ENZYME IMMOBILIZATION AND PERMSELECTIVITY ANALYSIS OF AN INTERFERENCE FREE PEROXIDE BASED GLUCOSE BIOSENSOR

WONG FUI LING

UNIVERSITI TEKNOLOGI MALAYSIA

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ABSTRACT

The performance of a biosensor sensing layer is mostly determined by the immobilization method and the materials used to immobilize the enzyme. In this work, the performances of four types of glucose oxidase immobilization materials based on poly(vinyl alcohol) (PVA) for peroxide-based glucose biosensor were compared. The matrices of interest were glutaraldehyde cross-linked PVA (GAPVA), freeze-thawed PVA cryogel (FTPVA), tetramethoxysilane sol-gel-PVA hybrid material (TMOS-PVA), and alumina sol-gel-PVA hybrid material (Al-PVA). Only GAPVA showed short period of enzyme leaking and high value of K_m^{app}. However, its sensitivity was poor. With the same enzyme loading, the other three types of membranes showed good sensitivity and stability. FTPVA and TMOS-PVA, which showed satisfactory sensitivity and adequate value of K_m^{app} were quite promising as the support materials for immobilizing glucose oxidase (GOD). The enzyme leakage of FTPVA which had shown highest leaking was improved by reducing the enzyme loading. Even though peroxide-based sensor is very simple and easy to construct, it suffers from electrochemical interferences from common electroactive species present in blood such as acetaminophen. Hence, a photocured poly(hydroxyethyl methacrylate) (pHEMA) layer was investigated as a potential permselective inner membrane to eliminate the interferences based on size exclusion. PHEMA membrane with the cross-linking ratio of 0.043 was found to be able to achieve a selectivity of 10, while maintaining an acceptable degree of hydrogen peroxide response. In a two-layer glucose biosensor, where the FTPVA-GOD and cross-linked pHEMA were combined, acetaminophen and ascorbic acid at 0.2 mM were eliminated completely. 0.2 mM uric acid still gave a bias of approximately 6.6% relative to 5 mM glucose. Overall, the partial glucose biosensor showed promising performance.

ABSTRAK

Prestasi sesuatu penderia glukosa banyak bergantung kepada cara penyekatgerakan enzim dan jenis bahan sokongan yang digunakan. Dalam kerja ini, prestasi empat jenis bahan penyekatgerak glukosa oksida (GOD) berdasarkan poli(vinil alkohol) (PVA) untuk penderia glukosa berasaskan hidrogen peroksida telah dibanding. Matriks yang dikaji adalah poli(vinil alkohol) (PVA) disambung-silang dengan glutaraldehid (GAPVA), kryogel beku-cair PVA (FTPVA), bahan hibrid sol-gel (tetrametoksi)silane-PVA (TMOS-PVA), dan bahan hibrid sol-gel alumina-PVA (Al-PVA). Hanya GAPVA menunjukkan jangka masa kebocoran enzim yang pendek dan nilai K_m^{app} yang tinggi. Walau bagaimanapun, kepekaannya adalah rendah. Dengan kuantiti enzim yang sama, tiga jenis membran yang lain menunjukkan kepekaan dan kestabilan yang baik. FTPVA dan TMOS-PVA yang menunjukkan kepekaan yang memuaskan dan nilai K_m^{app} yang memadai sesuai untuk dijadikan bahan penyekatgerak GOD. Kebocoran enzim bagi membran FTPVA telah diperbaiki dengan mengurangkan kuantiti enzim yang ditambahkan ke dalam proses penyekatgerakan. Walaupun penderia yang berasaskan hidrogen 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 poli(hidroksietil metakrilat) telah dikaji untuk dijadikan membran dalaman berasaskan ketertelapan memilih bagi menyingkirkan gangguan melalui penyisihan saiz. PHEMA dengan nisbah sambung-silang 0.043 yang menunjukkan selektiviti 10 berjaya menyingkirkan gangguan asetaminofen, sementara mengekalkan gerak balas terhadap hidrogen peroksida yang agak munasabah. Dalam penderia glukosa dua-lapisan yang menggabungkan FTPVA-GOD dan pHEMA yang disambung-silang, 0.2 mM asetaminofen dan asid askorbik telah disingkirkan dengan lengkap. 0.2 mM asid urik masih menghasilkan lebih kurang 6.6% ralat arus berbanding dengan 5 mM glukosa. Secara keseluruhan, penderia glukosa separa tersebut menunjukkan prestasi yang memuaskan.

