-gels and the amperometric response agreed well with theoretical predictions (Audebert, 1993). Sol-gel is a low-temperature process that involves the hydrolysis and polycondensation of suitable precursors to form ceramic materials (Wu *et al.*, 1999). The low temperature gel synthesis facilitates the encapsulation of biorecognition elements within the gel, by adding the biological compound to the reaction mixture at the onset of polymerization. The porous inorganic sol-gel matrix possesses physical rigidity, chemical inertness, high photochemical, biodegradational, tuneable porosity, and experiences negligible swelling in both aqueous and organic solutions (Liu *et al.*, 1999)

The sol-gel process involved the initial hydrolysis and polycondensation of alkoxides in localized regions, leading to the formation of colloidal particles, which is called sol. As the interconnection between these particles increases, the viscosity of the sol starts to increase and this leads to the formation of the porous gel, which is used as enzyme encapsulation matrix (Wu *et al.*, 1999). When dried near room temperature, the dried sol-gel matrix provides an aqueous environment inside the pores, which host the enzymes (Gudeman and Peppas, 1995). Due to the porous

nature of the matrix, an analyte can interact easily with immobilized enzyme (Lilis *et al*., 2000).

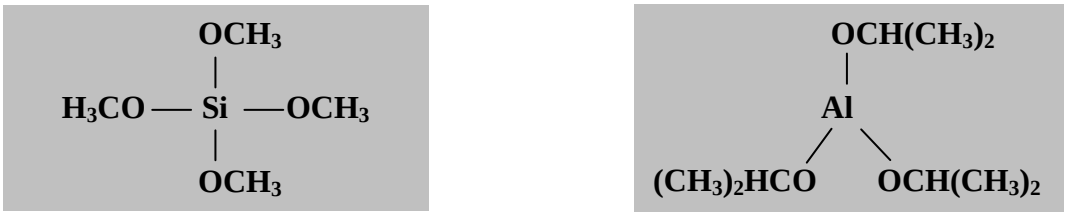
The properties of the porous sol-gel matrix are affected by various process factors (Wu *et al*.,1999). Rapid hydrolysis occurs under basic condition, which gives rise to a more particulate sol-gel, with a larger average pore size (that able to give higher initial enzyme activity), but results in a brittle and easily cracked film upon drying at room temperature (Lilis *et al*., 2000). Cracking occurs due to capillary stresses generated by evaporation of water and solvent molecules from the porous network (Lilis *et al*., 2000). Slower hydrolysis occurs under acidic condition creating a polymeric gel with a smaller average pore size, which may lead to diffusional restraints in the sol-gel matrix, resulting in a lower initial enzyme activity but more rigid enzyme layer (Lilis *et al*., 2000). Under acidic condition, aprotic solvents such as dioxane promote initial hydrolysis while protic solvents such as ethanol retard initial hydrolysis (Lilis *et al*., 2000).



In a typical procedure, tetramethoxysilane (or tetraethoxysilane) is mixed with water in a mutual solvent such as methanol followed by the addition of suitable catalyst. As the sol becomes interconnected, a macroscopically rigid, hydrated gel is formed. Specific reagents such as proteins, organic dyes, and redox species can be trapped into this optically transparent, stable host matrix by simply adding them to the sol prior to its gelation. These materials have been used in numerous applications including solid-state electrochemical devices, chemical sensors, catalysts, and nonlinear and optic applications (Howells *et al*., 2000). An *R* value, which is the water/alkoxide ratio, of 1:3.7 was seen to be optimal (Lilis *et al*., 2000). Higher *R* value causes increase in the rate of hydrolysis resulting in a more particulate gel.

The utmost important point is the extent to which the entrapped reagents maintain their chemical and physical properties when immobilized in this solid host. The silica gel matrix is not a completely inert support while stable. The surface of the pore walls contain several kinds of functional groups including siloxane (SiOSi), silanol (SiOH), siloxide (SiO-), and possibly unreacted silicon alkoxide groups ($SiOCH_3$). Furthermore, the walls will be negatively charged with p*I* of silica is approximately 2 under most condition. The degree of surface interactions between an entrapped dopant and the walls of the silica host and the extent of surface confinement can strongly affect the rotational and translational mobility of the entrapped guest and impact the overall performance of sol-gel-based devices. The size, charge, and functionality of the entrapped species as well as the average pore size, pore connectivity, tortuosity, and interfacial polarity of the pore walls are important variables that need to be considered (Howells et al., 2000).

As mentioned earlier, two types of alkoxides will be applied, the silica alkoxide, tetramethoxysilane (TMOS) ($C_4H_{12}O_4Si$) (Chen *et al*, 2002, Wu *et al*., 1999; Sapsford, and Ligler, 2004; Wolfbeis *et al*., 2000), and metal alkoxides, alumina (Aluminium isopropoxide) ($Al[OCH(CH_3)_2]_3$) (Liu *et al*., 1999; Wei *et al*., 2001; Chen *et al*., 2002) are of interest.



(a) **(b)**

Figure 2.6 Structure of (a) Tetramethoxysilane (b) Aluminium isopropoxide

The process is based on the inorganic polymerization of silica alkoxide $Si(OR)_4$ for which the hydrolysis and condensation concerted reactions are known to be relatively slow. The need for a catalyst is due to the lower reactivity of silicon alkoxide as compared to the reactivity of other metal alkoxides such as aluminum, titanium, zirconium (Griesmar *et al.*, 2003). Different alkoxides may give different properties to the resulted sol-gel matrix.

2.4.3 Covalent Coupling

The formation of covalent bonds between enzyme and an insoluble support is the most frequently used techniques. This technique consists of forming a covalent bond between one or more of the enzyme's amino acid residues and a functional group on the insoluble support (Saburo and Tanaka, 1990). The strength of binding is very strong and very little leakage of enzyme from the support occurs. The usefulness of the various functional groups for covalent link formation depends on their availability and reactivity (nucleophilic). The reactivity of the protein side-chain nucleophiles is determined by their state of protonation, which is the charge status, and roughly follows the following relationship where the charges may be estimated from the pK_a values of the ionizing groups and the pH of the solution.

$$-S^- > -SH > -O^- > -NH_2 > -COO^- > -OH >> -NH_3^+$$

The functional groups of enzymes that can be utilized for covalent attachment include (Saburo and Tanaka, 1990):

(a) the ε-amino groups of lysine and arginine, and α-amino groups of the polypeptide chains
(b) the ε-carboxyl groups of the aspartate and glutamate residues and the α-carboxyl groups of the chains
(c) the hydroxyl groups of the serine and threonine residues
(d) the aromatic ring of the tyrosine residues
(e) the imidazole ring of histidine
(f) the indole ring of tryptophan
(g) the sulfhydryl groups of the cysteine residues

Lysine residues are found to be the most generally useful groups for covalent bonding of enzymes to insoluble supports due to their widespread surface exposure and high reactivity. They also appear to be only very rarely involved in the active sites of enzymes (Chaplin, 1990). The amino groups of a protein can react with a large number of functional reagents such as acylating and alkylating agents,

aldehydes, diazonium salts, and isocyanates. Compared to the amino groups, the carboxyl groups of proteins are much less reactive groups. Covalent coupling will be quite generally applied, even if little is known about the structure or active site of the enzyme (Saburo and Tanaka, 1990).

2.4.4 Cross-linking

In many cases the immobilization of enzymes has been achieved by cross-linking the enzyme molecules to each other or to some functional groups on a carrier matrix. The result is a coupling one enzyme molecule to another, thus forming large matrices of enzyme molecules. The cross-linking is accomplished with bifunctional reagents, which may either contain two identical functional groups or two different functional groups (Saburo and Tanaka, 1990). Of these reagents, glutaraldehyde is by far the most widely used. Glutaraldehyde is used to cross-link enzymes or link them to supports. It is particularly useful for producing immobilized enzyme membranes for use in biosensors by cross-linking the enzyme plus a non-catalytic diluent protein within a porous sheet. Carbodiimides are very useful bifunctional reagents as they allow the coupling of amines to carboxylic acids. Careful control of the reaction conditions and choice of carbodiimide allow a great degree of selectivity in this reaction. The use of trialkoxysilanes allows inert materials as glass to be coupled to enzymes (Chaplin, 1990).

2.4.4.1 Chemically Cross-linked Poly(vinyl alcohol)

Chemically cross-linked PVA involves the cross-linking of glucose oxidase and the support by using a bifunctional cross-linking agent. As discussed before, the most commonly employed bifunctional reagent for cross-linking is glutardialdehyde, simply called glutaraldehyde. The reaction aldehyde groups at the two ends of the glutaraldehyde react with free amino groups (ε-amino groups, N-terminal amino groups) of enzymes.

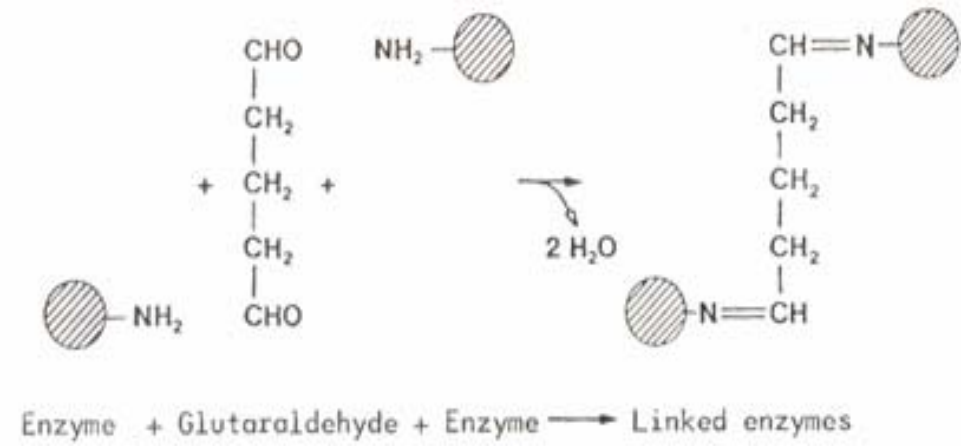


Figure 2.7 Cross-linking of enzyme with glutaradehyde (Hartmeier, 1986)

The cross-linking method is to stabilize the immobilized enzyme and to minimize enzyme leaking from the matrix. At the same time, the access to the substrate binding sites cannot be block by direct reaction with the sites or by burying the sites under excess cross-linking rope. Cross-linking bridges can be formed by two mechanism, include the slow cross-linking, and the fast cross-linking. The slow cross-linking involves aldol condensation between two or more α,β-unsaturated aldehydes. Figure 2.8 shows the aldol condensation equations. The condensation products of glutaraldehyde provided the "glue" for cross-linking in the sense that nucleophiles add to α,β-unsaturated aldehydes irreversibly.

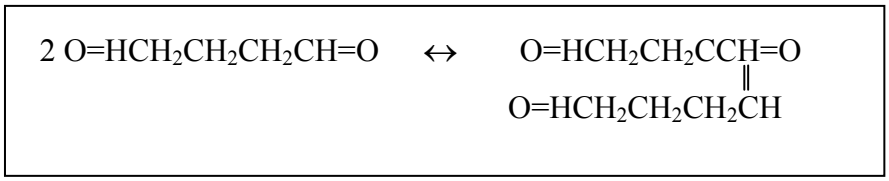


Figure 2.8 Aldol condensation of glutaradehyde

The fast cross-linking process requires glutaradehyde and other precursors, containing amine groups. When the latter are added to glutaraldehyde solutions, a complex set of pyrimidine products in a range of sizes are rapidly generated. These products are the cross-linking bridges and thus provide structural "glue" for cross-linking (Johnson, 1993).

2.4.4.2 Freeze-thawed PVA

The chemically cross-linked PVA modifies the immobilized enzyme drastically and leads to conformational changes and thus results in loss of enzyme activity (Braun, 1976). Physically cross-linked PVA may be a good choice of immobilizing the enzyme. While minimizing the chemically cross-linked PVA problem, at the same time maintaining the good properties of PVA. The exposition of aqueous PVA solutions to several freezing-thawing cycles leads to reinforced gels owing to a densification of the macromolecular structure (Chen *et al*., 2002) which is function of the cycling time and temperatures. After the freezing-thawing process, fine crystallites are formed due to the slow heat treatment. The chains are physically cross-linked by semipermanent entanglements, molecular associations or crystalline (Doretti *et al*., 1997). Formations of crystallites serve as physical cross-links to render the material insoluble in water.

Some characteristics of the physically cross-linked PVA gels include high degree of swelling in water, a rubbery and elastic nature, and high mechanical strength because the mechanical load can be distributed along the crystallites of the three-dimensional structure (Chen *et al*., 2002). The properties of gel may depend on the molecular weight of the polymer. the concentration of the aqueous PVA solution, the temperature and time of freezing and thawing, and the number of freezing-thawing cycles (Chen *et al*., 2002).

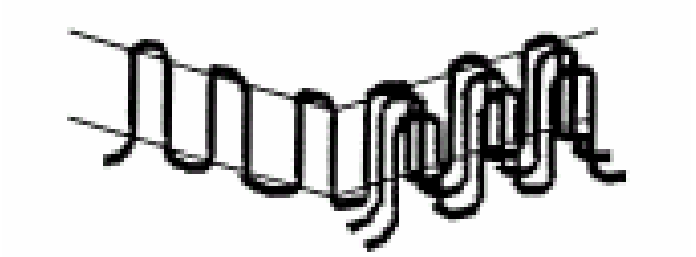


Figure 2.9 Freeze-thawed crystallite structure. A double layer of molecules is held together by hydroxyl bonds while weaker van der Waals forces operate between the double layers. A folded chain structure of PVA chains leads to small order regions (crystallites), scattered in unordered, amorphous polymer matrix (Peppas *et al*., 1985)

The freezing-thawing method us characterized by the absence of chemical cross-linking agents that could compromise its biocompatibility or of physical agents, such as γ radiation that could deactivate the biological substrates, due to damage caused mostly by the indirect effect of water radiolysis (Doretti *et al*., 1997). Generally, physical entrapment of enzyme molecules in polymeric membranes is one of the most advantageous methods because it is rapid and simple, and the retained activity is high (Doretti *et al*., 1998). The novel networks are of significant interest in the biomedical field because they are nontoxic for organisms, contain no impurities, and their water content matches that of biological tissue (Doretti *et al*., 1997). In this work, the parameters that could affect the enzymatic layer of a glucose biosensor, such as freezing-thawing cycle and PVA concentration will be evaluated.

2.5 Methods of Tethering the Mediator to the Enzymatic Membrane

Various methods of tethering the mediator to the enzymatic membrane for the second generation sensor have been reported. Cross-linking of the enzyme and the redox polymer using glutaraldehyde was reported by Koide and Yokoyama, 1999. Redox hydrogel polyallylamine ferrocene was prepared by crosslinking polyallylamine hydrochloride with glutaraldehyde and attaching the ferrocene covalently. Amino group of cross linked polyallylamine and carboxyl group of ferrocene carboxylic acid was activated by using carbodiimide reagents.

The use of a load protein like albumin improves enzymatic activity because of the better mass distribution of the various protein, but it does not alter the mechanical properties of the membrane produced. Koide and Yokoyama, 1999 suggested that BSA addition prevented the polymer matrix from over-swelling. There were decrease in the redox response resulted from the electrode without BSA. These results indicate that such a decrease in the redox response resulted from swelling of the polymer protein hydrogel. It was because the distance between the

redox site of the polymer was extended. Therefore, the electron transfer rate among neighbouring redox redox sites decreased. Stable cyclic voltammograms were obtained for electrode with a greater BSA content than the ratio of redox polymer.

For ferocene mediator, the leaching problem is less severe if electroactive or ion exchange polymers, such as nafion, are used to contain the mediator. In a simple Nafion–ferrocene film, where entrapment is provided by nafion only, the oxidized and the reduced forms of ferrocene are believed to interact differently with the hydrophilic and hydrophobic phases of nafion (Niu and Lee, 2002). Thus, the use of polyelectrolytes (PE) incorporated SGS to fabricate reagentless mediator-based enzyme was firstly reported by Niu and Lee, 2002. SGS-PE membrane is an excellent matrix for the immobilization of enzyme and mediator in the development of mediated reagentless biosensors. The electrode is fabricated by casting in sequence of Nafion- ferrocene solution, enzyme solution and PE loaded silica sol. Weakly held species as well as leached ferrocene derivatives from the inner Nafion mediator layer will be retained by the outer PE-SGS network layer. The presence of hydrophilic PVA and the relatively hydrophobic network of sol gel silica will modify the environmental for ferrocene carboxylic acid retention. The result is a consolidation of the effects of polymer, ionomer and sol gel network.

The simultaneous presence of the polyelectrolyte and sol–gel silica has greatly improved the selectivity and stability of the sensors. High stability originated from the effective entrapment of mediator and enzyme by the three-dimensional interpenetrating network of the PE–SGS matrix. The co-operative effect from the hydroxyl groups of PVA and the sol–gel environment sustain the rotational freedom for the enzyme molecules to adopt the active configuration typical under physiological conditions. The active matrix environment prolongs the life span of the enzyme to result in high sensitivity. Biosensors based on PE–SGS immobilization is simple to fabricate, work under lower operating potentials, and provide good responses over a wide range of concentrations.

2.5.1 Redox Polymer

A promising strategy in biosensor design is the immobilization of both enzyme and mediator, which generally require polymeric material. One of the approaches to the electrical contacting of polymer-bound enzymes involves the use of polymers that are functionalized with redox-units. The advantages of using the redox polymer are several with the main advantage is more stable biosensors since leaking of mediator from the electrode is minimized and higher and faster responses are observed due to proximity between the enzyme and the mediator (Rondeau *et al*., 1999).

Polyelectrolytes represent the best choice for the optimization of interactions with enzymes and electrodes. Hydrophilic, charged, flexible chains of polyelectrolytes can easily surround protein molecules, and even penetrate inside the protein matrix, providing good contact between the protein structures and polymer backbone. Each unit of a polyelectrolyte is weakly adsorbed on an electrode surface, but the cooperative effect of the entire polymer chain leads to strong adsorption, while some parts of the chain remain unattached, providing binding domains for protein molecules. Three-dimensional redox polyelectrolyte networks that electrically connect enzyme redox centers to electrodes have been formed in several systems, of which enzyme 'wired' hydrophilic epoxy cements are an excellent example.

A popular approach has been made to polymerize 4 vinylpyridine on the surface (Lyons, 1991). A similar approach has been used with polypyrroles, poly N methylenepyrroles and polythiophenes (Grimshaw and Perera, 1990) using mainly covalently attached quinines as the redox group. In this case, the polymeric chain consists of a poly(vinylpyridine) backbone of which approximately one-sixth of the pyridine units are complexed to $[Os(bpy)_2Cl]^{2+}$ and about one-fifth of the pyridines have been reacted with 2-bromoethylamine to form pyridinium-*N*-ethylamine polycationic domains. This redox polyelectrolyte interacts with enzymes easily and 'wires' their redox centers by penetrating into the protein shell (e.g. of lactate oxidase, glycero-3-phosphate oxidase, or cellobiose oxidase) (Heller, 1992; Heller and Khatakis, 1992). Although negatively charged enzymes can strongly interact

with this polycationic polymer even without crosslinking, crosslinking with the water-soluble diepoxide poly(ethylene glycol) diglycidyl ether can further stabilize the system.

A similar positively-charged copolymer of allylamine and ferrocene-functionalized acrylic acid can interact with negatively charged proteins and be crosslinked with glutaric dialdehyde in the presence of GOD to yield stable electrically 'wired' biocatalytic matrices (Koide and Yokoyama. 1999; Calvo et. al, 1994). Figure 2.10 shows the ferrocene containing crosslinked polyallylamine.

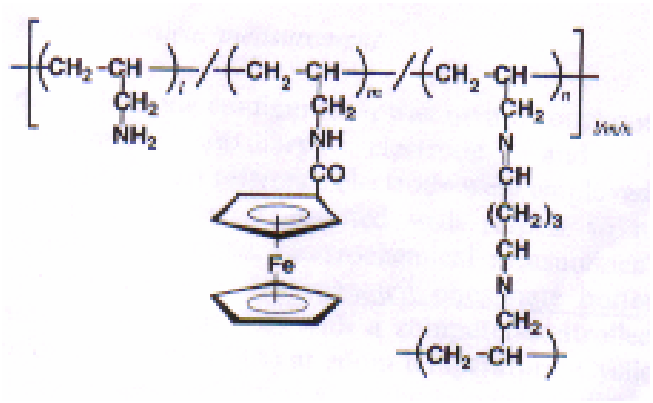


Figure 2.10 Ferrocene containing cross-linked polyallylamine ((Koide and Yokoyama, 1999)

These enzyme electrodes also demonstrate an electrocatalytic current for glucose oxidation. Koide and Yokoyama, 1999 have investigated a cross-linked redox polymer that can be prepared readily and characterized mediated enzyme electrode using this redox polymer. Polyallylamine was cross-linked with glutaraldehyde and modified successively with ferrocene carboxylic acid (Koide and Yokoyama, 1999). Figure 2.11 shows the cyclic voltammograms of the electrode modified with ferrocene-containing crosslinked polyallylamine containing glucose oxidase. Successive additions of glucose at a fixed oxidative potential result in increases in the current.

