Jurnal Teknologi, 52 Mei 2010: 181–192
© Universiti Teknologi Malaysia

IMMOBILIZATION OF GLUCOSE OXIDASE IN FREEZE –THAWED PVA AND PVA/TMOS MEMBRANES

AZILA ABDUL-AZIZ[1], NORHANA JUSOH[2], MOHD. AZIZUL ASHARI[3] & NORHASLIZA HASSAN[4]

Abstract. In this work, two methods of glucose oxidase (GOD) immobilization for peroxide based glucose biosensor had been investigated. For the first immobilization method, GOD was immobilized in freeze-thawed poly (vinyl alcohol) (PVA) at three different PVA concentrations (5%, 10% and 15% PVA). The higher the PVA concentration used for immobilization, the better the retention of the enzyme in the membrane. Nevertheless, higher PVA concentration did not necessarily correlate well with enzyme activity. Freeze-thawed PVA-GOD membranes with 10% PVA showed the highest sensitivity to glucose. The performances of freeze-thawed PVA-GOD membranes with 15% and 5% PVA was comparable. For the second immobilization method, GOD was immobilized within PVA/tetramethoxysilane (TMOS) matrix with different (3-glycidoxypropyldimethylethoxy)silane (3GPDES) concentrations. For the membranes prepared with 1:1 (TMOS: 3GPDES) volumetric ratio, the percentage of enzyme activity remaining at day 40 was about 51%. Meanwhile, membrane prepared with 1:2 (TMOS: 3GPDES) and 1:3 (TMOS: 3GPDES) were quite stable with 69% and 58% of enzyme activity remaining at day 40, respectively. Overall, the glucose biosensor showed very promising performances.

Keywords: Biosensor; peroxide; temperature; concentration

Abstrak. Dalam projek ini, dua kaedah penyekat-gerakan enzim glukosa oksidase (GOD) bagi biosensor glukosa amperometrik berdasarkan peroksida telah dikaji. Bagi kaedah penyekat-gerakan yang pertama, glukosa oksidase telah disekat-gerak dalam polivinilalkohol (PVA) dibeku-cair pada tiga kepekatan PVA yang berbeza (5%, 10% dan 15% PVA). Kepekatan PVA yang lebih tinggi mampu menyekat-gerak enzim di dalam membran dengan lebih baik. Namun, kepekatan PVA yang lebih tinggi tidak semestinya mempunyai korelasi yang baik dengan aktiviti enzim. Membran PVA-GOD dibeku-cair dengan 10% PVA menunjukkan sensitiviti yang paling baik kepada glukosa. Prestasi membran PVA-GOD dibeku-cair dengan 15% PVA dan 5% PVA adalah hampir sama. Bagi kaedah penyekat-gerakan yang kedua, GOD disekat-gerak dalam PVA/tetrametoksisilan (TMOS) pada kepekatan (3-glisidoksipropildimetiletoksi)silan yang berbeza. Bagi membran yang disediakan menggunakan 1:1 (TMOS: 3GPDES), peratusan enzim aktiviti yang tinggal selepas hari ke 40 ialah lebih kurang 51%. Membran yang disediakan menggunakan 1:2 (TMOS: 3GPDES) dan 1:3 (TMOS: 3GPDES) adalah agak stabil dengan masing-masing sebanyak 69% dan 58% peratusan enzim aktiviti yang tinggal selepas hari ke 40. Keseluruhannya, biosensor glukosa telah menunjukkan prestasi yang memberangsangkan.

Kata kunci: Biosensor; glukosa; peroksida; kepekatan

[1–4] Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia
Tel: +60-7-5535525. Fax: +60-7-5581463. Email: azila@fkkksa.utm.my

1.0 INTRODUCTION

Over the years, a wide range of methods for measuring blood glucose concentration has been studied. Basic principles of glucose sensing are according to the following reaction:

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-gluconic acid} \quad (1)$$

Glucose concentration can be measured by electrochemically following the decrease in oxygen concentration as the reaction proceeds [1–2], the production of hydrogen peroxide [3–5] or the change in pH with the production of D-gluconic acid electrochemically [6]. To date, the most widely researched methods of detection are the monitoring of oxygen or hydrogen peroxide at an electrode.

The hydrogen peroxide-based enzyme glucose sensor [3–5] has found wide application in the development of a glucose biosensor, especially as an implantable biosensor, due to its simple sensor configuration that facilitates ease of miniaturization. Unlike oxygen, hydrogen peroxide is not present in the sample to be analyzed, so no differential set-up is needed. However, it suffers the interference problem from analytes such as urate and ascorbate and some common drugs such as acetaminophen. These species are electro-active at the detection potential of hydrogen peroxide.