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LIST OF SYMBOLS/ABBREVIATIONS

3-GPDES	-	(3-Glycidoxypropyldiethoxy)silane
A	-	Electrode surface area (cm^2)
AA	-	Ascorbic acid
AC	-	Acetaminophen
Ag/AgCl	-	Argentum/Argentum chloride
Al	-	Aluminium
B	-	Boron
BSA	-	Bovine serum albumin
C^*_{dl}	-	Diffusion layer interfacial concentration
C^*_i	-	Concentration at the interface
C^*_m	-	Membrane interfacial concentration
C_b	-	Bulk concentration (mM)
CE	-	Counter electrode
CR	-	Cross-linking ratio
DCCT	-	Diabetes Control and Complications Trial
d_{dl}	-	Diffusion layer thickness
D_i	-	Diffusion coefficient ($cm^2\ s^{-1}$)
d_i	-	Layer thickness
d_m	-	Membrane thickness (cm)
DMPP	-	2,2-dimethoxy-2-phenylacetophenone
EGDMA	-	Ethylene glycol dimethacrylate
F	-	Faraday constant (96487 A s mol^{-1})
FTPVA	-	Freeze-thawed PVA
FW	-	Formula Molecular Weight (g $mole^{-1}$)
GA	-	Glutaraldehyde

GAPVA	-	Glutaraldehyde cross-linked PVA
GDH	-	Glucose dehydrogenase
GOD	-	Glucose oxidase
H	-	Water content (%)
H_2O_2	-	Hydrogen peroxide
HCl	-	Hydrochloric acid
HEMA	-	Hydroxyethyl methacrylate
HRP	-	Horseradish peroxidase
i	-	Steady-state current (μA)
IDDM	-	Insulin-dependent diabetes mellitus
I_{max}	-	Maximum current measured under the conditions of enzyme
J_i	-	Flux (mol m^{-2} s^{-1})
KCl	-	Potassium chloride
K_m^{app}	-	Apparent Michelis constant (mM)
LSV	-	Linear sweep voltammetry
m	-	Mass
MW	-	Molecular weight
n	-	Freezing and thawing cycle
n	-	Number of electron involved in the electrode reaction
NADH	-	Reduced nicotinamide adenine dinucleotide
NIDDM	-	Non-insulin-dependent diabetes mellitus
P	-	Permeability
P_{ac}	-	Permeability of acetaminophen (cm^2/s)
PBSA	-	Protein based stabilizing agents
PEG	-	Polyethylene glycol
PEI	-	Polyethyleneimine
PEO	-	Poly(ethylene oxide)
PHEMA	-	Poly(hydroxyethyl methacrylate)
P_{hp}	-	Permeability of hydrogen peroxide (cm^2/s)
PPO	-	Polyphenol oxidase
PQQ	-	Pyrroloquinoline quinine
PSS	-	Poly(styrene sulfonate)
PVA	-	Poly(vinyl alcohol)
PVP	-	Polyvinylpyrrolidone

PVS	-	Poly(vinyl sulfate)
R	-	Molar ratio of water: alkoxide
RDE	-	Rotating disk electrode
RE	-	Reference electrode
S	-	Substrate concentration (mM)
Si	-	Silica
TEOS	-	Tetraethoxysilane
Ti	-	Titanium
TMOS	-	Tetramethoxysilane
TTF-TCNQ	-	Tetrathiafulvalinium tetracyanoquinodimethanide
UA	-	Uric acid
V	-	Vanadium
V_{max}	-	Maximum reaction rate
W_d	-	Dehydrated weight of the membrane
WE	-	Working electrode
WHO	-	World Health Organization
W_w	-	Fully hydrated weight of the membrane
Zr	-	Zirconium
α	-	Partition coefficient
ω	-	Rotating rate of RDE (rps)
σ	-	Selectivity
υ	-	Viscosity of bulk solution ($cm^2 s^{-1}$)

CHAPTER 1

INTRODUCTION

1.1 The Need for Glucose Biosensor

Diabetes mellitus is a group of metabolic diseases that resulted from failure in the regulation of blood glucose levels due to defects in insulin production, or action, or both. The disorder in insulin secretion or reduced function can cause hyperglycemia (elevated levels of blood glucose) and hypoglycemia (low levels of blood glucose). Compared to normal blood glucose levels which range from 3.5 to 6.5 mM, a diabetic's blood sugar range can vary between 1 to 30 mM (Miller, 2003).

According to the World Health Organization (WHO) Report 2006, nearly 2.9 million deaths per year are as a result of diabetes. It is estimated that more than 180 million people from all over the world suffer from diabetes. WHO predicts more than 50% increment of diabetes deaths in the next 10 years. In general, diabetes mellitus can be classified into 3 categories. Type I diabetes, also called insulin-dependent diabetes mellitus (IDDM), normally occurs in young individuals. The sufferers are not able to produce insulin because of the auto-immune destruction of beta cells, which are responsible in insulin secretion. Type II diabetes made up of 90% of diabetics worldwide and commonly affects middle-aged or older patients.