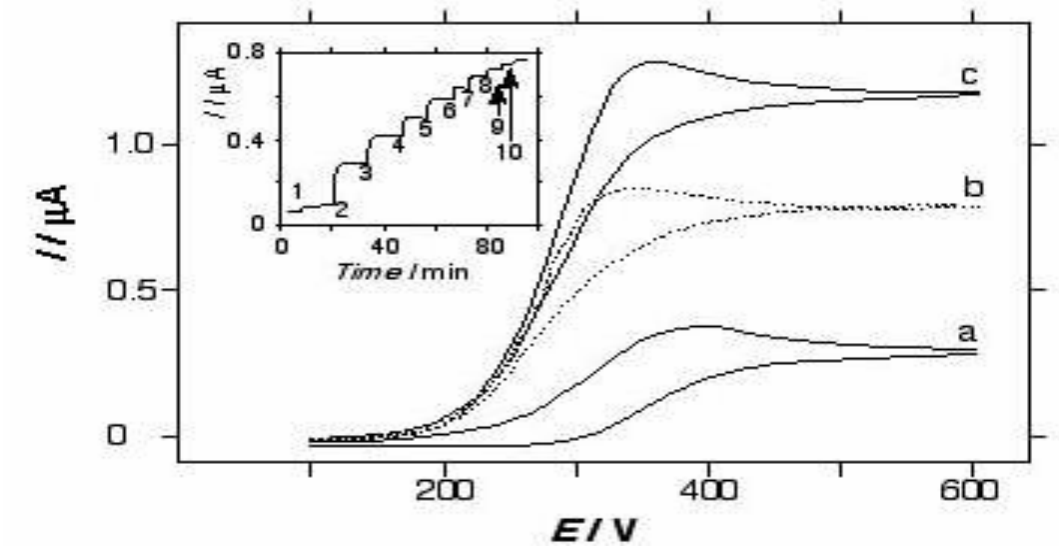


Figure 2.11 Cyclic voltammograms of the electrode modified with ferrocene-containing cross-linked polyallylamine containing glucose oxidase in the polymer matrix: (a) in the absense of glucose, (b) with glucose, 1 mM, and (c) with glucose, 3 mM. Potential scan rate 5 mV s^{-1}. Inset: amperometric responses of the enzyme electrode (at 0.6 V) upon successive additions of glucose. Numbers show glucose concentration in mM

2.5.2 Multilayer Systems

Enzymes deposited in ordered monolayers and multilayer systems have been described using different assembling techniques for enzyme immobilization such as Langmuir-Blodget, self-assembled monolayers, step by steps electrostatic adsorption of alternate multilayers, antigen-antibody interaction, avidin-biotin interaction, surfactant films and electrostatic adsorption of hyperbranched polyelectrolytes (Calvo *et al*., 2001). The enzyme content in monolayers is low, however, and electrical contact in the presence of a diffusional mediator does not usually result in a detectable amperometric response. Thus, an increase of the enzyme content is essential to obtain the detectable current when diffusional mediators are applied. The stepwise deposition of a multilayer assembly results in the increase of the enzyme content, resulting in a significantly larger current.

Layered construction of proteins into organized systems has attracted considerable attention in recent years due to its potential application in the areas of bioelectronic and biooptical devices, biosensors, etc. There have been a number of approaches for constructing multilayer protein films on the surface of solid matrices, including a layer-by-layer deposition of proteins on the surface of an electrode through a coupling reagent and consecutive adsorption of positively and negatively charged polyelectrolytes and proteins on a solid surface through an electrostatic force of attraction. Above method have proven to be effective and successful ways to fabricate multilayer thin films containing proteins. However, these procedures are complex and somewhat tedious, and the latter are not stable enough (Zhang *et al*, 2004).

Multilayered construction of glucose oxidase and redox poly(allylamine) ferrocene utilizing layer-by-layer covalent attachment has been reported by (Zhang *et al*, 2004, Yoon *et al*., 2000 and (Hodak *et al*., 1997). In that method, glucose oxidase iwas immobilized on a cystamine modified gold (Au) electrode by layer-by-layer covalent attachment of periodate-oxidized glucose oxidase and poly(allylamine) ferrocene complex (PAA-Fc). The key to produce the multilayer is by covalent bonding through the formation of Schiff base bonds between aldehyde groups of periodate-oxidized GOD and amino groups of PAA-Fc on a gold electrode. In addition the formation of Schiff bond is also applied in the preparation of polyallylamine ferrocene using ferrocene carboxaldehyde and polyallylamine hydrochloride.

As it is well known that the reaction between amino group and carbaldehyde group easily proceeds in a moderate condition. It is not necessary to introduce other material or energy to the system and avoid the contamination and deactivation of the enzyme (Zhang *et al*., 2004). The method has proven to be an efficient and experimentally simple way to produce complex layered enzyme structure with precise control of layer composition and thickness (Zaborsky *et al*., 1974, Yoon *et al*., 2000a, Yoon *et al*., 2000b and Zhang *et al*., 2004).

The deposition of variable numbers of the enzyme layers also allows the tuning of the enzyme electrode amperometric output by the control of the number of layers. The enzyme content of monolayer assemblies may also be increased by the application of rough electrode surfaces. Treatment of Au surfaces with Hg results in a roughening of the conductive support by the generation and dissolution of an Au-amalgam. Typically, Au surfaces with initial roughness factor of 1.2-1.5 can be roughened to exhibit a roughness factor of 15-25. Multilayers of GOD were linked to smooth and rough Au electrodes by coupling to cystamine-functionalized surfaces, and ferrocene monocarboxylic acid was applied as a diffusional mediator to contact the enzymes.

2.6 Permselective Layer for Hydrogen Peroxide-Based Glucose Sensor

The amperometric detection of hydrogen peroxide via electro-oxidation requires high over potential (+700mV) and may cause interferences including ascorbic acid, urate acid, and acetaminophen in biological fluids due to easily oxidable substances presented in the fluid at the measuring potential (Sirat *et al*., 1992). In order to minimize the interference effects, five approaches have been adopted.

The first strategy is to replace the natural electron acceptor, which is oxygen, with redox mediators that are able to transfer electrons from the GOD reduced active sites to the electrode surface at lower potentials. However, the mediators for in vivo use is limited due to the leaching of the mediator from the electrode, the sensitivity to oxygen, and the catalytic oxidation of electroactive interference by the mediator (Poyard *et al*., 1998).The second approach is to prepare a bienzyme glucose sensor combining GOD with a wired peroxidase [8] or to incorporate an interference-removing enzyme (Wan *et al*., 1990).

The third alternative is to apply a permselective membrane, which can exclude interferent species through molecular size or charge effects (Palmisano *et al*., 1993; Groon and Luong, 1993). However, such permselective membranes lead to the diffusional constraints, which result in low analyte sensitivity [8]. Another two methods are the development of optical-based sensors (Gunasingham and Tan, 1992) and by applying differential measurements (Vincke *et al*., 1985).

A variety of materials have been used for this purpose including Nafion (Harrison *et al*., 1988; Hu and Wilson, 1997; Matsumoto *et al*., 1998), cellulose acetate (Zhang *et al*., 1994), silane film (Jung and Wilson, 1996), alternately adsorbed polyions films (Mizutani *et al*., 1998), and electropolymerized membranes (Bartlett and Cooper, 1993; Cosnier, 1997). The issue with the deposition of Nafion and other conventional polymers on the electrode surface is difficult to control so as to produce thin, homogeneous, reproducible, and strongly adhesive films by coating methods. Electropolymerized films are attractive for its ability to carefully control deposition conditions even with complex electrode shapes (Emr and Yacynych, 1995; Jung and Wilson, 1996) but the main problem is the maintenance of high permselectivity with repeated use (Barlett and Cooper, 1993; Cosnier, 1997; Christie *et al*., 1993; Eddy *et al*., 1995).

Permselectivity membrane using poly(2-hydroxyethyl methacrylate) (pHEMA) has been demonstrated in conjunction with optical glucose affinity sensors (Kumar and Chaudhari) pHEMA also prepared together with a redox hydrogel, polypyrrole, in clinically important biosenors, for its biocompatility and high degree of swelling (Brahim *et al*., 2002).

Another method demonstrated was to utilize selective electroanalysts. Metal based electrodes, such as palladium (Sampath and Lev, 1996), ruthenium (Wang and Pamidi, 1997), and iridium (Wang *et al*, 1997; Tian and Zhu, 2002), exhibits a strong and preferential electrocatalytic action towards the enzymatically produced hydrogen peroxide, while display no response to coexisting oxidizable substances (Tian and Zhu, 2002).

Two of the main interference substances, which are urate acid and ascorbic acid are charged molecules and thus can be excluded by ionic charge. While acetaminophen, a neutral interference molecules, has to be excluded by molecular weight. In this work, the third alternative is in concern and poly (2-hydroxyethyl methacrylate) (pHEMA) is chosen to be the permselective layer due to its specific characteristics.

2.6.1 pHEMA

Poly (2-hydroxyethyl methacrylate) (pHEMA) was firstly prepared for biological use by Wichterle and Lim. Its well-tolerated safety, good biocompatibility, non-toxicity, and non-antigenic properties contribute for its wide applications in biomedical field (Hsiue *et al*., 2001). Moreover, pHEMA is a kind of hydrogel, which is a class of polymeric material. It has the ability to hold substantial amount of water, showing soft and rubbery-like consistency and low interfacial tension (Kudela, 1976). These structural features dominate its surface properties, permselectivity, and permeability that gives pHEMA their unique and interesting properties and similarity of their physical properties to those of living tissue (Seidel and Malmonge, 2000). The physical properties of the pHEMA can be adjusted according to a specific application since it can be fabricated and easily altered in various geometric forms (Seidel and Malmonge, 2000).

The bulk polymerization of HEMA can result in a glassy and transparent material in dense form that is considered non porous (*Chirilla et al*.). In opposite, the solution polymerization of this monomer allows the formation of porous structures by deciding the type and amount of diluent used. For water amount less than 55.7%, the solution polymerization technique led to formation of non-porous hydrogel (Seidel and Malmonge, 2000).

Dense hydrogels show the behaviour of a rigid and fragile material when dried and and elastomeric consistence when swelled in water. Generally dense hydrogels show an amorphous structure since it is very difficult the arrangement of

the macromolecules and formation of crystallites is in the presence of crosslinks (Seidel and Malmonge, 2000).

The non-porous pHEMA membrane has appropriate permselectivity and biocompatibility (Peppas *et al*., 1985). The diffusion in pHEMA membrane can be investigated based on two factors, which are the cross-linking density of the network or mesh size, and the degree of swelling (Peppas *et al*., 1985). The mesh size of the hydrogel is varied through different cross-linking ratio and in order to exclude larger species while maintaining the small solute permeability (Peppas *et al*., 1985).

2.7 Protective Membrane for Mediator-Based Glucose Biosensor

Finally, the electrode is coated with a protective layer that renders the sensor response to be limited by mass transfer rather than kinetically controlled and which also provides a biocompatible interface with the surrounding environment. Another role of the outer membrane is to protect the enzyme layer and preserve the enzyme activity. Outer membrane is not an option when a sensor is continuously used in biological fluids. If amperometric enzyme based biosensors are to function successfully in vivo, they need biocompatible outer membranes that can prevent fouling by proteins in physiological fluids. The outer membrane is especially important for in vivo measurement because of its ability to make the enzymatic reaction essentially independent of the oxygen partial pressure over a wide range while excluding erythrocytes, tissue, catalase and other oxidizable interfering substances at the electrodes.

The outer layer is applied to control glucose fluxes in order to optimize linearity of sensor response and minimize dependence on oxygen tension. A variety of different polymer coatings were employed in order to attempt to extend the linear range of the prepared sensor. The stability of the sensors life's time is recognized as one of the most important factors with respect of their practical application. Ihab *et*

al., 1995, investigated different concentrations of polyurethane, polyvinylchloride and cellulose acetate coating solutions. The polymer coatings were obtained by dipping the face of the sensor in the polymer solution for 20 s.

Increasing the polymer coating solution concentrations extends the linear range of the sensor response further, but is accompanied by a corresponding decrease in the sensor sensitivity. This can be explained by assuming that increasing the polymer coating concentration produces a thicker or less porous coating. Increasing the coating thickness or decreasing its porosity limits the flux of glucose and thus reducing the response of a given glucose concentration. Yang *et al*., 1998 have used Nafion as external diffusion and additional interfering eliminating layer, which extend the linear range of the sensor response to over 25mM glucose concentration.

Nafion is a negatively charged polymer. It can be used to reduce the diffusion of negatively charged ions such as ascorbic acid and uric acid to the catalytic electrode surface. The use of Nafion layer casted from solutions of the ionomer as an external diffusion control membrane is an attractive approach for reducing the interferences caused by small neutral or cationic electroactive molecule present in biological media. The reproducibility of permeability of this layer in whole blood and the ease of the film preparation are important features of this material.

Also what is significant is the fact that Nafion has many of the features that have been suggested as desirable for biocompatibility. These include having both hydrophilic and hydrophobic properties and being chemically inert. Another advantage of choosing Nafion as an external membrane is the resulted fast sensor response. The response time (estimated as time to reach 95% of the steady-state value of the current signal) is about 10s. The shorter stabilization period required following electrode polarization (1–3 min) and low background current (10–40 nA) are also advantageous. This is achieved as a result of Nafion as the coating membrane which effectively limit the access of the larger molecules and anions to the electrode (Yang *et al*., 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger* were purchased from Sigma (England). Hydroxyehtyl methacrylate (HEMA), (3-glycidoxypropyldimethoxy) silane (3-GPDES), Aluminium isopropoxide 98+%, ethylene glycol dimethacrylate (EGDMA), 2,2-dimethoxy-2-phenylacetophenone (DMPP), ferrocene carboxylic acid (97%) and ferrocene carboxaldehyde (98%) were purchased from Aldrich (Germany). Cystamine dihydrochloride (98%) were purchased from Aldrich (China). Peroxidase horseradish (E.C. 1.11.1.7, type VI from Horseradish), glucose (corn sugar, 99.5%), sodium borohydride (98%), etylene glycol, poly (allylamine hydrochloride) (Average MW CA:70 000), triethlamine, sodium m-periodate, HEPES (99.5%, pH 6.8-8.2), bovine serum albumin (BSA), polyvinyl alcohol (PVA, Average MW 70 000-100 000), glutaraldehyde, lysozyme (Grade 1 from chicken egg, 58,100 units/mg solid, and *N*-cyclohexyl-*N*%-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate were purchased from Sigma (USA). Nafion solution (5% in a mixture of lower aliphatic alcohols and water) were bought from Fluka (USA). Tetramethylorthosilicate (TMOS), kalium di-hydrogen phosphate, di-kalium hydrogen phosphate, kalium chloride, acetic acid,

methanol, sulfuric acid and hydrochloric acid were purchased from Merck (Germany).

3.2 Instrumentation

Electrochemical measurements were carried out using three-electrode configuration. The working electrode (WE) used was a gold or platinum electrode. A platinum auxiliary electrode was used as the counter electrode (CE). A Ag/AgCl/ KCl was employed as the reference electrode (RE). Electrochemical measurement was carried out in normal or deoxygenated phosphate buffer solution with a computer controlled potentiostat (Metrohm μAutolab Type III).

3.3 Methodology for Hydrogen Peroxide-Based Glucose Sensor

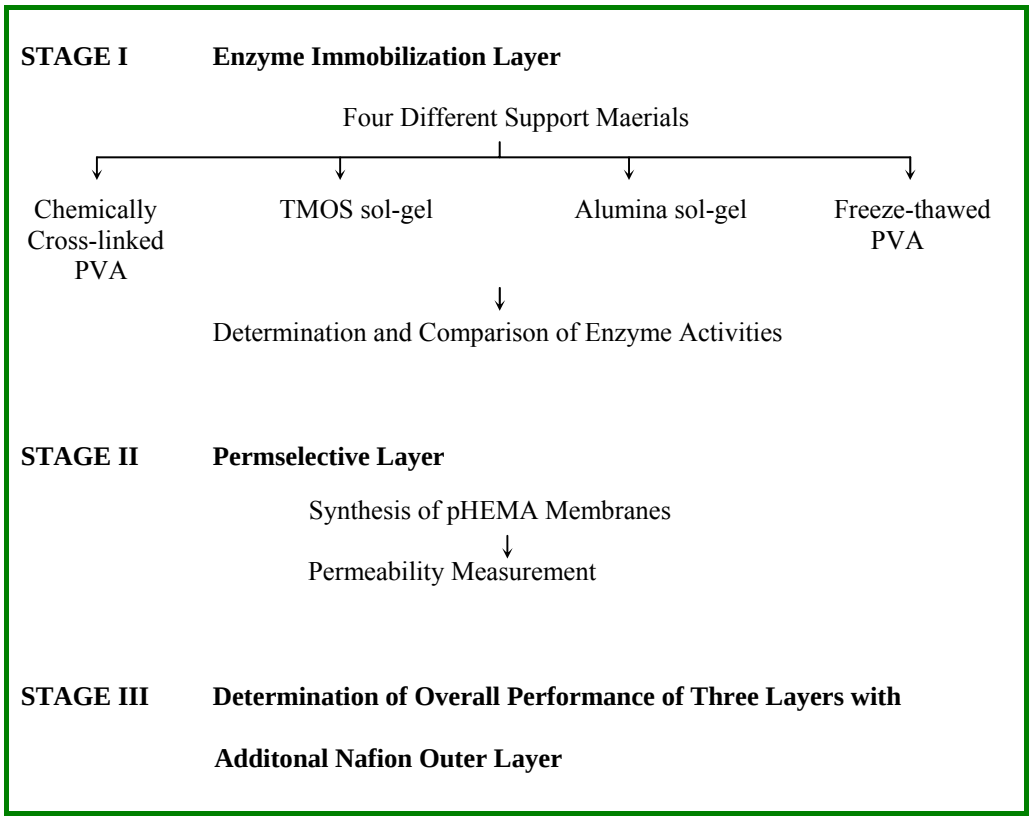


Figure 3.1 Flow chart of research methodology of hydrogen peroxide-based glucose biosensor

3.3.1 General Casting Method of Free-standing Membrane

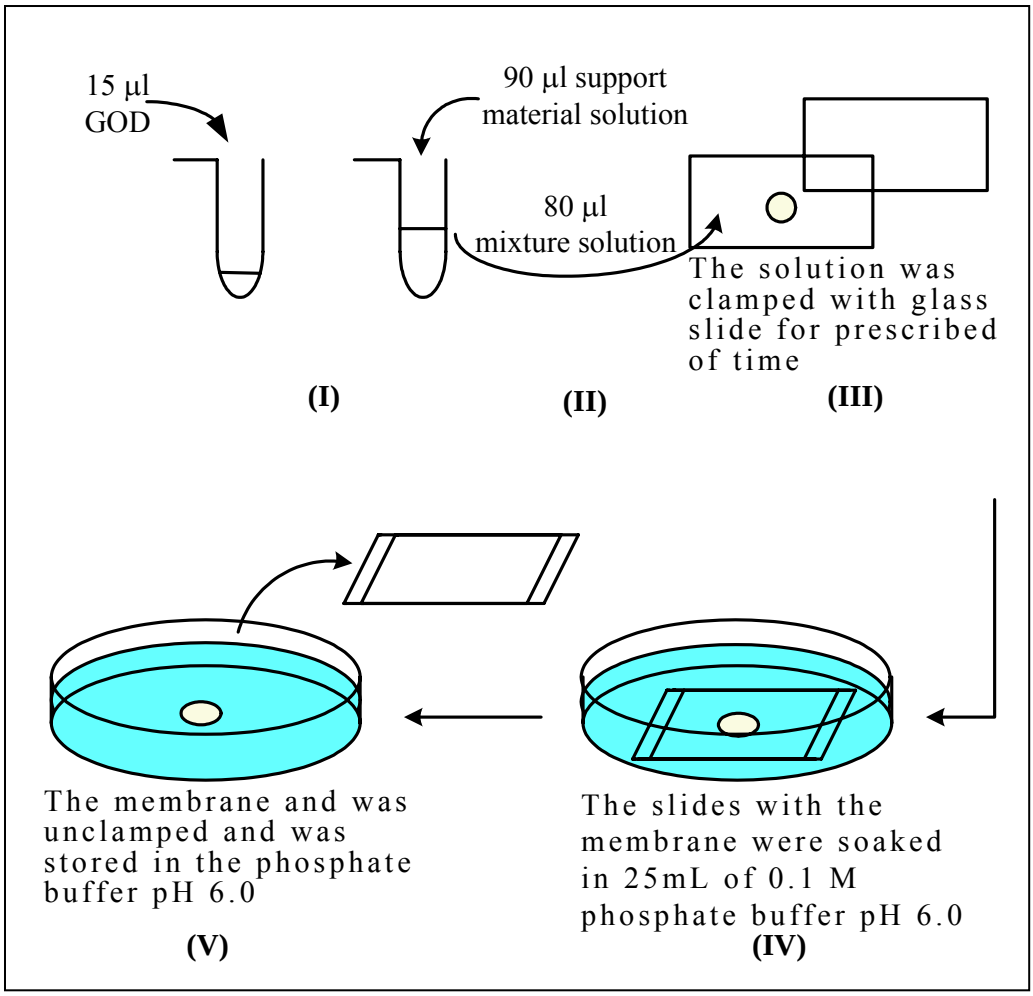


Figure 3.2 General casting method of free-standing membrane

To prepare 280mg/ml GOD aqueous solution, 70mg of GOD lyophilized powder was dissolved in 250µl distilled water. The solution was then stored at 4ºC for later use. The general membrane casting procedures were as shown in figure 3.1. An amount of 280mg/ml GOD solution was added to the support material solution at a volume ratio of 6:1. The final enzyme loading was 40mg/ml. Then, an aliquot of the mixture was transferred quickly onto a glass slide and air-dried for 10 minutes before it was covered with another glass slide. The two glass slides were clamped together and left for a prescribed amount of time at room temperature. The thickness of the membrane was controlled with 3 layers of aluminium spacer tape which were stuck on the first glass slide. Following the gelation of the membrane, the slides

were soaked in 0.1 M phosphate buffer pH 6.0. The slides were then unclamped and the membrane layer was stripped carefully from the slide. Finally, the membrane was washed and stored in phosphate buffer at 4°C for further analysis.