Some of the conventional methods of immobilization that have been investigated include covalent attachment to a reactive insoluble support, physical adsorption to a solid surface, physical entrapment in polymeric gels, and cross-linking with a bifunctional agent such as glutaraldehyde in combination with adsorption or physical entrapment [7]. Poly(vinyl alcohol) (PVA), one of the most widely used polymers, can be cross-linked chemically or physically to form a hydrogel. One physical technique of cross-linking PVA is through repeated freeze and thaw process to form a cryogel.

Sol-gel matrix is also frequently used to immobilize GOD. Due to the porous nature of the matrix, an analyte can interact easily with immobilized enzyme. Sol gel process involved the initial hydrolysis and polycondensation of suitable precursors to form ceramic materials, leading to the formation of colloidal particles, which is called sol. 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 [8]. Two types of alkoxides are usually applied, the silica alkoxide, tetramethoxysilane (TMOS) ($C_4H_{12}O_4Si$), metal alkoxides, alumina (aluminium isopropoxide) ($Al[OCH(CH_3)_2]_3$).

In this work, glucose oxidase was immobilized in freeze-thawed PVA matrix and PVA/TMOS matrix using (3-glycidoxypropyldimethylethoxy)silane (3GPDES) as a cross-linker. The effect of PVA and cross-linker concentration on enzymatic membrane performance was investigated to determine the optimized method for GOD immobilization.

2.0 MATERIALS AND METHODS

2.1 GOD Immobilization in Freeze-thawed PVA Matrix

2.1.1 Membrane Preparation

60 μl of PVA solution (5%, 10% or 15% w/v) was added to 10 μl of 280 mg/ml GOD solution. An aliquot of the mixture was pipetted quickly onto a polymer block and covered with another polycarbonate block. The two blocks were clamped together in order to prevent the membrane from contracting. The membrane was then frozen at -20°C for 12 hours. After freezing, the membrane was thawed at 4°C for 12 hours. The process was repeated 5 times as the exposure of aqueous PVA solutions to several freezing-thawing cycles leads to reinforced gels owing to a densification of the macromolecular structure which is a function of the cycling time and temperatures [9]. The membrane thickness was controlled with aluminum spacer tapes. After the freezing and thawing process, the blocks were soaked in 40 ml of phosphate buffer (pH 6.0) at room temperature. The blocks were slowly unclamped and the membrane was gently stripped from the block. 1 ml of the washing solution was collected to determine the protein contents in the washing solution.

2.1.2 Measurement of Protein Concentration in Washing Solution

Bicinchoninic acid (BCA) method was used to determine the total protein contents in the washing solution. This method was used to infer the concentration of enzyme in the washing solution. A standard working solution (SWR) was prepared by mixing reagent A (0.04 g/ml $CuSO_4.5H_2O$) and reagent B (0.01 g/ml BCA, 0.02 g/ml $Na_2CO_3.H_2O$, 0.0016 g/ml $Na_2C_4H_4O_6.H_2O$, 0.004 g/ml NaOH and 0.0095 g/ml $NaHCO_3$) in a ratio of 50:1. Bovine serum albumin (BSA) was used as the protein standards. The concentrations used were in the range of 0 mg/ml – 1.0 mg/ml. 2 ml of SWR was added into each test tube that contained 0.1 ml of either the BSA solution or the washing solution. The tubes were incubated at 37°C for 10 minutes. The absorbance for the standards and unknowns were recorded at a wavelength of 450 nm using a Hitachi V-100 UV spectrophotometer.

2.1.3 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 55°C 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 (2):

$$H = \frac{(W_w - W_d)}{W_w} \times 100 \tag{2}$$

where W_w is the weight of the fully hydrated membrane and W_d is the weight of the dehydrated membrane.

2.1.4 Measurement of Immobilized Enzyme Apparent Activity

Apparent activity of immobilized enzyme in freeze-thawed membrane was measured by colorimetric method. A chromogen solution was prepared by diluting 0.1 ml of 1% w/v O-dianisidine in 12 ml of phosphate buffer at pH 6.0. 450 µl of 25 %w/v of aqueous glucose solution and 150 µl of 200 µg/ml peroxidase were added to 3.75 ml of the chromogen solution. The mixture was incubated at room temperature for temperature equilibration for 10 minutes. 300 µl of 4M HCl was added to stop the reaction. The membrane was removed prior to the addition of the acid. The amount of colour formed was measured by reading the absorbance value at 450 nm. For enzyme activity calibration curve, 150 µl of GOD with the activity ranging from 0 to100 mU/ml was mixed with the mixture.