Also referred as non-insulin-dependent diabetes mellitus (NIDDM), this type of patients do not produce insulin according to the body needs due to the lack of sensitivity of the body to insulin. Another group of diabetes is gestational diabetes that occurs during pregnancy as a result of hormonal changes. Women who have had gestational diabetes stand 20-50% chance of developing diabetes in later life (Miller, 2003; Newman and Turner, 2005; WHO, 2006).

Diabetes is long-standing and untreatable. The long term complications arisen from diabetes include damage of the heart, blood vessels, eyes, kidneys, and nerves. It is widely accepted that proper control of blood glucose levels within the normal range has direct effect in delaying and preventing diabetes complications (American Diabetes Association, 2005). The findings of Diabetes Control and Complications Trial (DCCT) clearly show that intensive control of blood glucose levels in diabetics was the key element to decrease eye complications by 76%, kidney complications by 50%, nerve complications by 60%, and to lower the risk of developing high cholesterol and stroke. To achieve optimal glucose control without undue risk, it is commonly agreed that diabetes mellitus patients must monitor their blood glucose four or more times a day to adjust insulin dosage, diet and physical activity. Home blood glucose testing thus becomes an important aspect in the tight control of blood glucose levels.

Recently, different types of biosensor technologies and products have emerged in the market. As diabetes is prevalent worldwide, glucose biosensor comprises about 85% of the biosensors market, which is equivalent to approximately $5 billion (Newman *et al.*, 2004). Recently, significant market growth has been observed in developing nations relative to United State and these countries have appeared to be the key market for glucose monitoring devices (Newman and Turner, 2005).

1.2 Research Background

Since urine glucose test has been found to be ineffective, the diabetic self-care technology has been taken over by blood glucose testing devices (Miller, 2003). Clark and Lyons have reported the first prototype biosensor in 1962, which was an enzyme electrode. They immobilized glucose oxidase (GOD) on an oxygen-sensing electrode to measure the concentration of glucose in solution. This prototype enzyme electrode later has brought about the development of the first commercialized enzyme electrode and glucose analyzer by Yellow Springs Instrument Company (YSI) in the seventies (Taylor, 1991). Since then, the search for an ideal biosensor with a suitable membrane that results in an enzymatic electrode that is reproducible, sensitive, accurate, and simple continues to be the goal in glucose detection.

Enzymes for glucose detection contain redox groups that change redox state during the biochemical reaction. The most familiar enzyme is glucose oxidase (GOD) that oxidizes β-D-glucose as shown in equation (1.1).

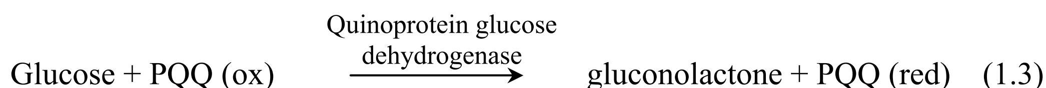
$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{D-Gluconic acid} + H_2O_2 \qquad (1.1)$$

GOD is reduced in the process by accepting electrons and returned to their active oxidized state by transferring these electrons to molecular oxygen that is dissolved in the surrounding fluid, resulting in the production of hydrogen peroxide (H_2O_2). Glucose dehydrogenase (GDH) can also oxidize glucose with NAD^+ as cofactor and NADH is produced.

$$\text{Glucose} + NAD^+ \xrightarrow{\text{Glucose dehydrogenase}} \text{gluconolactone} + NADH \qquad (1.2)$$

This process is oxygen independent, but the cofactor is expensive and relatively unstable. The third group is quinoprotein glucose dehydrogenase, which relies on

pyrroloquinoline quinine (PQQ) as cofactor. This system has rapid electron transfer rate but is relatively costly (Newman and Turner, 2005).



The majority of works on glucose measurements use GOD for substrate conversion. Although it is oxygen dependent, it is highly selective towards glucose in the concentration range corresponding to human blood, inexpensive, and robust. It is able to withstand greater extremes of environment such as pH, ionic strength, and temperature. This allows less stringent conditions during the manufacturing process and also relatively care-free storage and use by the home-user of the biosensor.

One of the major issues in biosensor research is the enzyme immobilization techniques (Chen and Lin, 1994; Masaro *et al.*, 1998). Due to the instability of biomolecule, the choice of immobilization material is crucial for the operational stability and long-term use of biosensor. It is important that the immobilization method can retain a high percentage of the enzymes while preserving as much catalytic activity as possible (Everse *et al.*, 1979). Numerous works have been done using various methods, mainly physical and chemical immobilization of enzymes. It is quite difficult to create a fine and uniform mesh so as to prevent the immobilized enzymes from leaking out of the matrices while on the other hand, impose low mass transfer resistances for both the substrate and the product. Different immobilization methods impose different limitations for optimum preservation of enzyme activity.