3.3.2 Preparation of Chemically Cross-linked PVA-GOD Membrane (Abdul-Aziz, 2001)

This type of enzymatic layer was prepared by entrapment of GOD in 10% PVA (w/v) aqueous solution using the methods established by Abdul-Aziz (2001) with modifications. The PVA solution was prepared by adding 5 g of PVA powder in 50 ml distilled water. Then, the solution was heated up and stirred at a temperature of approximately 90°C. Subsequently, the well-dissolved solution was filtered and kept at 4°C. 10 wt% aqueous PVA solution was mixed with 10% acetic acid, 50% methanol, and 10% sulfuric acid, in a volume ratio of 5:3:2:1. An appropriate amount of glutaraldehyde was added to this mixture to make up a solution with a cross-linking ratio, CR of 0.06 where CR is defined as the moles of glutaraldehyde per moles of PVA repeat unit. Following step III to IV as depicted in figure 3.2, the two glass slides were clamped together, and left for 1-8 days at 4°C and 24 hours at 25°C.

3.3.3 Preparation of Freeze-thawed PVA-GOD Membrane (Hickey and peppas, 1995)

The preparation of the freeze-thawed PVA-GOD membranes was as described in the general free-standing casting method. The preparations was based on the methods established by Hickey and Peppas (1995) with modifications. The mixture solution of PVA and GOD was pipetted onto the plates and maintained at -20°C for 12 hours to induce crystallization. Following the freezing process, it was allowed to thaw at 25°C for 12 hours. The freezing and thawing cycle, n, was

repeated for 5 times. The preparation conditions were varied as shown in Table 3.1 to investigate the effect on the enzyme leakage and apparent enzyme activity.

Table 3.1 Different preparation conditions of freeze-thawed PVA-GOD membranes

Notation	Period (h) / process	$T_{Freezing}$ (°C)	$T_{Thawing}$ (°C)	No of cycle, n
A	12	-20	25	5
B	6	-20	25	5
C	12	-20	4	5

3.3.4 Preparation of Alumina-PVA-GOD Membranes (Chen *et al*., 2002)

Alumina sols were prepared according to the method established by Chen *et al*. (2002) with modifications. Appropriate amount of Al(*i*-PrO)$_3$ was added to deionized water at 80°C and was stirred for 1h. Then, 1M of HCl was added into the mixture. The molar ratio of Al(*i*-PrO)$_3$: water: HCl was 1:100:0.07. The mixture was then heated to 90°C and kept under reflux condition for 24h. Later, the clear sol was decanted and stored at 4°C. Prior to membrane casting, the sol was dried at 100°C for 5h to evaporate part of the water and alcohol. 10% PVA solution was mixed with the resulting alumina sol (1:2 v/v) to prepare the casting solution. Enzyme immobilization was done following the general method in section 3.3.1. In this case, polystyrene petri dish was used instead of glass slide to facilitate the peeling of the membrane from the support.

3.3.5 Preparation of TMOS-PVA-GOD Membranes (Wu *et al.*, 1999)

This type of enzymatic layer was prepared using the methods established by Wu *et al*. (1999) with modifications A defined amount of TMOS, acidic water, methanol, and pre-calculated volume of (3-glycidoxypropyldimethylethoxy)silane were mixed, and stirred for 30min at 300rpm. Then, a defined amount of aqueous acid was added into the mixture, and stirred for 1h to obtain a transparent sol solution. 120µL of sol solution was mixed with 20µL of GOD solution. After that,

an aliquot of the mixture was pipetted onto the substrate surface, and spread evenly. Gelation occurred within 5min and the ensuing membranes were sealed with parafilm and left to age for 24 hours.

3.3.6 Determination of Water Content

The weight of the swollen membrane was recorded until equilibrium hydration was achieved. Subsequently, the membrane was left to dry at approximately 55ºC in the oven in order to remove residual water in the membranes. The weight of the dehydrated membrane was recorded until constant weight was obtained. Consequently, the water content, H, was determined according to equation (3.1):

$$\mathbf{H} = \frac{(W_w - W_d)}{W_w} \times 100 \qquad (3.1)$$

Buffer solutions that were used to store the ensuing membranes were changed every 6 hours for the first day, 12 hours for the second day, and every 24 hours thereafter. These washing solutions were collected and analyzed for the amount of enzyme released from the membrane into the solutions.

3.3.7 Determination of Enzyme Leakage

The free enzyme activity assay was performed using GOD-HRP coupling colourimetric method as described in the Worthington Enzyme Manual. To prepare the chromogen solution, 0.1 ml of 1% o-dianisidine was diluted in 12 ml of 0.1 M phosphate buffer pH 6.0. Then, 150 µl of 18% D-glucose solution and 50 µl of 200 µg/ml HRP were added to 1.25 ml of chromogen solution. The glucose solution was prepared 24 hours prior to use for mutarotation at room temperature. 50 µl of diluted enzyme (for the construction of calibration curve) or washing solution was then added to the mixture and the reaction was allowed to proceed for 10 minutes at room temperature. Upon termination of the reaction, 100 µl of 4 M HCl was added

(Horner, 1997). The formation of the colour was evaluated by reading the absorbance value at 450 nm and compared to the standard GOD calibration curve.

3.3.8 Determination of Apparent Enzyme Activity of Membrane

The apparent enzyme activity was carried out with amperometric method using a conventional three-electrode potentiostat system from Metrohm, Netherlands. The setup consisted of an Ag/AgCl reference electrode and a platinum sheet counter electrode. A platinum disk electrode with a surface area of 3.14 mm^2 was employed as the working electrode throughout the work.

Prior to every electrochemical measurement, the working electrode surface was polished with alumina powder on a polishing cloth supplied by Metrohm. Then, the enzyme-immobilized layer was secured tightly onto the working electrode surface with gauze and rubber ring before immersing into a cell with 10 ml of 0.1 M phosphate buffer pH 6.0 at room temperature under constant stirring.

A constant potential, +700 mV versus Ag/AgCl, was applied to the electrode. After the background current baseline was stable, 526 µl of 100 mM glucose solution was injected into the cell to give a final concentration of 5 mM. The current response based on the oxidation of enzymatically liberated H_2O_2 at +700 mV was recorded until steady state was reached.

3.3.9 Enzyme Kinetics

The modified kinetic parameters of the immobilized enzyme were determined by electrochemical measurements as outlined in the previous section. 100 mM glucose solution of different volumes was injected successively to obtain different concentrations and the response at +700 mV was monitored.

3.3.10 Casting of pHEMA Membrane as Permselective Layer

HEMA monomer with 30 vol. % was mixed with ethylene glycol methacrylate (EGDMA) at different cross-linking ratio. The photoinitiator, DMPP (2,2-dimethoxy-2-phenylacetophenone) was added to the mixture solution in a vial to achieve a final concentration of 1.6wt% (Lee *et al*., 2003)). An aliquot of the mixture was transferred onto a clean glass slide or on the electrode surface and spin coated at 150rpm for 1min. Then, the disc was placed under an UV light and irradiated for 5 min under continuous purging with nitrogen gas. The pHEMA layer was then soaked in 0.1M PBS pH6.7 for 48h to hydrate the layer. The water content of pHEMA membranes were determined as described in section 3.3.6.

3.3.11 Permeability Analysis (Abdul-Aziz, 2001)

Determination of permeability was performed with a rotating disc electrode (RDE) system. Prior to voltammetry experiments, the membrane-electrode surface was conditioned by applying a constant potential of 700mV vs Ag/AgCl electrode while rotating at 100rpm. 10mM acetaminophen solution was prepared and was saturated with nitrogen before run. 10mL buffer into the cell and voltammograms of the background current was recorded at rotation speeds from 100-400rpm by scanning from 200mV-1100mV (vs Ag/AgCl) at a scan rate of 1mV/s. Acetaminophen was added to the buffer solution to achieve a final concentration of 0.5mM. Experiments of hydrogen peroxide were performed with the same procedure. Koutecky-Levich graph was constructed by plotting $1/i_{lim}$ vs $1/\omega^{1/2}$. The permeability (αD_m) of the diffusing species through the pHEMA membrane was calculated from the intercept of the plot according to the following equation.

$$\frac{1}{i_{\lim}} = \frac{d_m}{nFA\alpha D_m C_b} + \frac{1}{0.62nFAD_{dl}^{2/3}\nu^{-1/6}C_b}\frac{1}{\omega^{1/2}}$$

where

i_{lim} ~ limiting current

d_m ~ membrane thickness

n ~ number of electrons involved

F ~ Faraday constant

αD_m ~ Permeability of the diffusing species through the membrane

D_{dl} ~ Diffusion coefficient of the diffusing species in solution

ν ~ Liquid viscosity

C_b ~ Concentration of diffusing species

ω ~ Rotating speed

3.3.12 Casting of Nafion Outer Membrane

5 wt% nafion was diluted to 2 wt% with mixture of water and methanol at a ratio of 1:9. Then,10 µL of the solution was deposited on the surface of enzymatic layer and spin coated at 300rpm for 2 min. The ensuing membrane was let dry in the air for 15min.

3.4 Methodology for Mediator-Based Glucose Sensor

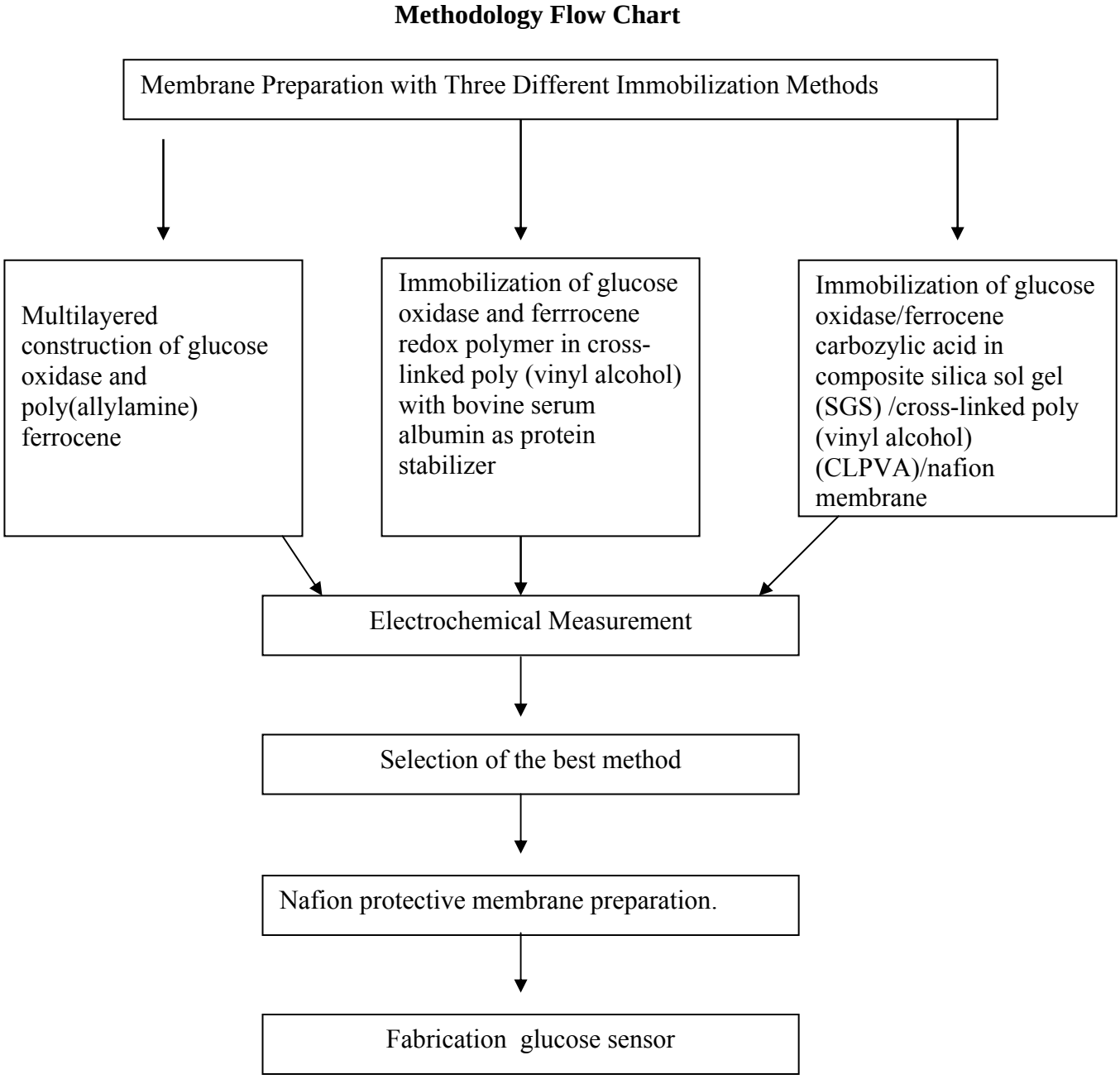


Figure 3.3 Methodology flow chart of mediator-based glucose biosensor

3.4.1 Immobilization of Glucose Oxidase and Ferrrocene Redox Polymer in Cross-linked Poly (vinyl alcohol) with Bovine Serum Albumin as Protein Stabilizer

3.4.1.1 Synthesis of Poly(allylamine) Ferrocene (PAA-Fc).

Preparation of ferrocene-containing redox polymer was done according to Koide and Yokoyama, 1999. 581 mg of polyallylamine hydrochloride and 5 mL of 20% glutaraldeheyde solution were dissolved in a HEPES buffer (50mM, pH6.8) to a total volume of 25mL in a beaker and the solution was then left to gelate. The cross linked gel was crushed through a sieve and freeze dried. 60mg of this polymer was suspended in 50mL of HEPES buffer (50mM, pH6.8) containing 115mg of ferrocene carboxylic acid. Water soluble carbodiimide was added drop wise during the first hour. The reaction was allowed to proceed for 4 days. Small particles of the ferrocene modified polyallylamine hydrogel were rinsed with a phosphate buffer solution. These particles were enclosed in dialysis tubes containing phosphate buffer. The outer buffer solution was stirred and exchanged many times with fresh buffer. This dialysis procedure was carried out for 3 days.

3.4.1.2 Cross linking with PVA and BSA Addition

10% PVA stock solution was prepared by dissolving PVA in water and heating the solution to 80–90 °C under stirring for about 30 minutes. Then, the 5% PVA stock solution was mixed with 10% acetic acid as a buffer, 50% methanol as a quencher, and 10% sulphuric acid as a catalyst in the volume ratio of 5: 3: 2: 1 (Abdul-Aziz, 2001). Appropriate amount of 2% glutaraldehyde was added to the solution in order to obtain a cross-linking ratio of 0.06. Cross-linking ratio is defined as the ratio of the moles of glutaraldehyde per moles of PVA repeat unit. Then, polyallylamine ferrocene, BSA and GOD were added to the CLPVA solution and an aliquot of the mixture was pipetted on a glass slide and air-dried for 20 minutes. Then, it was covered with another glass slide and the two glass slides were clamped together and left for 24hr at 4°C. The membranes obtained were swollen in phosphate buffer at 4°C.

3.4.2 Immobilization of Glucose Oxidase/Ferrocene Carboxylic Acid in Composite Silica Sol Gel (SGS) /Cross-linked Poly (vinyl alcohol) (CLPVA)/Nafion Membrane.

3.4.2.1 Preparation of Nafion–ferrocene Carboxylic Acid (Nafion-FcA)

Ferrocene carboxylic acid solution in absolute alcohol was mixed with 2% Nafion solution(diluted from 5% Nafion solution with absolute alcohol) in the volume ratio of 5:1 (Niu and Lee, 2002). The ethanol content in the final mixture was 91% (v/v). At such high ethanol content, the Nafion film cast on the electrode surface should be stable and capable of good mediator retention.

3.4.2.2 Preparations of Cross-link with PVA (CLPVA) solution

10% PVA stock solution was prepared by dissolving PVA in water and heating the solution to 80–90 °C under stirring for several hours. For preparation of CLPVA solution, 10% PVA stock solution was mixed with 10% acetic acid, 50% methanol, and 10% sulphuric acid in the volume ratio of 5: 3: 2: 1 (Abdul-Aziz, 2001). Later 2% glutaraldehyde was added in such a way that the cross-linking ratio was 0.06. Cross linking ratio is defined as the ratio of the moles of glutaraldehyde per moles of PVA repeat unit.

3.4.2.3 Preparation of SGS-CLPVA Solutions.

The TMOS stock sol gel was prepared by mixing TMOS, 50% methanol, hydrochloric acid (HCl) and water in the mole ratio of (1: 3: 0.0013: 3.7) at 4ºC for 2 hours, based on 3.7 as water/silicate mole ratio. The long mixing time was to make sure that the reaction occurred completely. Since TMOS and water were immiscible, TMOS was initially mixed with methanol under constant stirring at room temperature, followed by water and hydrochloric acid. Freshly prepared TMOS was stored in fridge when not in use.

Then, the TMOS sol gel solution was mixed with the CLPVA solution in a volume ratio of 1:4 (Cajlakovic et. al., 2001). Three – dimensional network

formation could be achieved using composition of PVA / TMOS equals to 80-90 / 20-90% to result in crack-free film (Cajlakovic et. al., 2001). Finally, nafion was added to the mixed silica sol solution based on 1:1 of optimal weight ratio of nafion and PVA (Shao et al., 2002). The performance of the membrane would be optimal by using this dry wieght ratio (Shao et al., 2002). The mixture was then stirred using vortex mixer until homogenus. The silica sol will only be prepared immediately before the fabrication of sensors.

3.4.2.4 Casting of SGS-CLPVA/nafion Membranes

Two types of membranes with different GOD concentrations, 40mg/mL and 20mg/mL, were fabricated separately by casting the following solutions in sequence: 36µL Nafion–FcA solution, 54µL of respective GOD aqueous solution, and 36µL SGS-CLPVA solution. Every layer was dried under ambient condition after each casting before storage in a refrigerator at 4 °C overnight. The enzymatic membranes were kept at 4 °C in the refrigerator when not in use.