2.2 GOD Immobilization in PVA/TMOS Matrix

2.2.1 Membrane Preparation

TMOS, (3-glycidoxypropyldimethylethoxy)silane, H_2O, methanol and 57.5 µl of HCI were mixed together, in a volume ratio of 1 : *x* : 3.7 : 3.7 : 0.0013, where *x* was 1, 2 and 3 respectively. The solution was stirred for 30 minutes. 115 µl of HCI was then added and the sol gel solution was stirred for 1 hour to form homogenous solution. 25 µl of PVA solution was mixed with 100 µl of the sol gel solution. 90 µl of the solution was then added to 15 µl of 140 mg/ml GOD solution. An aliquot of the mixture was pipetted quickly onto a glass slide. The membrane was air-dried for 20 minutes and then covered with another glass slide. The two glass slides were clamped together to

prevent the membrane from contacting and left for 24 hours at 25°C. Membrane thickness was controlled with aluminium spacer tapes. After 24 hours, the slides were soaked in 25 ml phosphate buffer solution in a petri dish at 25°C. The slides were carefully unclamped and the membrane layer was gently stripped from the slide. The membrane was swollen in 5 ml phosphate buffer solution and kept at 4°C. 1 ml of the washing solution was collected for enzyme assay purposes.

2.2.2 Measurement of Protein Concentration in Washing Solution

Protein concentration in washing solution was measured using Biuret method. This method was used to infer the concentration of enzyme in the washing solution. 6.0 g sodium potassium tartrate tetrahydrate ($NaKC_4H_4O_6.4H_2O$) was dissolved in 500 ml distilled water. 1.5 g of copper sulfate pentahydrate ($CuSO_4.5H_2O$) was then added and dissolved into the solution. After that, 300 ml of 10 %w/v sodium hydroxide (NaOH) was added slowly with stirring. Finally, the solution was diluted to 1 l with distilled water. The solution was stirred until homogenous. The Biuret reagent was stored in a Schott® bottle covered with aluminium foil and kept in shaded place for subsequent use due to its sensitivity to light. Bovine serum albumin (BSA) was used as the protein standards. The concentrations used were in the range of 0–12.5 mg/ml. For the protein assay, 3 ml of biuret reagent was added into each test tube that contained 2 ml of either BSA solution or the washing solution. Then the tubes were incubated at 37°C in a water bath for 15 minutes. The absorbance of each solution was measured against a blank (biuret solution without BSA) at a wavelength of 550 nm.

2.2.3 Measurement of Immobilized Enzyme Apparent Activity

Apparent enzyme activity for PVA/TMOS membranes was measured amperometrically using three-electrode electrochemical cell. A platinum electrode and a platinum auxiliary electrode were used as the working electrode (WE) and the counter electrode (CE), respectively. In this electrochemical cell, an Ag/AgCI was employed as the reference electrode (RE). The enzymatic membrane was attached to the surface of the working electrode. Before it was ready to be used, the electrode was rinsed with deionized water and immersed in 15 ml of phosphate buffer with pH 7.0. Voltage at 0.7 (V) vs Ag/AgCl was applied to the system. After the current had stabilized, a prescribed amount of stock glucose solution was injected into the cell to make up a 5 mM glucose solution.

3.0 RESULTS AND DISCUSSION

3.1 GOD Immobilization in Freeze-thawed PVA Matrix

Previous works on PVA were usually based on 10% PVA as the selected PVA concentration [10–12]. In this work, 5% PVA, 10% PVA and 15% PVA were investigated to see whether the concentration of PVA had any effect on the ability of freeze-thawed PVA matrix in retaining GOD and maintaining its activity.

Figure 1 shows the total protein concentration in the washing solutions for freeze-thawed PVA-GOD membranes against time. 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 well within the solid support and leached out easily from the membrane into the washing solution that resulted in big deviation of enzyme the leaking for all membranes.