The development of biosensor has relied on biomolecule immobilization and stabilization techniques, and the refinement and miniaturization of transducers (Calvo and Danilowicz, 1997). The immobilized biological molecule has to be integrated with a transducer to convert a biochemical reaction into a measurable signal that is proportional to analyte concentration. Over the years, electrochemical and optical transducers have been intensively applied to convert the biochemical reaction into a readable signal. Some other techniques which have been developed

are piezoelectric crystals, quartz crystal microbalance, acoustic wave, and thermistor. Owing to their sensitivity and possibility of miniaturization, electrochemical approaches have found remarkable applications in this research field. Such devices can be divided into conductometry, potentiometry, and amperometry. In conductometric transducers the biological recognition event is converted into conductance. Potentiometric devices monitor potential associated with biochemical reaction at zero current. Amperometric transducers that operate at a constant potential measure the current related to the redox process involved in the biorecognition process. Among all, amperometric glucose biosensors have dominated the market of biosensors due to their high sensitivity, excellent selectivity, simplicity, low cost and rapid response (Wang, 1999a).

First generation amperometric glucose biosensors are based on glucose biosensors that use molecular oxygen as electron acceptor. Glucose concentration can be evaluated by following pH change due to gluconic acid production, oxygen depletion, or the formation of hydrogen peroxide. The later two approaches have received the most interests. The oxygen-based sensor measures the difference in oxygen concentration of the ambient oxygen level and the resulted oxygen level along with the enzymatic reaction as a linear function of glucose concentration. For this type of sensors, the advantage is that cathodic potential is required for oxygen reduction, which is -0.6 to -0.9 V versus Ag/AgCl, thus resulting in only minor interference. However, a differential setup is necessary to monitor the ambient oxygen and hence making the sensor construction complex and the miniaturization difficult (Atanasov and Wilkins, 1994). On the other hand, the peroxide-based sensors oxidize hydrogen peroxide at the electrode surface.

$$H_2O_2 \xrightarrow{\text{700 mV vs Ag/AgCl}} O_2 + 2H^+ + 2e^- \qquad (1.4)$$

The most important advantage of hydrogen peroxide electrode-based sensors is their ease of fabrication and the possibility of miniaturization with simple technology (Wilkins and Atanasov, 1996). However, the major drawback is that it is susceptible to electrochemical interferences by oxidable species exist in the blood at

the required oxidizing potential. Different approaches have been investigated to minimize the interference effects. A selective layer is often placed between the enzymatic active layer and the electrode to filter out interfering species either by surface charge or pore size. Another approach is by lowering the oxidation potential by using a mediator to replace oxygen to shuttle electrons in the redox process between the enzyme and the electrode.

This has led to the development of the second generation biosensors. The key research was performed in the early 80's by Cass *et al*. (1984) in collaboration with Cranfield Institute of Technology and the University of Oxford. The most common mediators included ferrocene and its derivatives, tetrathiafulvalene, methylene blue, ruthenium compounds, and quinines (Eggins, 1996). However, the major downside of the mediator-based glucose sensors is that most mediators have short operational lifetime thus limiting long-term-use for *in vivo* applications. Besides, the leaching of inherently toxic mediator remains an issue for implantable sensors (Wilkins and Atanasov, 1996). Carbon electrode dispersed rhodium, ruthenium and iridium particles were also found to be useful in measuring hydrogen peroxide at low overvoltage potential (Crumbliss *et al.*, 1986; Wang *et al*., 1993; Sampath and Lev, 1997).

The third generation biosensors, omitting any co-substrate and mediator, directly oxidize or reduce the enzyme on the electrode surface. The design is simple and involves direct electron transfer from the electrode and enzyme active sites. In this approach, sufficiently close contact between the redox centre and the electrode is required to allow rapid electron transfer (Eggins, 1996). Conducting organic salts such as tetrathiafulvalinium tetracyanoquinodimethanide (TTF-TCNQ) had been used as electrode materials (Albery *et al*., 1987).

In this work, hydrogen peroxide-based amperometric glucose biosensor, which has simple configuration, was studied from the aspects of enzyme immobilization and interference elimination. Both physical and chemical

immobilization methods were investigated to determine the most appropriate support material for GOD immobilization. In addition, the characteristics of a permselective layer were studied to develop an interference-free hydrogen peroxide-based glucose biosensor.

1.3 Project Objective

The objective of this study was to systematically select materials for an interference-free peroxide-based glucose biosensor.

1.4 Project Scopes

(i) Develop a suitable support material for glucose oxidase immobilization.

(ii) Develop a permselective layer for interference elimination.

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