3.4.3 Multilayered Construction of Glucose Oxidase and Poly(allylamine)ferrocene

3.4.3.1 Preparations of Surface Carbaldehyde Groups

Carbohydrate groups on the peripheral surface of the glucose oxidase molecule was oxidized with periodate to carbaldehydes according to established procedure (Yoon et al., 1997). 20µM GOD solution in 5mL of 0.1 M phosphate buffer solution (pH 6.8) was stirred slowly with 30 mg of sodium metaperiodate for 1 h at 4 °C in the dark. The reaction was stopped with the addition of 25mM of ethylene glycol for 30min at 25 °C. The product was purified by membrane dialysis against water and freeze-dried.

3.4.3.2 Synthesis of Poly(allylamine)ferrocene (PAA-Fc).

Poly(allylamine) ferrocene (PAA-Fc) used was synthesized according to the method established by Zhang et al 2004 with modifications. 16mg ferrocene carboxaldehyde was dissolved in 10mL methanol and was added drop wise within an hour to 60mL of anhydrous methanolic solution of 80 mg of poly(allyamine) containing 0.52mL of triethylamine. The mixture was stirred for another hour at room temperature. Then sodium borohydride was carefully added in portions at 0 °C, and the stirring was continued for 90 min. Finally the mixture the mixture was dried at 35˚C and the residue was extracted with distilled water (Hodak et al., 1997). The aqueous solution was further purified by membrane dialysis against water.

3.4.3.3 Production of Covalently Linked Enzyme Multilayer Films

Prior to the construction of enzyme multilayer films, amino functionalities was introduced on the glass slide by pipetting 40μL aqueous solution of cystamine dihydrochloride (10mM, 2h). Then, 40μL of periodate-oxidized GOD solution in 0.1M phosphate buffer (pH 6.8) was added to the modified glass slide containing amino groups at room temperature. The GOD/PAA-Fc bilayer was formed by pipetting 40μL PAA-Fc solution in 0.1 M phosphate buffer on the resulting enzyme monolayer. A covalently attached enzyme multilayer film was fabricated by repeating the last two steps in a cyclic fashion. Then, it was followed by rinsing with buffer solution. Schiff bond was reduced by dipping the membrane in 5mM solution of cyanoborohydride at 4 ºC in the dark for 30 minute. The remaining carbaldehyde on the periphery was blocked with 10mM ethanolamine (pH 9.5, will be titrated with concentrated HCL) for 30 minute to avoid self-polymerization (Yoon et al., 2000). .

3.4.4 Electrochemical Measurement

Electrochemical measurement was carried out for all three types of enzyme electrodes separately. Before use, the electrode was rinsed with doubly distilled water, and immersed in the 0.1 M phosphate buffer (pH 7.0) until a stable electrochemical response is produced by the immobilized ferrocene. Generally, freshly prepared biosensors could attain a stable electrochemical response after 5–10 min of rinsing. Glucose stock solutions were allowed to mutarotate at room temperature overnight before use. The electrolyte solutions were deoxygenated with nitrogen bubbling for 90 min before each voltammetric run. The amperometric studies were run at 363 mV vs Ag/AgCl. All experiments were performed at a temperature of 25±1 °C and under nitrogen atmosphere, unless otherwise specified.

3.4.4.1 Kinetics Properties of Ferrocene Based Membrane

For kinetic studies and response time studies, the amperometric studies were run at 363 mV vs Ag/AgCl. Following background current stabilization, an appropriate amount of the stock 0.1M glucose solution was injected into the cell to give a pre-selected concentration.

3.4.4.2 Stability of Ferrocene Based Membrane

To investigate the effect of storage on the stability of the membrane, all membranes were prepared and than stored in buffer at 4ºC for different time periods. The electrochemical response of the membranes to 5mM glucose solution was then measured.

3.4.5 Ferrocene Leakage Detection

Leakage of ferrocene derivatives mediator was measured electrochemically. The washing solution was subjected to cyclic potentials from 600mV to -100mV with scan rate $10mVs^{-1}$. The concentration of the mediator was determined using a calibration curve.

3.4.6 Enzyme Leakage Detection

Leakage of enzyme was measured colorimetrically. The chromogen solution was prepared by diluting 0.1 mL of 1% O-dianisidine in 12 mL of 0.1 M phosphate buffer, pH 6.7. Then, 150μL of 18% aqueous glucose solution and 50μL of 200μg/mL peroxidase solution were added to 1.25 mL of the chromogen solution. The mixture was then placed in a water bath at 25˚C for temperature equilibration. Then, 50μL of the washing solution was added to the mixture. The reaction was allowed to proceed for 5 minutes before 100 μL of 4 M HCL was added to stop the reaction. The amount of colour formed was measured by reading the absorbance value at 450nm (Abdul-Aziz, 2001).

3.4.7 Preparation of Nafion Protective Membrane

The nafion layer preparation was based on the description in section 3.3.12.

3.4.8 Fabrication of Glucose Sensor

This part of the research has not been completed yet. Empty strips with three-electrode configuration will be used in the fabrication of mediated glucose biosensor. The electrodes are a working electrode, a counter electrode and reference electrode. Prepared enzymatic membrane solution will be coated onto the empty strips. After that, the enzyme strips will be connected to the potentiostat for electrochemical measurements.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Hydrogen Peroxide-Based Glucose Sensor

4.1.1 Chemically cross-linked PVA-GOD Membrane

In this work, the effects of immobilization temperature, and the content of protein-based stabilizing agent (PBSA) have been investigated.

4.1.1.1 Temperature of Immobilization

In order to compare the apparent enzyme activity of membranes immobilized at different temperatures, one of the conditions of the membranes that must be fixed was the equivalent cross-linking density, which was evaluated by its water content at both temperatures. Different duration of membrane clamping process that allows the cross-linking reaction and dehydration or vaporization of water and other volatile components contained in the membrane mixture solution would affect the water content of a membrane and thus the cross-linking density. Membranes that are immobilized at higher temperature would have lower water content relative to membranes that are immobilized at lower temperature. In this work, water content for membranes prepared at 25°C was compared to water content for membranes prepared at 4°C with different clamping period.

Table 4.1 Water content of membranes clamped for different period

Temperature (°C)	Clamping Period (Day)	Water content, H (%)	% Difference
25	1	76.73±3.07	-
4	1	81.07±1.73	5.66
	2	84.33±2.00	9.91
	3	86.86±1.57	13.20
	4	85.66±1.98	11.65
	5	86.69±2.29	12.99
	6	81.62±5.98	6.38
	7	77.18±1.63	0.59
	8	74.39±1.07	3.05

From the result obtained, the membranes immobilized at 25°C had the lowest water content, which was 76.73%. There was no significant difference in water content for membranes clamped for 2-6 days, which ranged from 81.0%-86.0%. While lower water content was observed for membranes clamped for 7-8 days. This is expected, as water content is inversely proportional to the period of dehydration of immobilized membranes. From the percent difference in water content of GOD-PVA membranes immobilized at 4°C, it was clear that GOD-PVA membranes dehydrated for 7 days had identical water content with the GOD-PVA membranes immobilized at 25°C

However, water content for membranes immobilized at 4°C, which were clamped for 1 day, had 81.07% of water content, which was an unexpectedly low value. It should be pointed here that the membranes that were clamped for 1 day were only partially gelled and very watery. Therefore the weights of the fully swollen membrane obtained were less than a completely gelled membrane and thus might result in inaccurate water content determinations.

Thus, it was concluded that membranes immobilized at 4°C have to be clamped for 7 days for dehydration in order to have comparative cross-linking density with membranes immobilized at 25°C and left dehydrated for 1 day.

In this study, the effect of immobilization temperature on the ability of the PVA-GOD membranes to retain the immobilized enzyme was investigated. Washing solutions were collected at certain period for enzyme activity determination.

Figure 4.1 shows that the enzyme activities of the washing solutions for the PVA-GOD membranes demonstrate a declining profile for the whole period of investigation both for membranes immobilized at 4°C and 25°C.

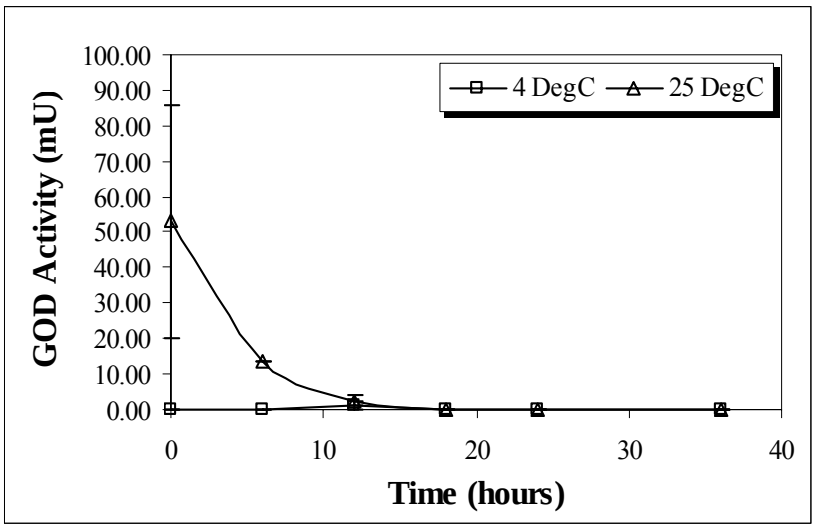


Figure 4.1 Comparison of leaking profile of membranes immobilized at different temperature

As expected, the enzymes activities reached zero within 18 hours for membranes immobilized at both temperatures. This clearly states that the chemically cross-linked PVA is effective in retaining the enzyme within the matrix.

Colourimetrical enzyme assay based on the oxidation of o-dianisidine through a peroxidase-coupled system had been performed on the PVA-GOD membranes to investigate and compare the apparent enzyme activity of membranes immobilized at 4°C and 25°C (L. Doretti *et al*., 1997). Furthermore, the stability of the repeated-use PVA-GOD membranes was examined as well since the decay of apparent enzyme activity over time and limited lifetime of the enzyme layer of a biosensor have been reported (L. Doretti *et al*., 1996, Azila Abdul Aziz, 2001). The enzyme activity of the membranes was tested at 5 days after the membranes were unclamped and until 22 storage days.

The comparison of apparent enzyme activity for membranes immobilized at both temperatures is represented in Figure 4.2.

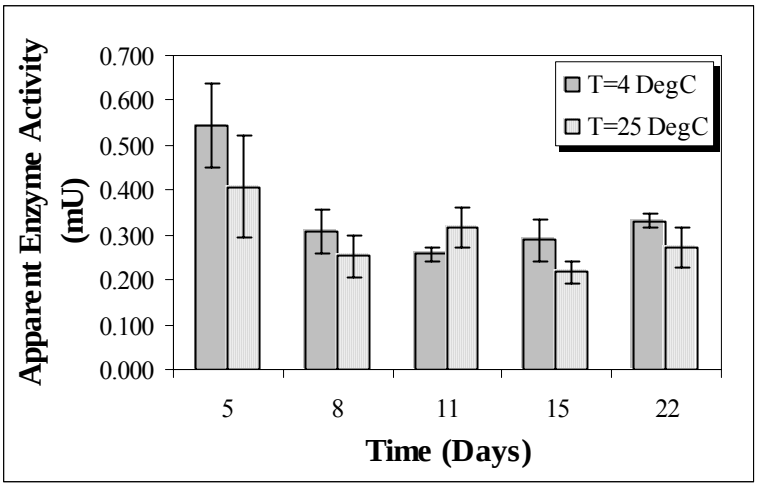


Figure 4.2 Apparent enzyme activities for membranes immobilized at different temperature

Membranes fabricated at 4°C showed initial decline in apparent enzyme activity followed by stabilization until day 22. This demonstrates that there was slow deterioration and denaturation of the immobilized GOD in the chemically cross-linked PVA membrane. Hydrogen peroxide generated during the enzymatic reaction was observed to be retained on the membranes surfaces, which can result in poisoning of the membranes (C.E. Hall and E.A.H. Hall, 1993). As mentioned before, the retention of hydrogen peroxide could be a result of high mass transfer resistance to substrates and products imposed by the extensive cross-linking imposes mass transfer resistance. The diffusional resistance encountered by the product molecules results in the product to accumulate near the center of the gel to an undesirable high level, leading to product inhibition for the enzyme.

The activity of the membranes formed at 25°C seemed to be stable throughout the 22 days and did not show the same initial declining profile in apparent enzyme activity such as that demonstrated by PVA-GOD membranes been cast at 4°C.

In conclusion, it can be said that membranes immobilized at 4°C and 25°C with comparable cross-linking density showed comparable long term activities. However, membranes formed at 25°C seemed to be more stable than the membranes formed at 4°C.

4.1.1.2 Content of Protein-based Stabilizing Agent

It is well known that glutaraldehyde is a very effective cross-linking agent in immobilizing enzyme. However, as compensation, it causes serious conformational changes to the enzyme and decreases the sensitivity as well as the operational life of the enzyme (Broun, 1976). One of the solutions for this obstacle is the addition of a protein-based stabilizing agent. The choice of the stabilizing agent must be inert to the biological reaction involved, and provide complimentary surface (Chang and Mahoney1995). Aside from bovine serum albumin (BSA), lysozyme has been found to be efficient for this purpose (Gouda *et al*, 2002).

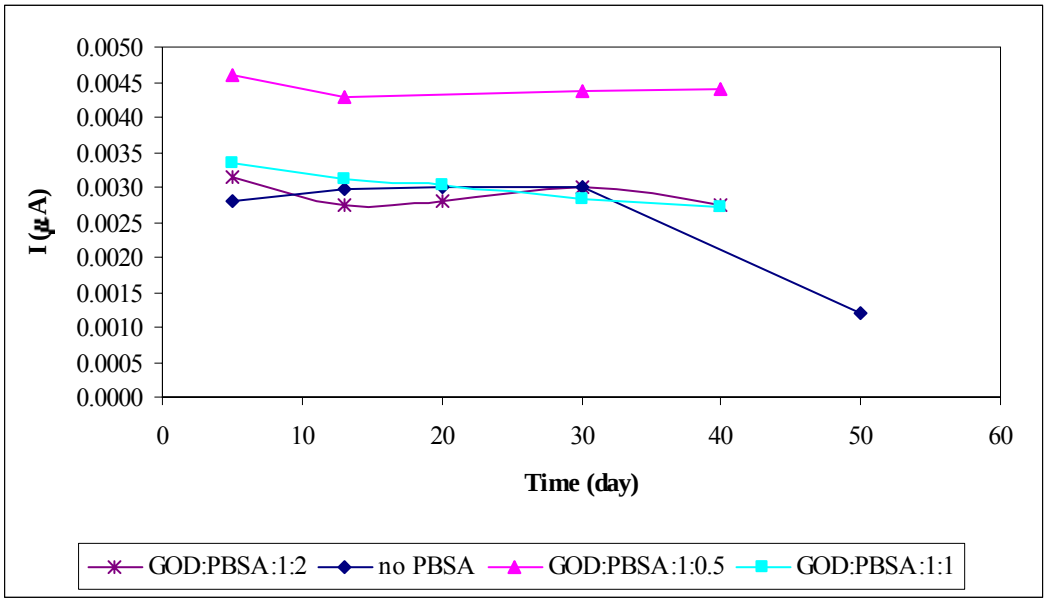


Figure 4.3 Comparison of current response of chemically cross-linked PVA-GOD membranes with different concentration ratio of lysozyme upon 5mM glucose

The comparison of apparent enzyme activity of chemically cross-linked PVA-GOD incorporated with different ratio of lysozyme is shown in Figure 4.3. The current was generated from the electrochemical oxidation of hydrogen peroxide at

the surface of the platinum working electrode at 700mV. It can be clearly seen that lysozyme at concentration ratio of 0.5 to GOD concentration has revealed the highest current response. This might be attributed to the formation of intermolecular bonds between GOD and the stabilizing protein. As a result, the linkages functioned as a protective barrier to the enzyme and intramolecular bonds between GOD and glutaraldehyde could be avoided (Gouda *et al*, 2002). On the other hand, higher ratio of lysozyme may impart a diffusive obstruction to the substrate and thus did not able to improve the current response towards 5mM glucose.

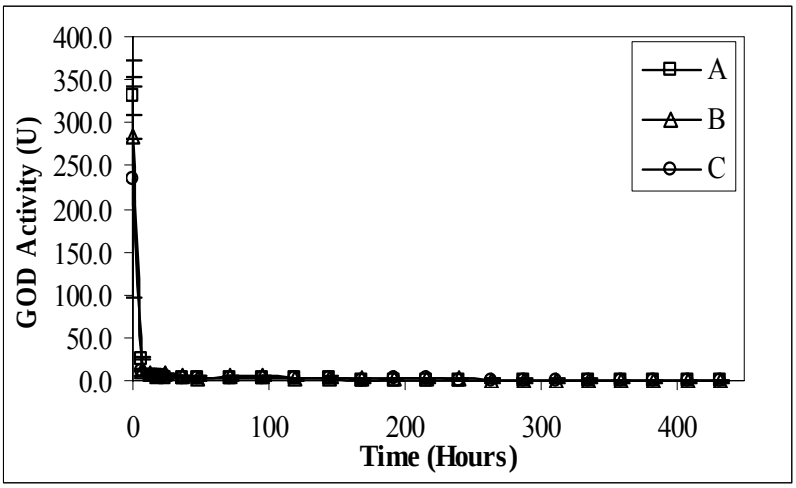
4.1.2 Freeze-thawed PVA-GOD Membrane

PVA prepared from freeze-thawing method has been studied extensively for drug delivery. In this work, the period of freeze and thaw process was investigated for the feasibility as an immobilization support. The effect of thawing temperature was also evaluated.

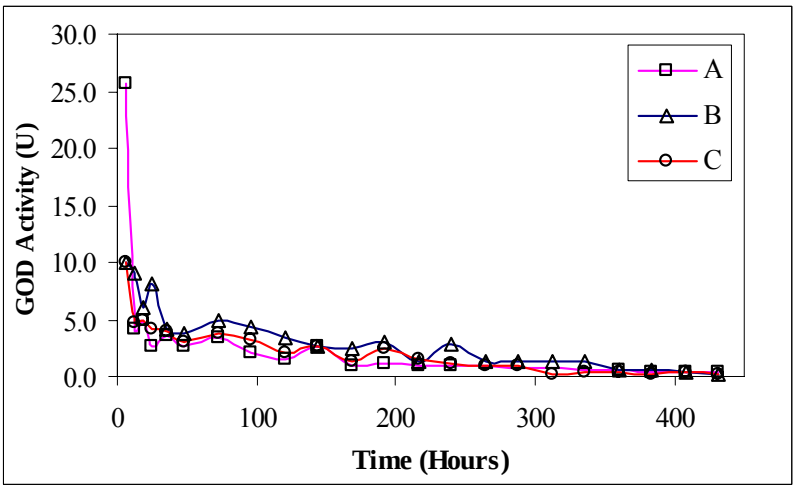
Table 4.2 Freeze-thawed PVA-GOD prepared at different conditions

Notation	*Period(h)/process*	$T_{Freezing}$ *(°C)*	$T_{Thawing}$ *(°C)*
A	12	-20	25
B	6	-20	25
C	12	-20	4

The comparison of the enzyme leaking for freeze-thawed PVA-GOD membranes is as in Figure 4.4.



(a)



(b)

Figure 4.4 Comparison of leaking profile of freeze-thawed PVA membranes at different conditions. (a) Enzyme leakage from 0 hour (b) A clearer picture of enzyme leakage omitting the 0 hour leakage

Compared to PVA-GOD membranes that were cross-linked with a bifunctional agent, enzyme leakage from freeze-thawed PVA had taken more than 2 weeks. From the freeze and thaw process, the PVA-GOD membrane was cross-linked with a physical means while entrapping GOD within its matrix. The insufficient capacity of the matrix to effectively retain the enzyme loaded might be the main explanation for the inadequate immobilization. Among the 3 types of the membranes, membrane A has shown the highest leaking while membrane C showed the least. It is suspected that enzyme leaked out during the thawing process at 25°C for membrane A and has shown an excessive leaking after the membranes were

unclamped. As for membrane B, unexpectedly the short hours of freezing and thawing process might resulted in a looser matrix and the enzyme leaked out slowly throughout the leaking period.

From the aspect of apparent enzyme activity, membrane A distinctly shows a lower current response towards 5 mM glucose in 60 days of storage period as shown in Figure 4.5. This might be due to longer period of exposure of membrane A to higher temperature that may denature the immobilized enzyme.