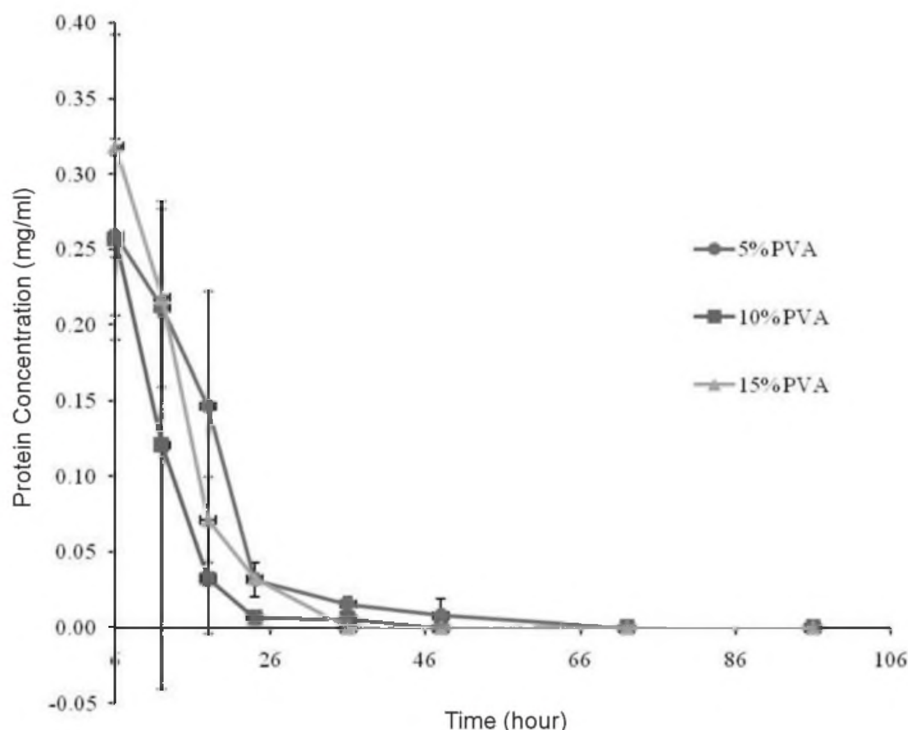

Figure 1 Total protein concentration (mg/ml) in the washing solutions for freeze-thawed PVA-GOD membranes against the time

PVA-GOD freeze-thawed membranes prepared with 5%, 10% and 15% PVA stopped leaking after 72 hours, 48 hours and 36 hours, respectively. This indicates that the higher the concentration of PVA used for the matrix, the higher the cross-links that were formed and consequently the higher the amount of enzyme that was retained in the matrix. On the other hand, the lower the concentration of PVA used for the matrix, the lower the cross-links that were formed. At moderate cross-linking density, the loss of active enzyme through leakage would be minimal but at the same time an acceptable micro-environment for the enzyme would be expected [10].

Cross-link density of a cross-linked membrane can be estimated by measuring the water content. Figure 2 shows the relationship between water content and PVA concentrations of the PVA-GOD freeze-thawed membranes. As expected membranes prepared using 5% PVA have higher water contents than membranes prepared using 10% and 15% PVA. This suggest that membranes made with 15% PVA had the highest cross-link density followed by membranes prepared with 10% PVA and 5%

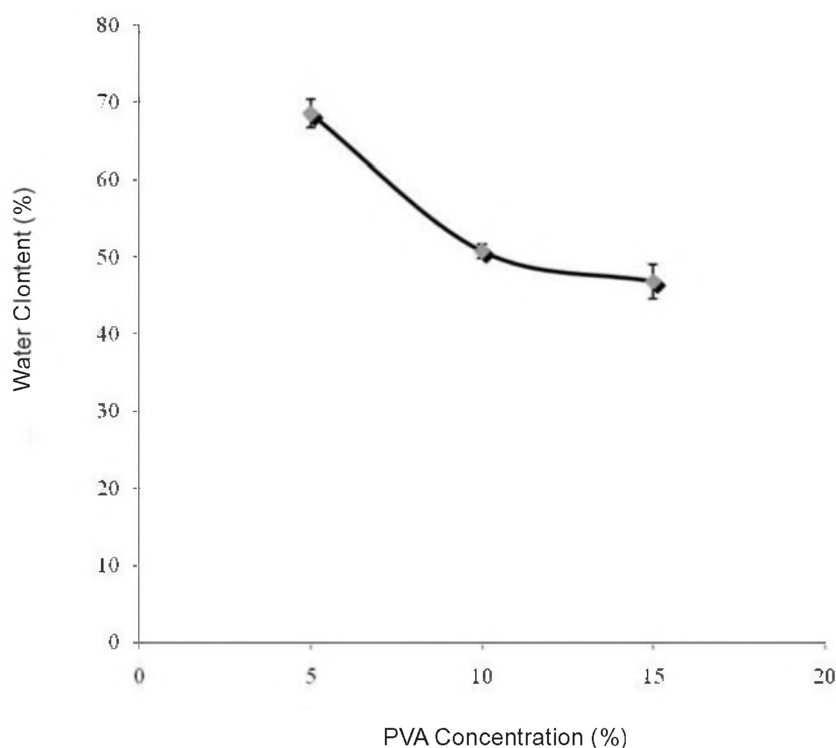

Figure 2 The relationship between the concentration of the PVA and water content (%) for freeze-thawed PVA-GOD membranes

PVA respectively. Water content is also a good indicator of the permeability of the membranes. Membrane permeability is generally proportional to membrane water content [10].