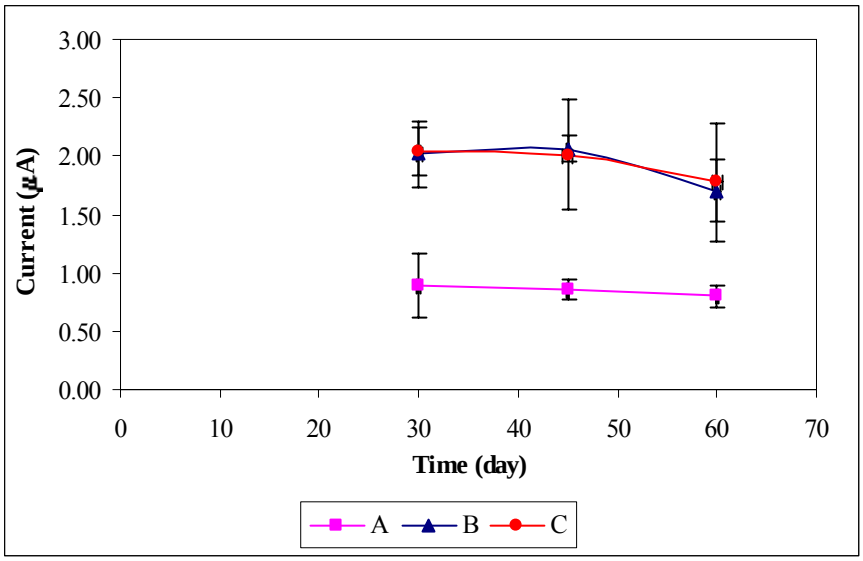


Figure 4.5 Comparison of current response of freeze-thawed PVA-GOD membranes at different conditions upon 5mM glucose

4.1.3 Alumina-PVA-GOD Sol-gel Derived Organic/Inorganic Membrane

In this work, in the beginning only alumina was used to cast the sol-gel material. Free-standing and crack-free alumina-GOD membranes were obtained. An obvious problem with sol-gel derived material is the brittleness of the matrix formed (Xu *et al.*, 2006). As low mechanical strength was observed in alumina membranes, a binder was required to strengthen the matrix. With the compatibility of alumina sol and PVA which both possessed hydroxyl group, PVA appeared to be a good choice of the binder (Yang *et al.*, 1996). Addition of PVA into the casting solution was able to reduce the surface tension of the membranes and thus avoid cracking (Yang *et al.*, 1996). This allowed the membranes to be peeled off from the support easily.

Figure 4.6 shows that the enzyme activities of the washing solutions for the alumina-PVA-GOD membranes which decreased with time.

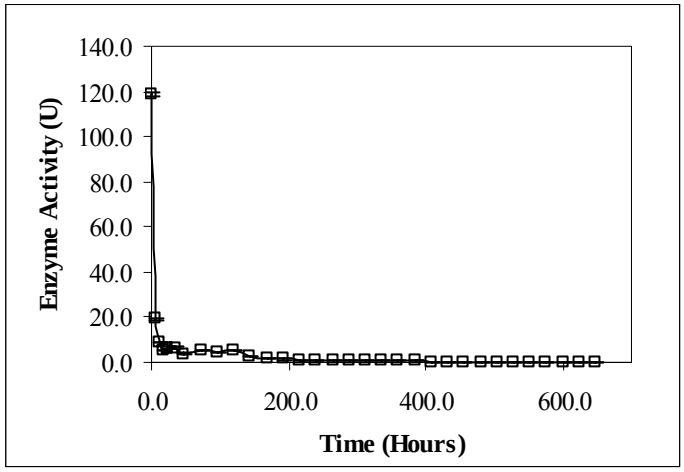


Figure 4.6 Leaking profile of alumina-PVA-GOD composite membrane

However, the enzyme leaked out for more than 16 days. A very small amount of enzyme activity still can be observed until day 25. Moreover, for the first ten days high leakage was shown. The amount of enzyme leakage and the leaking period were so much higher compared to chemically cross-linked PVA-GOD membranes with the same concentration of GOD immobilized as reported before. The results might be attributed to high concentration of enzyme added to the membranes, which exceeded the immobilization capacity of the membranes. Without a cross-linker such as glutaraldehyde, the enzymes were merely adsorbed within the matrix. Though this will contribute to the retention of the apparent enzyme activities, it resulted in poor retention of the enzymes. Compared to freeze-thawed PVA membrane, the enzyme leakage was lower in amount but dragged for a longer period. Lower concentration of enzyme might be employed to investigate the immobilization ability of such material. Besides, addition of an appropriate coupling agent such as (3-aminopropyl)trimethoxysilane might be able to enhance the matrix formation as well as to increase the enzyme immobilization capacity (Chen et al., 2002).

Long term stability of the alumina-PVA-GOD membranes was investigated to determine the shelf life of the sensor. The apparent enzyme activities of the membranes were tested for 65 storage days.

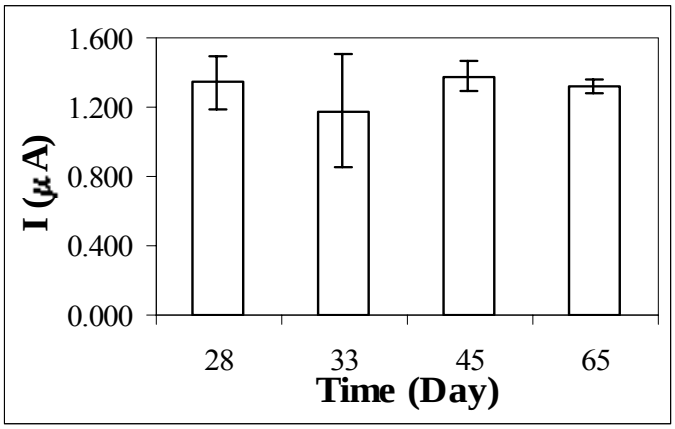
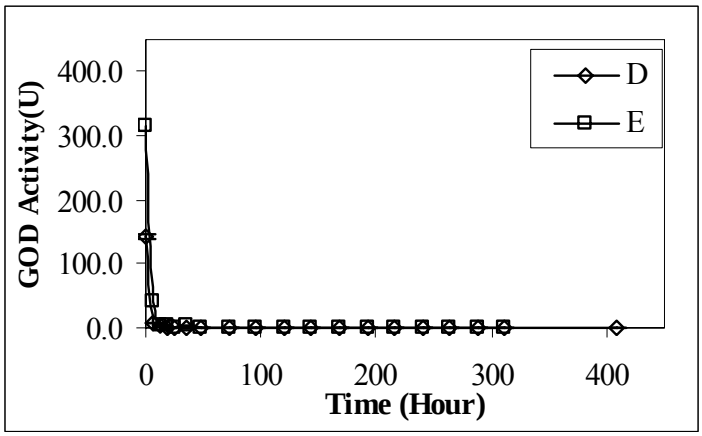


Figure 4.7 Current response of Alumina-PVA-GOD composite membrane upon addition of 5mM glucose

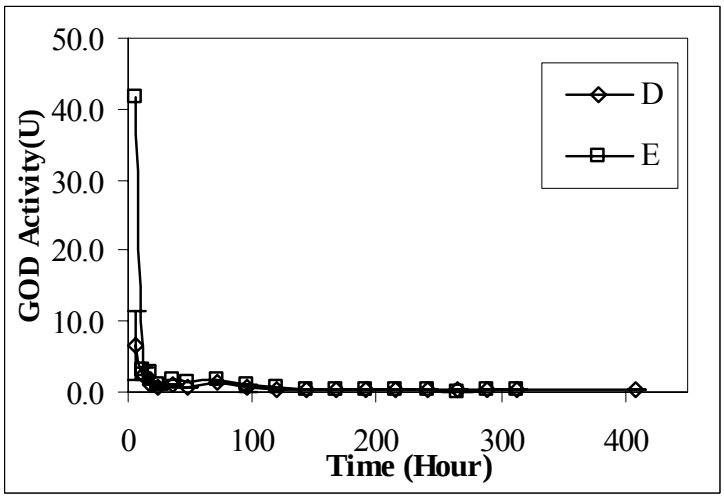
From Figure 4.7, it was shown that the alumina-PVA-GOD membranes were stable over a period of 60days. This agrees with the study reported by Chan *et al*. For a period of 65 days, it was observed that the apparent enzymatic activities of the membranes immobilized were quite high compared to the chemically cross-linked PVA-GOD membranes. This can be attributed to the relatively mild immobilization condition. Though the leaking problem with alumina-PVA membranes was more serious, the large amount of the remaining enzymes immobilized within the alumina sol-gel matrix was still able to give higher response. As compared to freeze-thawed PVA-GOD membrane, it is observed that the current response of alumina-PVA sol-gel membrane was relatively lower.

4.1.4 TMOS-PVA-GOD Sol-gel Derived Organic/Inorganic Membrane

By using a different precursor, tetramethoxysilane (TMOS), the effect of cross-linker was investigated in the effort to reduce enzyme leakage. PVA was also incorporated for the purpose of increasing mechanical strength. An optimum volume ratio of cross-linker to silane of 0.6 has been reported (Jian Wu *et al*., 1999) based on the current response. In this work, the same ratio was investigated on the effect of enzyme leakage.



(a)



(b)

Figure 4.8 Comparison of leaking profile of TMOS-PVA membranes D (without cross-linker) and membranes E (with cross-linker) (3-glycidoxydiethoxysilane) (a) Enzyme leakage from 0 hour (b) A clearer picture of enzyme leakage omitted 0 hour leakage

Figure 4.8 shows the comparison of the leaking profile of TMOS-GOD membranes with addition of a cross-linker, (3-glycidoxypropyl)dimethylethoxysilane. The cross-linker was included in the sol-gel system to covalently couple the backbone of the sol-gel matrix through a condensation reaction. The hydroxy group of the cross-linker can also react with the enzyme amine group (Jian Wu *et al*., 1999). Thus, it was expected the enzyme not only be physically entrapped but also covalently coupled to the sol-gel matrix. In addition, it was also expected that denser gel would be formed to reduce enzyme

leaking. However, addition of the cross-linker did not show improvement in enzyme retention even though higher ratios of cross-linker were investigated.

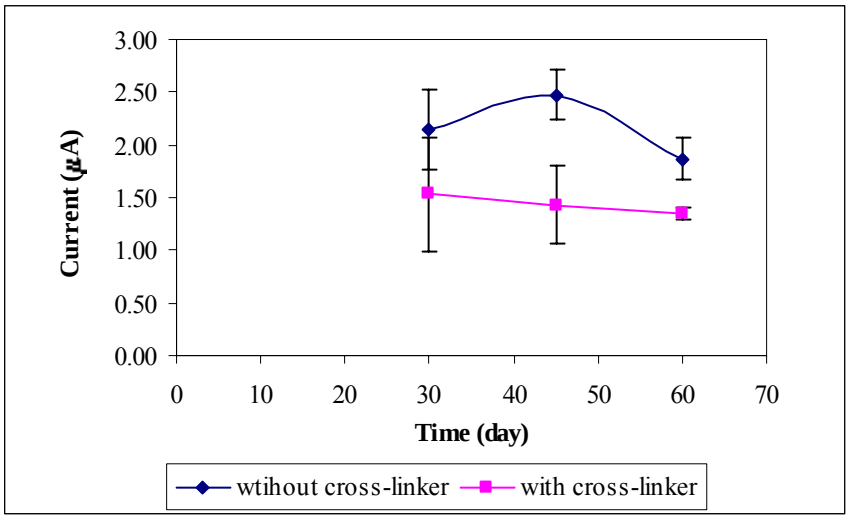


Figure 4.9 Comparison of current response of TMOS-PVA-GOD membrane at different conditions upon addition of 5mM glucose

From Figure 4.9, TMOS-PVA-GOD membrane without cross-linker has shown higher response to 5mM glucose. The cross-linker, (3-glycidoxypropyl)dimethylethoxysilane, might lead to the denaturation of the immobilized enzyme at some extent similar to the characteristic shown by glutaraldehyde although it was not as serious.

4.1.5 Overall Comparison of Performance of Different Membranes

From the four types of membranes with immobilized GOD, it can concluded that chemically cross-linked PVA membrane has the highest ability in retaining the enzyme within the matrix which stopped leaking within 1 day after being unclamped. TMOS-PVA sol-gel leaked for 1 week while alumina-PVA and freeze-thawed PVA leaked for about 15 days. From the aspect of biosensor response, however, the lowest response was observed in chemically cross-linked PVA membrane. With the optimum PBSA ratio of 0.5, the current response was approximately 4.5nA whilst other membranes were able to achieve more than 1μA. In contrast, apparent Michaelis-Menten constant, K_m^{app}, of the chemically cross-linked PVA was very

high, which was around 70-90 mM. K_m^{app} of sol-gel materials were less than 5mM. In terms of response time, sol-gel derived materials in general have shown faster response compared to PVA membranes. Overall, high loading of enzyme for immobilization may attribute to the good stability (Pfeifer D., 1997). From the advantages and disadvantages reviewed, freeze-thawed PVA-GOD membrane has been selected as the best choice.

4.1.6 Permselectivity Analysis

With the risk of electrochemical interference commonly faced by hydrogen peroxide-based biosensor, a permselective membrane fabricated from poly(hydroxyethyl methacrylate) (pHEMA) was studied in order to eliminate the interference. The electro-active substance which was represented by acetaminophen in this work can be oxidized at 700mV, the potential where oxidation of hydrogen peroxide took place, thus generating interfering current. At certain cross-linking ratio, the passage of acetaminophen through the pHEMA membrane was expected to be restricted.

As discussed previously, water content of a membrane revealed the cross-linking density as well as the mesh size of the membrane. Using ethylene glycol dimethacralyate (EGDMA) as cross-linker, pHEMA membrane was prepared with 30 vol.% of HEMA monomer at different cross-linking ratio to provide different mesh size (Kermis *et al*., 2003). The membranes were rubbery after swelling and were transparent. As shown in Figure 4.10, water content of the membrane decreased with increased cross-linking ratio, which indicated that denser networks were obtained.

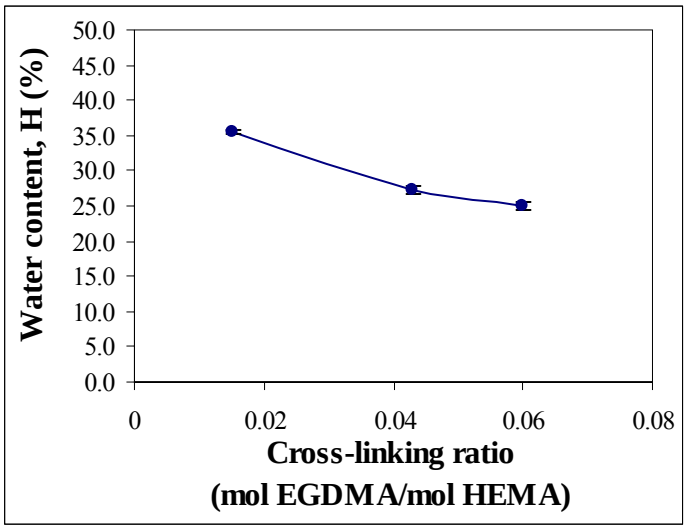


Figure 4.10 Water content of pHEMA permselective layer at different cross-linking ratio

The analysis of permeability was performed according to Koutecky-Levich plot with the following equation.

$$\frac{1}{i_{\lim}} = \frac{d_m}{nFA\alpha D_m C_b} + \frac{1}{0.62nFAD_{dl}^{2/3}\nu^{-1/6}C_b}\frac{1}{\omega^{1/2}}$$

A typical Koutecky-Levich plot was shown below.

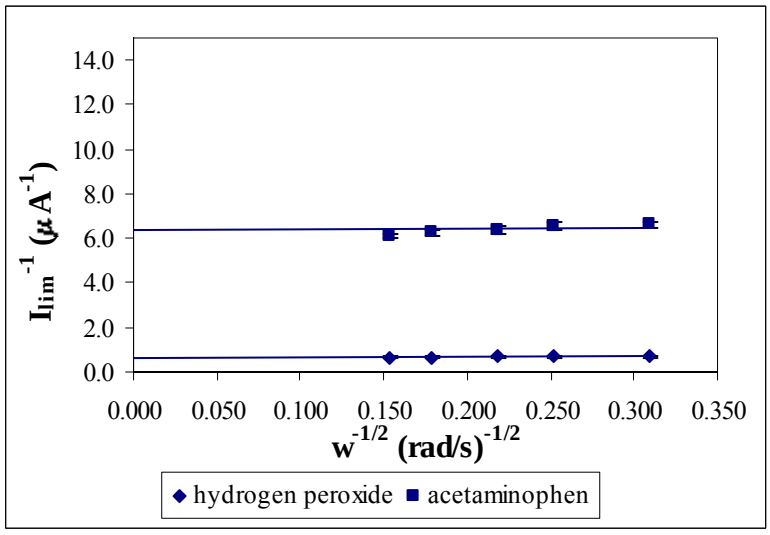


Figure 4.11 Typical Koutecky-Levich plot of acetaminophen and hydrogen peroxide through pHEMA membrane

The diffusion characteristics of pHEMA membranes are shown in Table 4.3. As shown in Figure 4.12, the permeability of acetaminophen dropped when cross-linking ratio was increased.

Table 4.3 Permeability performance of pHEMA membranes at different cross-linking ratio

Cross-linking Ratio	*Water Content, H (%)*	$P_{hp}x10^6$ (cm^2/s)	$P_{ac}x10^6$ (cm^2/s)	*Selectivity,* σ
0.015	35.6 ± 0.33	7.85 ± 0.75	1.28 ± 0.23	5.5 ± 0.7
0.043	27.2 ± 0.53	5.43 ± 0.46	0.53 ± 0.01	10.2 ± 0.6
0.060	24.9 ± 0.61	3.63 ± 0.02	0.30 ± 0.01	12.1 ± 0.5

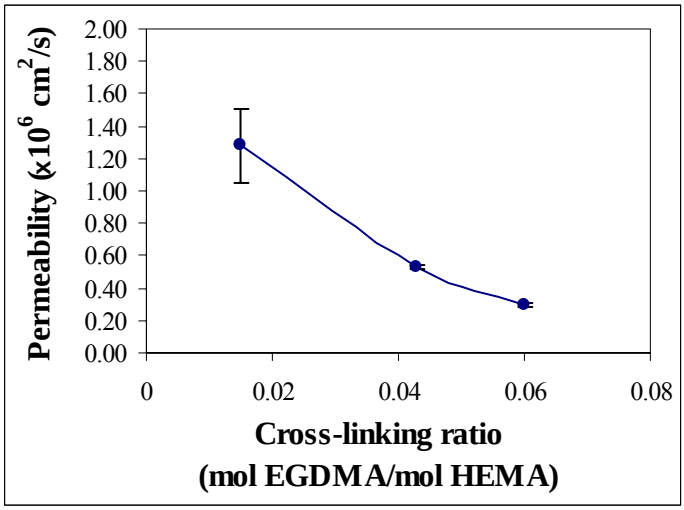


Figure 4.12 Permeability of acetaminophen at different cross-linking ratio

Although by decreasing mesh size of the network resulted in the diffusion of the interfering molecule, the permeability of hydrogen peroxide also declined in the same trend as depicted by Figure 4.13.

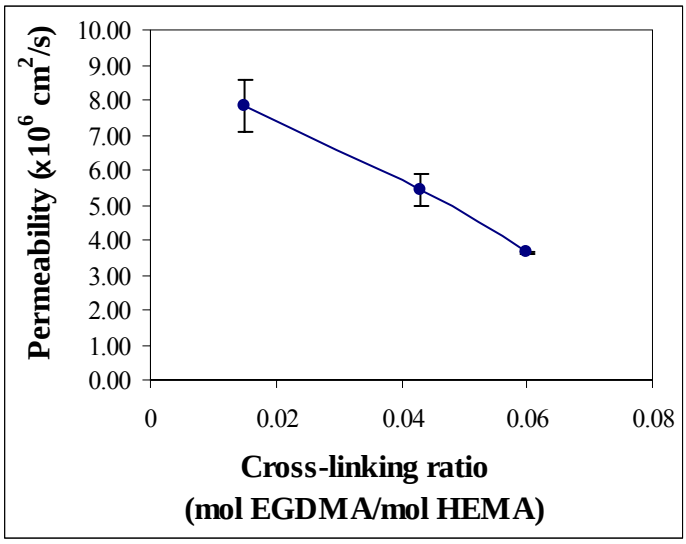


Figure 4.13 Permeability of hydrogen peroxide at different cross-linking ratio

To achieve the objective of rejecting acetaminophen while maintaining a reasonable passage of hydrogen peroxide, selection of an optimum cross-linking ratio was performed by calculating the permselectivity of hydrogen peroxide over acetaminophen. Figure 4.14 shows the dependence of the selectivity of pHEMA membrane to different cross-linking ratio.

$$\sigma = \frac{P_{peroxide}}{P_{acetaminophen}} = \frac{\alpha_{peroxide} D_{peroxide}}{\alpha_{acetaminophen} D_{acetaminophen}}$$

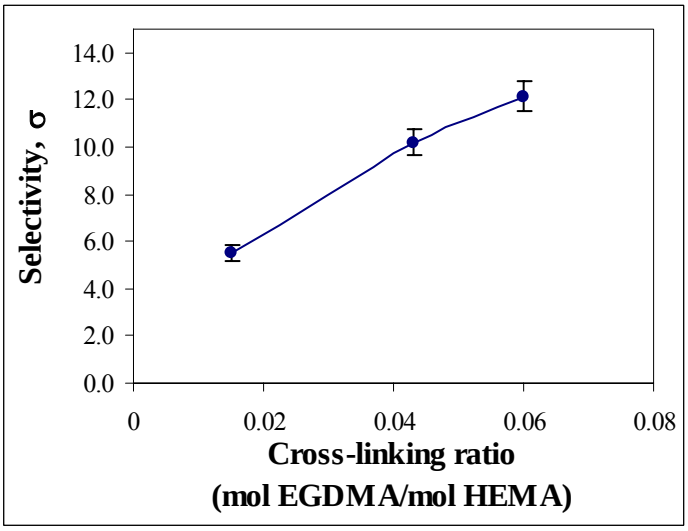


Figure 4.14 Selectivity of pHEMA membranes at different cross-linking ratio

By increasing the cross-linking ratio from 0.015 to 0.043, the selectivity was doubled. Further increment of cross-linker concentration resulted in only slight improvement of selectivity. As a conclusion, pHEMA membrane cross-linked at the ratio of 0.043 was selected.

4.1.7 Performance of Three Layers Biosensor

Freeze-thawed membrane type B was selected to be the enzymatic active layer. PHEMA membrane with the cross-linking ratio of 0.043 was selected to be the inner layer and a 2% Nafion layer was selected to be the outer layer. The complete lab-scale biosensor was tested for performance analysis. As shown in Figure 4.14, no significant current response of interference was shown with pHEMA membrane CR=0.043.

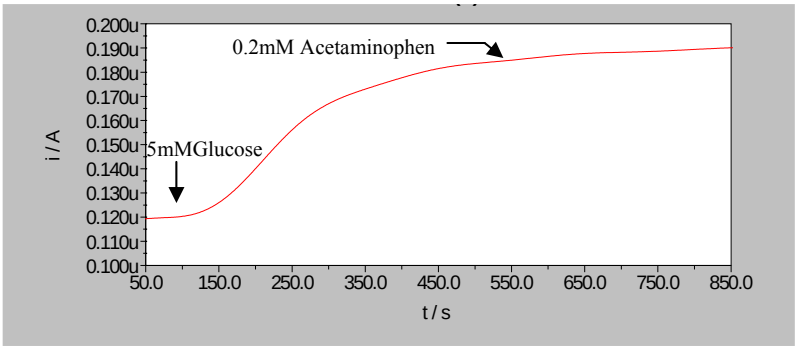


Figure 4.15 Amperometric current response with injection of 5mM glucose and 0.2mM acetaminophen

4.2 Mediator-Based Glucose Biosensor

4.2.1 Cyclic Voltammetry for Ferrocene Carboxylic Acid

For oxidation redox potential determination, ferrocene carboxylic acid solution was subjected to cyclic potentials from 600mV to -100mV with scan rate $10mVs^{-1}$. Figure 4.16 shows cyclic voltammograms at different concentrations of ferrocene carboxylic acid. Each cyclic voltammogram shows two peaks, one corresponding to the reduction of the original substrate and the second corresponding to the re-oxidation of the product back to the original substrate. The average of this the two peak potentials equals the standard redox regardless of the concentration of substrate or its diffusion coefficient or rates of electron transfer. The obtained oxidation redox potential was 0.363V. This is the same value obtained by Calvo and Danilowicz, 1997 for ferrocene/ferricinium couple in polyallylamine ferrocene hydrogel polymer. Meanwhile, the reduction potential obtained was 0.278V. Thus, the mean peak potential, E_0 was 0.321V for ferrocene carboxylic acid.

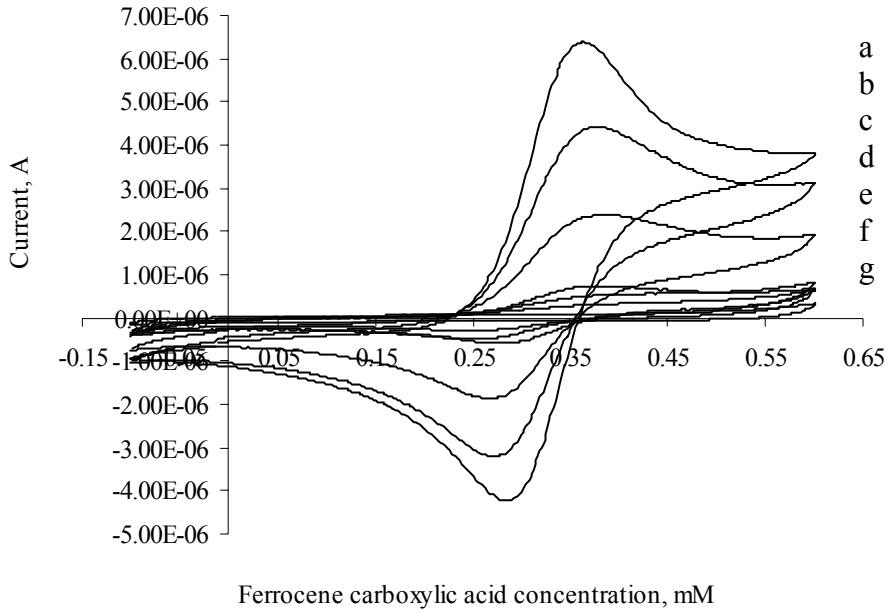


Figure 4.16 Height of the current peaks correspond to concentration of ferrocene carboxylic acid a) 1.4 mM b)1.0mM c) 0.5mM d) 0.2mM e) 0.1mM f) 0.05mM and g) 0 mM in phosphate buffer

The heights of current peak corresponded to the concentration of ferrocene carboxylic acid as seen in Figure 4.1. It can be seen that the anodic peak current increased with increasing concentration of ferrocene carboxylic acid. Solution with highest concentration of mediator gave highest current responds. Meanwhile, solution with lowest concentration of mediator gave lowest current response. Therefore, by doing cyclic voltammetry experiments to the solutions that were used to wash the immobilized enzyme and ferrocene, the concentration of the leaking ferrocene mediator, if any, can be detected using a calibration curve.

4.2.2 Glucose Oxidation

As shown in Figure 4.16, in the absence of glucose and enzyme, only the electrochemical behavior of ferrocene carboxylic can be observed. With the addition of glucose and glucose oxidase to ferrocene carboxylic acid in phosphate buffer, enzymatic reaction will occur. Figure 4.17 shows linear sweep voltammograms for ferrocene carboxylic acid with and without the addition of glucose and glucose oxidase. Increase in current response was observed when 0.1M glucose and glucose oxidase were added to ferrocene acid carboxylic in phosphate buffer solution.

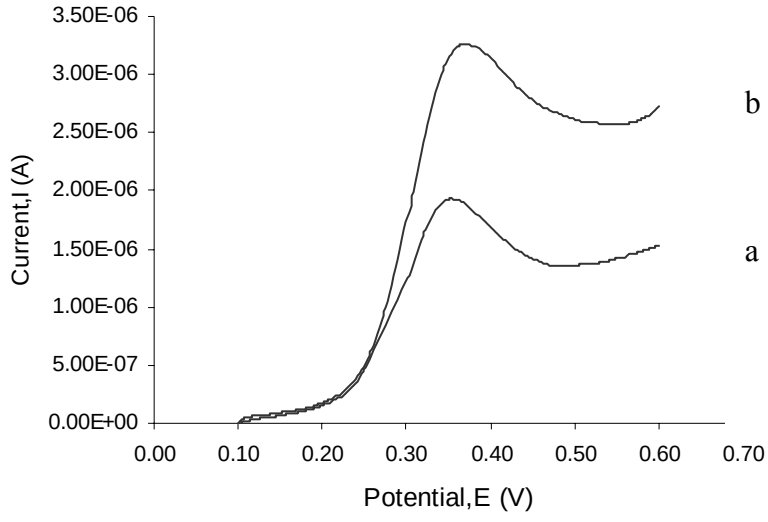


Figure 4.17 Linear sweep voltammograms for ferrocene carboxylic acid before (a), and after (b) addition of glucose and GOD in 0.1M phosphate buffer, ph 7.0 at scan rate 10mVs^{-1}

Before the addition of glucose and the enzyme, the current obtained was 1.91μA. After addition of glucose and GOD to ferrocene acid carboxylic solution, the current response increased about 65.96% to 3.59μA. It showed that the current signal was given by the reduced mediator that was re-oxidized at the electrode to generate the oxidized form of the mediator.

4.2.3 Immobilization Methods for Mediated Biosensor

4.2.3.1 Immobilization of Glucose Oxidase and Ferrocene Redox Polymer in Cross-linked Poly (vinyl alcohol) with Bovine Serum Albumin as Protein Stabilizer

1) Retention of enzyme and mediator in membranes

To investigate the ability of the membranes to retain GOD and ferrocene mediator, the washing solutions for the CLPVA-GOD/Fc membranes were assayed for any sign of enzyme activity and also leakage of the mediator. Figure 4.18 and 4.19 show the leaking profiles of GOD and ferrocene for the CLPVA-GOD/Fc membranes.

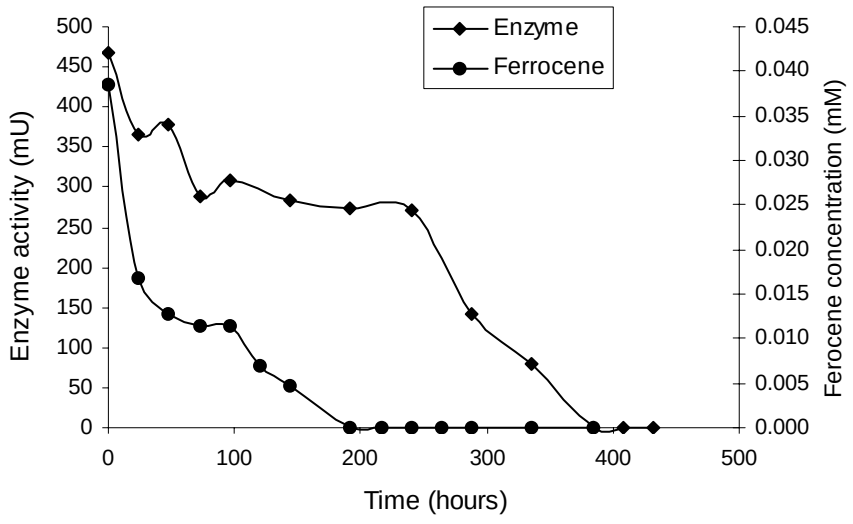


Figure 4.18 Leaking profile for CLPVA-GOD/Fc membrane with different GOD and BSA loading a) 1:1 (weight ratio of GOD: BSA)

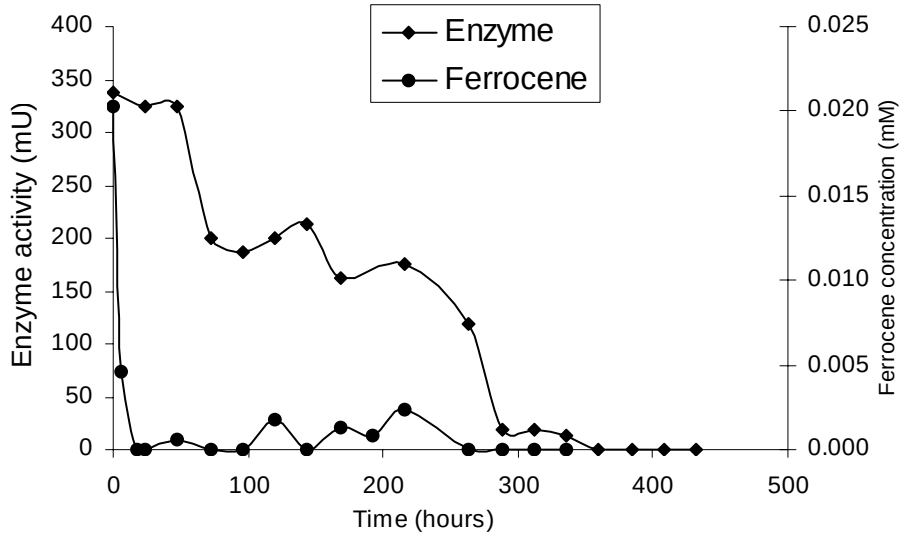


Figure 4.19 Leaking profile for CLPVA-GOD/Fc membrane with different GOD and BSA loading b) 1:3 (weight ratio of GOD: BSA)

As shown in Figure 4.18 and 4.19, the leaking of enzyme as well as mediator decreased with time. No sign of enzyme activity was observed in the washing solutions after 15 days for membranes with the weight ratio of 1:3 (GOD: BSA), which was 1 day earlier compared to membranes with the weight ratio of 1:1 (GOD: BSA). Meanwhile, leakage of ferrocene from membranes with the weight ratio 1:3 (GOD: BSA) stopped after 11days, which was 2 days later than the membranes with the weight ratio of 1:1 (GOD: BSA).The retention of enzyme and mediator in the membranes were very poor although CLPVA was applied as a solid support. For both membranes, the leakage of ferrocene stopped earlier compared to the enzyme. However, the leaking of ferrocene should not have occurred since ferrocene was covalently attached to the polyallylamine hydrogel. The leaking might be due to high concentration of enzyme as well as ferrocene redox polymer that might have exceeded the immobilization capacity of the membranes. The excess enzymes and mediator were not immobilized within the solid support and leached out easily from the membrane.

2) Kinetics properties of the membranes

The response time to arrive at 95% at the steady state current for CLPVA-GOD/Fc membranes with 1:1 and 1:3 (weight ratio of GOD: BSA) were approximately, 262s and 443s, respectively. Both membranes were quite thick, with 160 microns and 221 microns respectively, thus the distance between the electrode and the reaction center of the enzyme was large. As a result, the time required to reach 95% of the steady state current was relatively long. Typical calibration curves for CLPVA-GOD/Fc membranes at an applied potential of 360mV in aerated condition is shown in Figure 4.20. It shows the current – time response of CLPVA-GOD/Fc membranes for successive additions of 0.1M glucose stock solution in 10mL phosphate buffer.

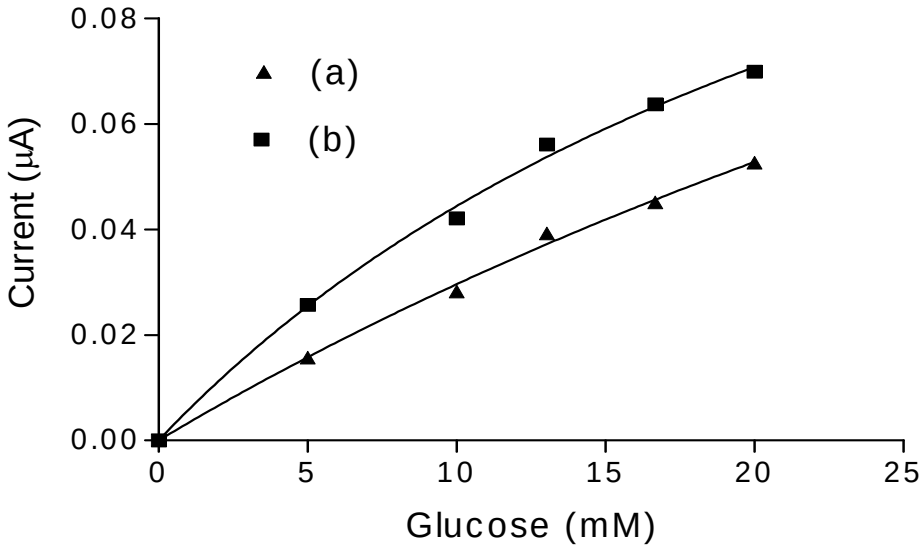


Figure 4.20 Typical glucose calibration curves for CLPVA-GOD/Fc membranes with different GOD and BSA loading a) 1:1 b) 1:3 (weight ratio of GOD: BSA)

The ferrocene functioned well as mediator, shuttling electrons between GOD and the platinum electrode. Current increased stepwise with addition of glucose. Michaelis-Menten kinetics were determined from steady state currents and the electrochemical version of the Lineweaver-Burk equation. Figure 4.21 shows the Lineweaver Burke plot for this membrane for enzyme kinetics study.

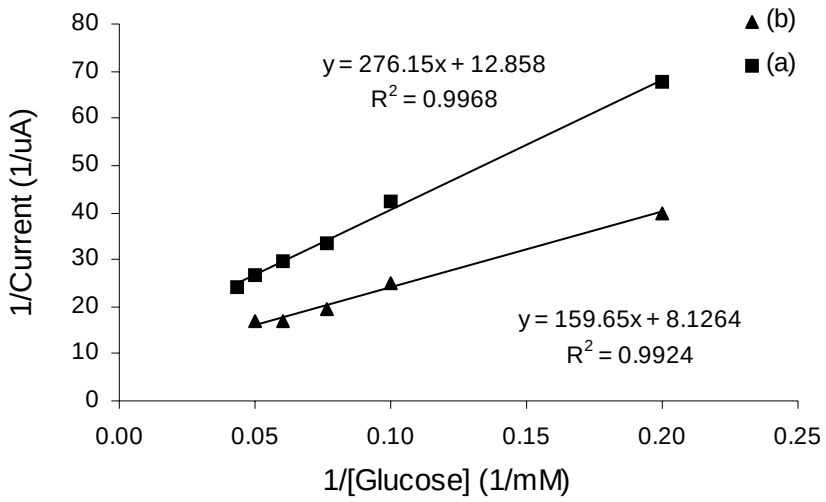


Figure 4.21 Double–reciprocal (Lineweaver Burke) plots of CLPVA-GOD/Fc membranes with different GOD and BSA loading a) 1:1 b) 1:3 (weight ratio of GOD: BSA in mg)

The apparent Michaelis-Menten constant, K_m^{app} for membranes with weight ratio (GOD: BSA) 1:1 and 1:3 were approximately, 21.48mM and 25.45mM, respectively. Meanwhile, the corresponding maximum current, I_{max} for both cases were 0.08μA and 0.16μA respectively. The membranes with higher BSA gave higher current response towards glucose. BSA stabilized the enzymes, creating a 'biological like' environment. Albumin improves enzymatic activity because of better mass distribution of the various proteins without altering the mechanical properties of the membrane. BSA could also prevent the polymer matrix from over-swelling (Koide and Yokoyama, 1999), which could extend the distance between the redox sites of the polymer. As the distance increased the electron transfer rate among neighbouring redox sites would decrease.

The apparent Michaelis-Menten constants, K_m^{app} were 21.48mM and 25.45mM respectively for membranes with weight ratio (GOD: BSA) of 1:1 and 1:3. These values were larger than the K_m^{app} of glucose oxidase in solution that has been reported to be approximately 12.43mM and 15.94mM at temperature 25°C and 30°C, respectively (Liu et al., 1996). Besides, the K_m^{app} of glucose oxidase in solution also

has been reported to be approximately 20mM (Wilson and Turner, 1992) Generally, the K_m^{app} of an immobilized enzyme will be larger than that of the free enzyme in solution due to the effect of the diffusion of substrate to the active sites (Abdul-Aziz, 2001). In this work, membranes with high loading of BSA had lower K_m^{app}. The low K_m^{app} suggested that the enzyme had a high affinity for the substrate (Shuler and Kargi, 2002).

3) Stability of CLPVA-GOD/Fc membranes

Stability of CLPVA-GOD/Fc membranes was investigated to determine the shelf life of the sensors. The current outputs of the membranes to 5mM glucose at certain period were measured. Figure 4.22 shows the effect of storage time on the stability of CLPVA-GOD/Fc membranes.

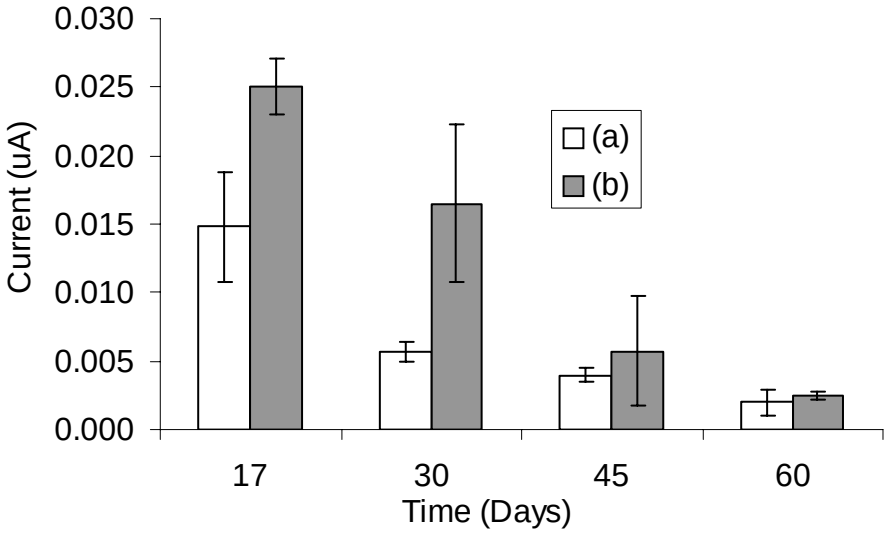


Figure 4.22 Stability of CLPVA-GOD/Fc membranes with different GOD and BSA loading a) 1:1 b) 1:3 (weight ratio of GOD: BSA)

As shown in Figure 4.22, the membranes retained approximately only 38.87% and 66.00% of the initial current after 1 month, for membranes with weight ratio (GOD: BSA) 1:1 and 1:3, respectively. Then, after 2 month, only 3.5% and 9.7% of the initial current remained, respectively for both membranes. The stability of CLPVA-GOD/Fc membranes was not good. This could be due to the deterioration

of the immobilized GOD or problems with the mediator. Brooks et al., however, reported that the loss of activity of ferrocene glucose sensors was more strongly influenced by the loss of enzyme by denaturation or detachment (Brooks et al., 1984). The addition of extra ferrocene to spent electrodes did not affect activity but the addition of more glucose oxidase rejuvenated the sensitivity to glucose. Thus, stability of CLPVA-GOD/Fc membranes could be improved if the immobilization process was more effective.

4.2.3.2 Immobilization of Glucose Oxidase/Ferrocene Carboxylic Acid in Composite Silica Sol Gel (SGS) /Cross-linked Poly (vinyl alcohol) (CLPVA)/Nafion Membrane

1) Retention of enzyme and mediator in membranes

Two types of enzymatic membranes were prepared. One contained 40mg/mL GOD and the other contained 20mg/mL GOD. To investigate the ability of the membranes to retain GOD and ferrocene mediator, the washing solutions for the SGS-CLPVA/nafion membranes were assayed for any sign of enzyme activity and also leakage of the mediator. Figure 4.23 and 4.24 show the enzyme and ferrocene leaking profile for SGS-CLPVA/nafion membranes.

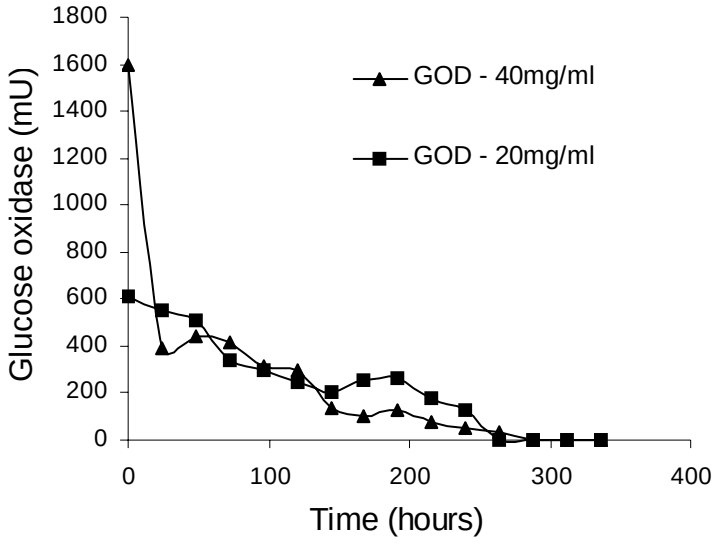


Figure 4.23 Enzyme leaking profile for SGS-CLPVA/nafion membranes

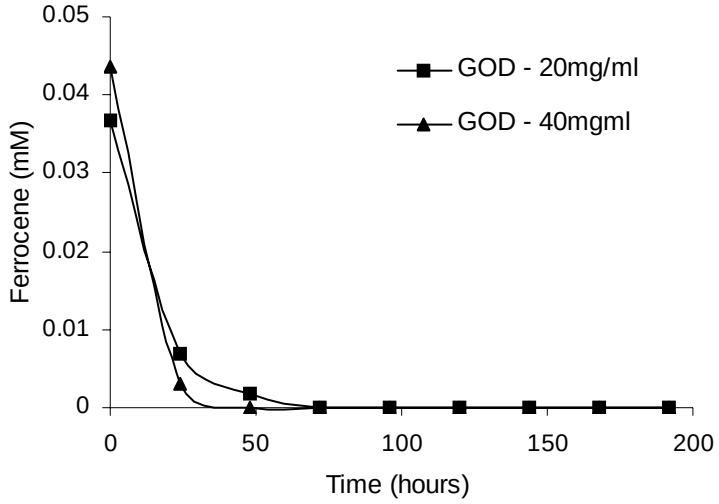


Figure 4.24 Ferrocene leaking profile for SGS-CLPVA/nafion membranes

As shown in Figure 4.23 and 4.24, the leaking of enzyme as well as mediator decreased with time for the two types of membranes with different GOD concentrations. No sign of enzyme activity was observed in the washing solutions after 12 days for both types of membranes. Meanwhile, leakage of ferrocene from membranes with 40mg/mL of GOD stopped after 2 days, which was 1 day earlier than the membranes with 20 mg/mL of GOD. For both membranes, the leakage of ferrocene stopped earlier compared to the enzyme. With high ethanol content, the Nafion film cast should be stable and capable of good mediator retention (Niu and Lee, 2002). However, if there were weakly held species as well as leached ferrocene derivatives from the inner Nafion mediator layer, they will be retained by the outer SGS-CLPVA network layer. However, the leaking of the enzyme still occurred for a long period for both of membranes. As shown in Figure 1, by reducing the enzyme concentration, the amount of leached enzyme was reduced instead of the leaking period. The leaking of enzyme might be due to the possibility that the enzyme concentration might have exceeded the immobilization capacity of the membranes. The excess enzymes were not immobilized within the solid support and leached out easily from the membrane.

2) Kinetics properties of the membranes

Figure 4.25 show the typical current response towards 5mM glucose solution.

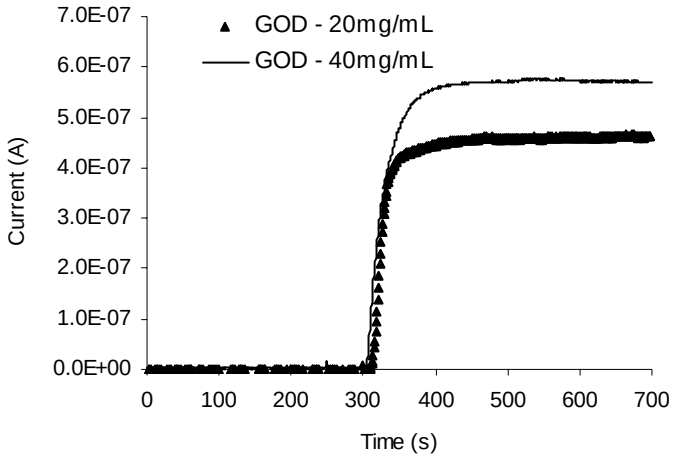


Figure 4.25 Typical current response of SGS-CLPVA/nafion membranes

As shown in Figure 4.25, the response time to arrive at 95% at the steady state current for membranes with GOD concentration of 40mg/mL and 20mg/mL were approximately, 87s and 73s, respectively. The response time for the two membranes was almost same. Both membranes were quite thin, 82 microns and 76 microns respectively, thus the distance between the electrode and the reaction center of the enzyme was small. As a result, the time required to reach 95% of the steady state current was relatively short. However, the contact between the redox site and reaction center of enzyme must be improved to get shorter response time of around 10s-20s.

I_{max} is the current at very high and saturated concentrations of substrate. Under these conditions, every enzyme molecule will have substrate attached to it and will be interacting with it to convert it to product as fact as it can. I_{max} for the membrane with 40mg/mL GOD was 0.51μA higher than I_{max} for membrane with 20mg/mL GOD. It shows that in this case I_{max} depended on enzyme concentration. Sato and Okuma (2006) reported that current response was found to increase with the amount of enzyme, but it would be constant after reaching a maximum unit of GOD. This effectively says that in the presence of sufficient amounts of GOD, the response

current is independent of the amount of GOD. Figure 4.26 shows typical calibration curves for both types of membrane for kinetics study.

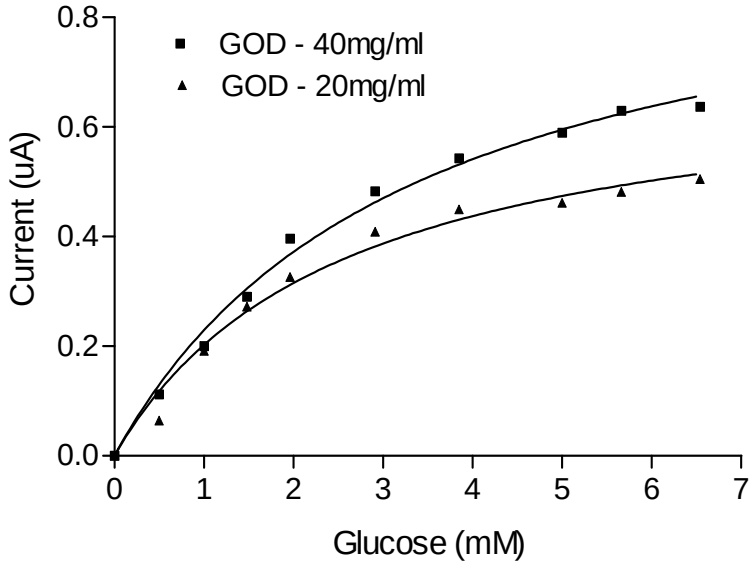


Figure 4.26 Typical calibration curves for SGS-CLPVA/nafion membranes

Michaelis-Menten kinetics were determined from steady state currents and the electrochemical version of the Lineweaver-Burk equation. Figure 4.27 shows the Lineweaver Burke plot for this membrane for enzyme kinetics study.

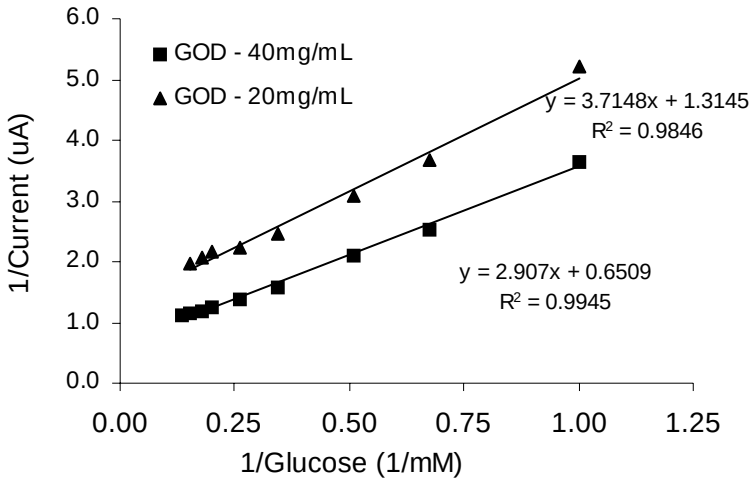


Figure 4.27 Double–reciprocal (Lineweaver Burke) plots of SGS-CLPVA/nafion membranes

The corresponding maximum current, I_{max}, for both cases was 1.23μA and 0.72μA, respectively. The apparent Michaelis-Menten constant, K_m^{app} for membranes with GOD concentration of 40mg/mL and 20mg/mL was approximately, 3.80mM and 3.08mM, respectively. The K_m^{app} obtained for both types of membranes were quite low and with only 0.72mM differences between the two of them. The high sensitivity and the small K_m^{app} mean that the immobilized glucose oxidase possessed a higher enzymatic activity. K_m^{app} is independent of enzyme concentration. The K_m^{app} value depends on the strength of the bonds between enzyme and substrate. If these bonds are strong, the K_m^{app} will be low, indicating that the immobilized enzyme retained its bioactivity and possessed high biological affinity to glucose. The high degree of affinity of the enzyme to the substrate may be explained by a favorable change in the structural organization of the enzyme due to the immobilization procedure (Arica et al., 1995). Consequently, the active sites of the enzymes could be more readily available for enzymatic interactions.

3) Stability of SGS-CLPVA/nafion membranes

The stability of SGS-CLPVA/nafion membranes was investigated to determine the shelf life of the sensors. The current outputs of the membranes when subjected to 5mM glucose at certain periods were measured. As shown in Figure 4.28, after 1 month, the membranes containing 40mg/mL and 20mg/mL GOD retained approximately 82.30% and 95.50% of the initial activities, respectively. After 2 months, only 59.50% of the activities of the membranes with 40mg/mL of GOD remained. On the other hand, the membranes with 20mg/mL GOD were still quite stable with 83.60% of the initial activity remained.

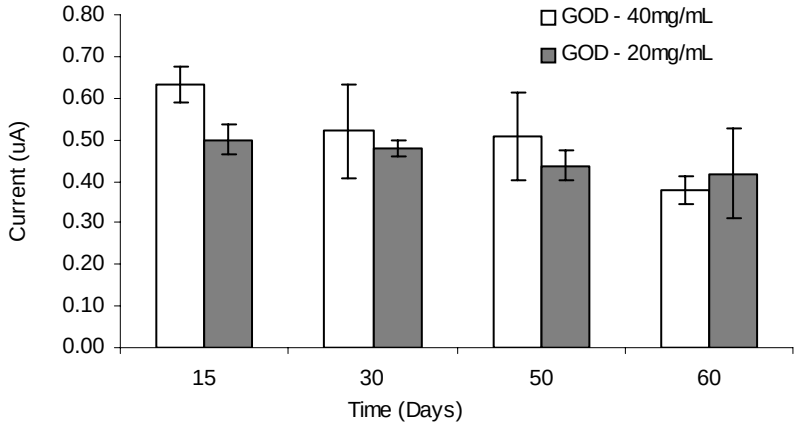


Figure 4.28 Stability of SGS-CLPVA/nafion membranes

As shown in Figure 4.28, the stability of membranes was quite good. This could be due to the excellent SGS-CLPVA/nafion matrix. CLPVA was applied as a solid support due to the ability to form very homogenous films with high quality. The presence of hydrophilic PVA and the relatively hydrophobic network of sol gel silica will modify the environment for ferrocene carboxylic acid retention. SGS was used to increase the encapsulation capacity for the enzyme and mediator. The presence of nafion, a negatively charged polymer, not only prevented the cracking of pure sol-gel derived silica film but also improved the sensitivity and stability of the enzyme/mediator membrane by minimizing the leaching of the mediator. The result is a consolidation of the effects of polymer, ionomer and sol gel network.

4.2.3.3 Multilayered Construction of Glucose Oxidase and Polyallylamine Ferrocene

1) Kinetics properties of the membranes

Figure 4.29 shows the typical current response towards 5mM glucose solution

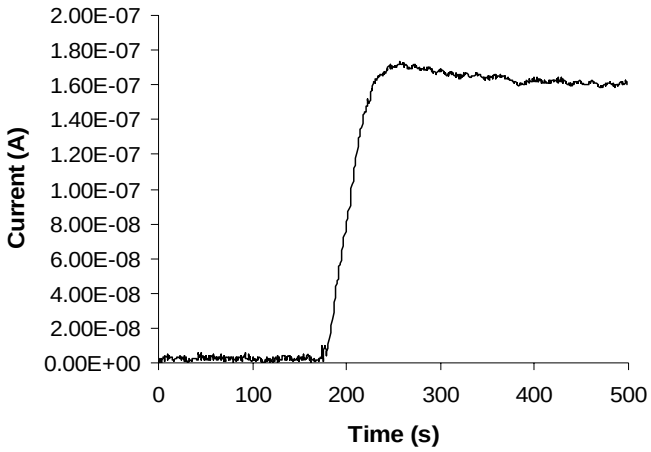


Figure 4.29 Typical current responses of multilayered membranes

As shown in Figure 4.29, the response time to arrive at 95% at the steady state current for multilayered membrane was approximately 80s. The thickness of multilayered membranes was 90 microns. Thus the distance between the electrode and the reaction center of the enzyme was small. As a result, the time required to reach 95% of the steady state current was relatively short. Figure 4.30 shows typical calibration curves for multilayered membrane for kinetics study.

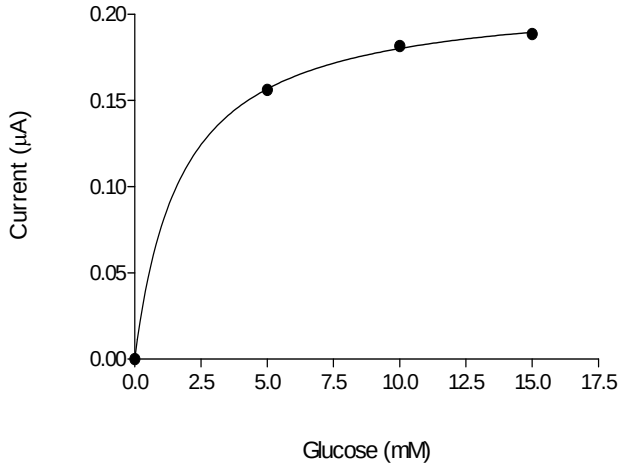


Figure 4.30 Typical calibration curves for multilayered membranes

The ferrocene functioned well as mediator, shuttling electrons between GOD and the platinum electrode. The current increased stepwise with addition of glucose. Michaelis-Menten kinetics were determined from steady state currents and the electrochemical version of the Lineweaver-Burk equation. Figure 4.31 shows the Lineweaver Burke plot for this membrane for enzyme kinetics study.

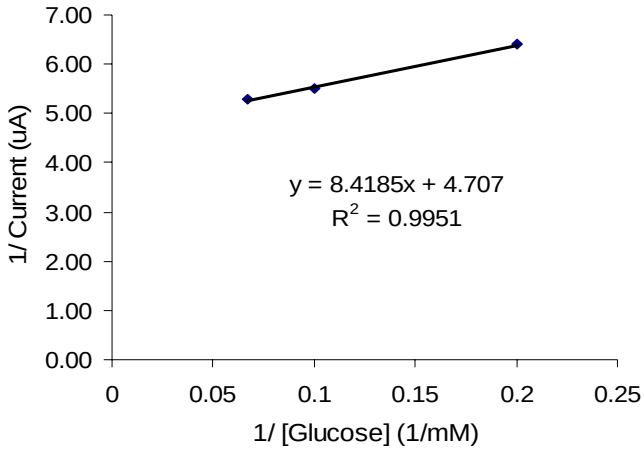


Figure 4.31 Double –reciprocal (Lineweaver Burke) plot of multilayered membranes

The corresponding maximum current, I_{max}, and apparent Michaelis-Menten constant, K_m^{app} for membranes were 0.21µA and 1.79mM respectively. The K_m^{app} obtained was very low. The high sensitivity and the small K_m^{app} mean that the immobilized glucose oxidase possessed a higher enzymatic activity. K_m^{app} is independent of enzyme concentration. The K_m^{app} value depends on the strength of the

bonds between enzyme and substrate. If these bonds are strong, the K_m^{app} will be low, indicating that the immobilized enzyme retained its bioactivity and possessed high biological affinity to glucose. The high degree of affinity of the enzyme to the substrate may be explained by a favorable change in the structural organization of the enzyme due to the immobilization procedure (Arica et al., 1995).

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Hydrogen Peroxide-Based Glucose Biosensor

For the selection of a suitable support material for the immobilization of glucose oxidase, four types of matrixes had been investigated. The selection was mainly based on the current response, enzyme leakage, long term stability, kinetic parameter, response time and sensitivity. Chemically cross-linked PVA-GOD membrane has shown the shortest leaking period, which indicated that the ability in retaining the enzyme within the matrix was high. The apparent Michaelis-Menten constant, K_m^{app}, shown was the highest too. However, its current response as well as sensitivity were very much lower compared to the others even when lysozyme was incorporated as a stabilizing agent. On the other hand, even though freeze-thawed PVA-GOD membrane leaked for a considerable longer period, approximately 2 weeks, it showed satisfactory current response. Besides, even though its K_m^{app} was low, it was still acceptable especially when combined with an appropriate external membrane. Both types of organic-inorganic sol-gel derived materials, tetramethoxysilane (TMOS), and alumina were brittle and PVA was added during membrane fabrication in order to increase the mechanical strength. The enzyme leakage was a problem with the mild immobilization condition and K_m^{app} was lower than freeze-thawed PVA membrane. However, both of them had shown good sensitivity and shorter response time as compared to PVA. In general, the

membranes were stable over a period of 60days. With an adequate level of sensitivity and K_m^{app}, freeze-thawed PVA-GOD membrane has been chosen as the support material for GOD immobilization. The enzyme leakage of this type of membrane has been overcome by reducing the enzyme loading. Moreover, the K_m^{app} can still be improved with a proper outer membrane.

To eliminate the interference suffered by hydrogen peroxide-based biosensor, an inner membrane fabricated from poly(hydroxyethyl methacrylate) (pHEMA) was studied to restrict the passage of acetaminophen, a model of an interfering molecule. This was done by controlling the mesh size of the resulted network. A pHEMA membrane with the cross-linking ratio of 0.043 was found to successfully obstruct the diffusion of acetaminophen at a selectivity of 10. Significant reduction of acetaminophen response was shown while maintaining reasonable diffusion of hydrogen peroxide.

5.2 Mediator-Based Glucose Biosensor

For the first method, immobilization of glucose oxidase and ferrocene redox polymer in CLPVA with the addition of BSA has been done. A membrane with greater BSA content gave higher current response with larger K_m^{app}. For both membranes, the large Km were obtained but with low current responses. However, retention of enzyme and mediator as well as the membrane stability were very poor. Therefore, further study must be done to improve the retention of enzyme and mediator as the CLPVA, which had been shown to be an excellent retainer of GOD (Abdul-Aziz, 2001) was not able to retain both GOD and ferrocene redox polymer effectively. This would ultimately influence the stability of the membranes.

For the second method, immobilization of glucose oxidase and ferrocene carboxylic acid in SGS-CLPVA/nafion was done. The immobilization technique resulted in an enzyme/mediator membrane that was simple to cast, resulted in

minimal mediator losses and very stable at lower operating potentials. A membrane with greater GOD concentration gave higher current response. However, K_m^{app} was independent of enzyme concentration. Furthermore, the low value of K_m^{app} might eventually decrease the detection limit of the biosensor. However, SGS-CLPVA/nafion is a good matrix for the immobilization of mediator as well as an enzyme. The co-operative effect from the hydroxyl groups of PVA and the sol–gel environment is hoped to sustain the rotational freedom for the enzyme molecules to adopt the active configuration typical under physiological conditions. The active matrix environment prolongs the life span of the enzyme to result in high sensitivity. The simultaneous presence of the sol–gel silica will greatly increase the selectivity and stability of the sensors.

Lastly, for the third method, multilayered construction of glucose oxidase and polyallylamine ferrocene has been done. Ferrocene was covalently bound to the pollyalylamine by aldehyde and amino groups. Layer-by-layer covalent attachment of periodate-oxidized glucose oxidase and poly(allylamine) ferrocene complex (PAA-Fc) did not allow the loss of ferrocene into solution. However, the current response and the Km were quite low. Thus the low value of K_m^{app} might eventually decrease the detection limit of the biosensor.

After comparing the three almost totally different approaches, the first method, which was the immobilization of glucose oxidase and ferrocene redox polymer in CLPVA with the addition of BSA was selected for the fabrication of disposable glucose biosensor, based on the Km value. Although the current response was low, the Km value obtained for this method was very high compare to the other method. By attaching the developed nafion external layer to the enzyme layer, this mediated glucose sensor will provide good responses over a wide range of concentration. However, implantable glucose biosensor could not be constructed using this method since the mediator and enzyme retention as well as the stability was not very good. But, the leaking was not a problem for disposable glucose sensor.

In the future, extensive study should be done on the external layer. Instead of using nafion, as in this study, many other polymer coatings can be use as an outer layer, such as polyurethane, polyvinylchloride, polycarbonates and cellulose acetate

coating solutions. By increasing the polymer coating solution concentrations, the linear range of the sensor response can be extended (Yang et al., 1998). Therefore, hopefully, with the new external layer, Km value for the second and the third method could be improved. It is important since those two methods display better membranes stability and mediator retention.

5.3 Recommendations

For hydrogen peroxide-based glucose biosensor, it is recommended that a more complete model should be investigated on each material including the optimization of each component's concentration in a membrane, such as the cross-linker and solvents used in membrane casting. Besides, a proper study on optimum enzyme loading for each type of membrane should be performed to overcome enzyme leakage problem. On the other hand, other types of outer membrane such as polycarbonate should be investigated in the effort to enhance the kinetic parameters of the immobilized enzyme.

One factor that can be studied in term of immobilization is diffusional limitation in immobilized enzyme systems. Diffusional resistances may be observed at different levels in immobilized enzyme. These resistances vary depending on the nature of the support material, hydrodynamical conditions surrounding the support material and the distribution of the enzyme inside or on the surface of the material. Whether diffusion resistance has a significant effect on the rate of enzymatic reaction depends on the relative rate of the reaction rate and diffusion rate, which is characterized by the Damkohler number (Da).

Da= maximun rate of reaction / maximun rate of diffusion

$$= V_m / k_L [S_b]$$

where k_L is the mass transfer coefficient (cm/s) and $[S_b]$ is the substrate concentration in bulk liquid (g/cm^3).

The rate of enzymatic conversion may be limited by diffusion of the substrate or reaction, depending on the value of the Damkohler number. If Da >> 1, the diffusion rate is limiting. For Da << 1, the reaction rate is limiting, and for Da ~ 1, the diffusion and reaction resistances are comparable. Diffusion and enzymatic reactions may be simultaneous, with the enzymes entrapped in a solid matrix, or may be two consecutive phenomena for adsorbed enzymes.

REFERENCES

Albery, W. J. and Cranston, D. H. (1987). In: Turner, A.P.F., Karube, I. and Wilson, G.S. eds. *Biosensors: Fundamentals and Applications*. Oxford: Oxford University Press. 180-210.

Arica, M. Y., Hasirci, V. and Aleeddonoglu, N. (1995). Covalent Immobilization of a-Amylase onto pHEMA Microsperes; Preparation and Application to Fixed Bed Reactor. *Biomaterials*. 15: 761-768.

Audebert, P., Demaille, C. and Sanchez, C. (1993). Electrochemical Probing of The Activity of GOD Embedded Sol Gel Matrixes. *Chemistry of Materials*. 5(7): 911- 913

Azila Abdul Aziz (2001). *Amperometric Glucose Biosensors: Systematic Material Selection and Qualitative Analysis of Performance.* The John Hopkins University, Baltimore, Maryland: Ph.D. Thesis.

Bartlett, P. N. (1987). The Use of Electrochemical Methods in The Study of Modified Electrodes. In: Turner, A.P.F., Karube, I. and Wilson, G.S. eds. *Biosensors: Fundamentals and Applications*. Oxford: Oxford University Press. 211-146.

Bartlett, P. N., Tebbutt, P. and Whitaker, R.C. (1991). *Prog. Reaction Kinetics.* 16: 55-155.

Boguslavsky, L., Kalash, H., Xu, Z., Beckles, D., Geng, L, Skotheim, J., Laurinavicius, V., Lee, H.S. (1995). *Anal. Chim. Acta.*311: 15.

Brahim, S., Narinesingh, D., and Guiseppi-Elie, A. (2002). Polypyrrole-hydrogel Composites for the Construction of Clinically Important Biosensors. *Biosensors and Bioelectronics*. 17: 53-59.

Brooks, S. L., Ashby, R. E., Turner, A. P. F., Calder, M. R. and Clarke, D. I. 1984. Development of an On-line Glucose Sensor for Fermentation Monitor. *Biosensors.* 3: 45-56

Cajlakovic, M., Lobnik, A. and Werner, T. (2001). Stability of New Optical pH Sensing Material Based on Crosslinked Poly(vinyl alcohol) Coploymer. *Analytica Chimica Acta*. 207-213.

Calvo, E. J., Danilowicz, C. and Diaz, L. (1994). *J. Electroanal. Chem.* 369: 279-282;

Calvo. E.J. and Danilowicz C. (1997). Amperometric Enzyme Electrodes. *Journal of The Brazilian Chemical Society.* 6: 563-574.

Calvo, E. J., Etchenique, R., Pietrasantha, L. and Wolosiuk, A. (2001). Layer by layer Self Assembly of Glucose Oxidase and $Os(Bpy)_2ClPyCH_2NH$-polyallylamine Bioelectrode. *Anal. Chem.* 73: 1161-1168.

Chaplin, M. (1990). Enzyme Technology. United Kingdom: Cambrigde University Press.

Chen, X. H., Hu, Y. B., and Wilson G. S. (2002). Glucose Microbiosensor Based on Alumina Sol-gel Matrix/Electropolymerized Composite Membrane. *Biosensors & Bioelectronics*. 17: 1005-1013.

Clark, L.C., Lyons, C. (1962). Electrode Systems for Continuous Monitoring in Cardiovascular Surgery. *Ann. New York Acad. Sci*. 102: 29–45.

Degani, Y. and Heller, A. (1987). *J. Phys. Chem. 91*: 1285-1289.

Doretti, L., Ferrara, D., Gattolin, P., and Lora, S. (1997). Amperometric Biosensor with Physically Immobilized Glucose Oxidase on a PVA Cryogel Membrane. *Talanta*. 44: 859-866.

Doretti, L., Ferrara, D., Gattolin, P., and Lora, S., Schiavon, F. and Veronese, F. M. (1998). PEG-Modified Glucose Oxidase Immobilized on a PVA Cryogel Membrane for Amperometric Biosensor Applications. *Talanta*. 45: 891-898.

Eggins, B. R. (1996). *Biosensors: An Introduction*. England: John Wiley & Sons Ltd and B.G. Teubner.

Griesmar, P., Ponton, A., Serfaty, S., Senouci, B., Gindre, M., Gouedard, G., Warlus, S. (2003). Kinetic study of silicon alkoxides gelation by acoustic and rheology investigations. *Journal of Non-Crystalline Solids.* 319: 57–64.

Gudeman, L.F., and Peppas N.A. (1995). *Journal of Membrane Science*. 107: 239.

Hartmeier, W. (1986). *Immobilized Biocatalyst, An Introduction*. New York: Springer-Verlag Berlin Heidelberg. 3-68.

Henning, T. P. and Cunningham, D. D. (1998). Biosensor for Personal Diabetes Management. In: Ramsay, G. ed. *Commercial Biosensors: Application to Clinical, Bioprocess and Environment Sample.* Canada: John Wiley & Sons. 5.

Hickey, A. S. and Peppas N. A. (1995). Mesh Size and Diffusive Characteristics of Semicrystalline Poly(vinyl alcohol) Membranes Prepared by Freezing/Thawing Techniques. *Journal of Membrane Science*. 107: 229-237.

Hodak, J., Etchenique, R., Calvo, E.J., Singhal, K., Bartlett, P.N. (1997). Layer-by-layer Self-assembly of Glucose Oxidase with a Poly(allylamine)ferrocene Redox Mediator. *Langmuir.* 13: 2708–2716.

Horner R.D. (1997). *Biochemistry Laboratory Workbook*. Dept. of Biology, Johns Hopkins University. Unpublished.

Howells, A. R., Zambrano, P. J,.and Collison, M. M. (2000). Diffusion of Redox Probes in Hydrated Sol-Gel-Derived Glasses. *Analytical Chemistry*. 72: 5265-5271.

Hsiue G. H., Guu, J. A., Cheng, C. C. (2001). Poly(2-hydroxyethyl methacrylate) film as a drug delivery system for pilocarpine. *Biomaterials*. 22: 1763-1769.

Johnson, T. J. .A. (1993). Glutaraldehyde Cross-Linking: Fast and Slow Modes. Forth Chemical Congress of North America. New York: American Chemical Society. 283-295

Kase, Y. and Muguruma, H. (2004). Amperometric Glucose Biosensor Based on Mediated Electron Transfer between Immobilized Glucose Oxidase and Plasma-polymerized Thin Film of Dimethylaminomethylferrocene on Sputtered Gold Electrode. *Analytical Sciences*. 20: 1143-1146.

Katakis, I. and Heller, A. (1992). *Anal. Chem.* 64: 1008-1013.

Kermis, H.R., Govind Rao, Timothy A. Barbari (2003). Transport Properties of PHEMA Membranes for Optical Glucose Affinity Sensors. *Journal of Membrane Science*. 212: 75-86.

Koide, S., and Yokoyama, K. (1999). Electrochemical Characterization of an Enzyme Electrode Based on a Ferrocene Containing Redox Polymer. *Journal of Electroanalytical Chemistry*. 468: 193-201.

Kudela (1976). V. Hydrogels. In: Jacqueline IK, eds (1976). *Encyclopedia of Polymer Science and Engineering*. Wiley Interscience. 783-807.

Kumar, H. V., and Chaudhari. A. Proteins Immobilized at the Galleries of Layered α-Zirconium Phosphate: Structure and Activity Studies. University of Connecticut.

Lee, Y. H, The, H. F., Loh, H. C., and Musa, A. (2003). A Hydrogen Ion-Selective Sensor Based on Non-plasticised Methacrylic-acrylic Membranes. *Sensors.* 2: 339-346.

Lillis, B., Grogan, C., Berney, H., and Lane, W.A. (2000). "Investigation into Immobilization of Lactate Oxidase to Improve Stability." *Sensors and Actuators B*. 68: 109-114.

Liu, Y., Zhang X., Liu, H. and Deng, J. (1996). Immobilization of Glucose Oxidase onto the Blend Membrane of Poly(vinyl alcohol) and Regenerated Silk Fibroin: Morphology and Application to Glucose Biosensor. *Journal of Biotechnology.* 46: 131-138.

Liu, Z. J., Liu, B. H., Zhang, M., Kong, J. L., and Deng, J. Q. (1999). Al_2O_3 Sol-gel Derived Amperometric Biosensor for Glucose. *Analytica Chimica Acta*. 392: 135-141.

Lyons, M. E. G. (1991). Electrochemistry-developing Technologies and Application. *Annu. Rep. Prog. Chem. Sect. C.* 88: 135.

Miller, V. G. (2003). Diabetes Mellitus: Care & Control-A review and current update.

Newman, J. D., Turner, A. P. .F. (2005). Home Blood Glucose Biosensors: A Commercial Perspective. *Biosensors and Bioelectronics*. 20: 2435-2453.

Niu, J. and Lee, J. L. (2002). Reagentless Mediated Biosensors Based on Polyelectrolyte and Sol-gel Derived Silica Matrix. *Sensors and Actuaturs B.* 82: 250-258.

Peppas NA, Moynihan HJ, Lucht LM (1985). The Structure of Highly Crosslinked Poly(2-hydroxyethyl methacrylate) Hydrogels. *J Biomed Mater Res*. 19: 397-411.

Poyard, S., Jaffrezic-Renault, N., Martelet, C., Cosnier, S., and Labbe, P. (1998). Optimization of Inorganic/Bio-organic Matrix for the Development of New Glucose Biosensor Membranes. *Analytica Chimica Acta*. 364: 165-172.

Reynolds, E. R., Geise, J. R. and Yacynych, A. M. (1992). In : *Biosensor & Chemical Sensors : Optimization Performance Through Polymeric Material*. Edelman, P. G and Joseph Wang. USA: Library of Congress. 106.

Rondeau, A., Larson, N., Boujtita, M., Gorton, L. and Murr, N. E. (1999). The Synergetic Effect of Redox Mediators and Peroxidase in Bienzymatic Biosensor for Glucose Assays in FIA. *Analusis*. 27:649-656.

Saburo Fukui, and Atfuo Tanaka (1990). Immobilization. In: Biocatalyst Design. New York: VCH Publisher. 63-74.

Sampath, S., and Lev, O. (1996). Inert Metal-modified, Composite Ceramic-carbon, Amperometric Biosensors: Renewable, Controlled Reactive Layer. *Analytical Chemistry*. 68:2015-2021.

Sapsford, K. E., and Ligler, F. S. (2004). Real-time Analysis of Protein Adsorption to a Variety of Thin Films. *Biosensors and Bioelectronics.* 19: 1045–1055.

Sato, N. and Okuma, H. (2006). Amperometric Simultaneous Sensing System for D-glucose and L-lactate Based On Enzyme-modified Bilayer Electrodes. *Analytica Chimica Acta*. 565: 250-254.

Seidel, J. M., and Malmonge, S. M. (2000). Synthesis of PolyHEMA for using as Biomaterials. Bulk and Solution Radical-initiated Polymerization Techniques. *Materials Research*. 3: 79-83.

Shao, Z. G., Wang, X. and Hsing, I. M. (2002). Composite Nafio/Poly (vinyl Alcohol) Membranes for the Direct Methanol Fuel Cell. *Journal of Membrane Science*. 210: 147-153.

Shuler, M. L. and Kargi, F. (2002). *Bioprocess Engineering Basic Concepts.* 2nd ed. USA: Prentice Hall.

Sirat, D. M., Velhot, G., and Reach, G. (1992). *Biosensors and Bioelectronics*. 7: 345.

Taylor, R. F. (1991). Introduction: The Current Status of Immobilized Protein Technology. In: *Protein Immobilization*. Volume 14. New York: Marcel Dekker, Inc. 1-9.

Tian, F.M. and Zhu, G.Y. (2002). Sol-gel Derived Iridium Composite Glucose Biosensor. *Sensors and Actuators B*. 86: 266-270.

Updike, S. J. and Hicks, G. P. (1967). The Enzyme Electrode. *Nature*. 214: 968-988.

Von Worthington ed. (1972). *Worthington Enzyme Manual: Enzymes, Enzyme Reagents, and Related Biochemicals*. Freehold, N.J.: Worthington Biochemical Corporation.

Wang, J., and Pamidi, P.V.A. (1997). Sol-gel Derived Metal Dispersed Carbon Composite Amperometric Biosensors. *Electroanalysis*. 9: 52-55.

Wang, J., Rivas, D., and Chicharro M. (1997). Glucose Microsensor Based on Electrochemical Deposition of Iridium and Glucose Oxidase onto Carbon Fiber Electrodes. *Journal of Electroanalytical Chemistry*. 439: 55-61.

Wei, Q., Wang, D. W., Zhang, S. G., and Chen, C. S. (2001). Preparation and Characterization of Sol-gel Derived Unsupported Al_2O_3-SiO_2 Composite Membrane. *Journal of Alloys and Compounds*. 325: 223-229.

Wichterle O, and Lim D (1960). Hydrophilic Gels for Biological Use. *Nature*. 185: 117.

Wilkins, E. and Atanasov, P. (1996). Glucose Monitoring: State of the Art and Future Possibilities. *Med. Eng. P/y*. 18:.273-288.

Wilson, R. and Turner, A. P. F. (1992). Glucose Oxidase – and Ideal Enzyme. *Biosensor and Bioelectronics*. 7:162-185.

Wolfbeis, O. S., Ines Oehme, Natalya Papkovskaya 1, Ingo Klimant (2000). Sol–gel Based Glucose Biosensors Employing Optical Oxygen Transducers, and a Method for Compensating for Variable Oxygen Background. *Biosensors & Bioelectronics.* 15: 69–76.

Wu, J., Jan Suls, and Willy Sansen (1999). Amperometric Glucose Sensor with Enzyme Covalently Immobilized by Sol-Gel Technology. *Analytical Sciences*. 15: 1029-1032.

Yoldas, B. E. (1975). Alumina Sol Preparation from Alkoxides. *Ceramic Bulletin* 54: 289-290.

Yoon, H. C. and Kim, H. S. (2000a). Multilayered Assembly of Dendrimers With Enzymes on Gold: Thickness-Controlled Biosensing Interface. *Anal. Chem*. 72:922-826.

Yoon, H. C., Hong, M. Y. and Kim, H.S. (2000b). Functionalization of a Polyamidoamine Dendrimer with Ferrocenyls and its Application to the Construction of a Reagentless Enzyme Electrode. *Anal. Chem*. 72: 4420-4427

Zaborsky, O. R. and Ogletree, J. (1974). The Immobilization of Glucose Oxidase via Activation of its Carbohydtrate Residues. *Biochem. Biophys. Res. Commun.* 61: 210-216.

Zhang, S., Yang, W., Niu, Y. and Sun, Y. (2004). Multilayered Construction of Glucose Oxidase and Poly(allylamine)ferrocene on Gold Electrodes by Means of Layer-by-layer Covalent Attachment. *Sensors and Actuaturs B*. 101: 387-393.