The influence of storage in buffer solution and the effect of repeated-use on the apparent activities of the enzymatic membranes were studied. The first enzyme assays were tested after 4 days. Figure 3 shows the stabilities of the 5%, 10% and 15% PVA of freeze-thawed PVA-GOD membranes against time. The apparent enzyme activities of the membranes decayed over time as expected [13]. Decrease in enzyme activity of glucose sensors was usually strongly influenced by the loss of enzyme by denaturation or detachment [14]. The denaturation of enzyme might be due to contamination by bacteria that can destroy enzyme activity since the membrane was not covered with any protective membrane or exposure to hydrogen peroxide [15].

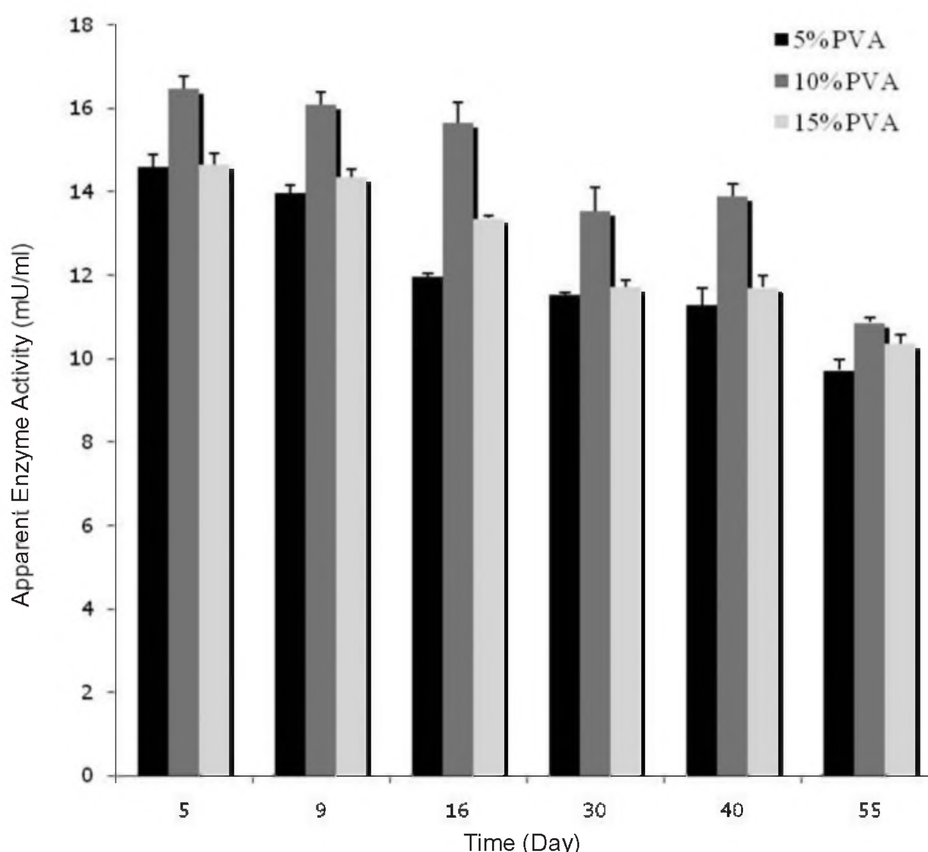

Figure 3 Comparison of apparent enzyme activity (mU/ml) for freeze-thawed PVA-GOD membranes against time

Freeze-thawed PVA-GOD membrane with 10% PVA has the highest sensitivities to glucose compared to the other two membranes. This might be due to the moderate cross-link density and also adequate permeability that effectively entrapped the GOD and maintained the appropriate micro-environment for the enzymes. The performances of freeze-thawed PVA-GOD membranes with 15% and 5% PVA was comparable. Freeze-thawed PVA-GOD membrane with 15% PVA has better enzyme retention since the membrane suppressed enzyme leakage earlier than the others. However, the membrane has very low water content and very high cross-link densities that might have adverse effects on membrane permeability and conformational configuration of the immobilized enzymes. As expected, the freeze-thawed PVA-GOD with 5% PVA membranes were not very effective in retaining the enzymes due to low cross-link density but its high water content promotes a good micro-environment for the immobilized enzymes.

Freeze-thawed PVA-GOD membranes with 5% PVA and 10% PVA display comparable stability for 55 days where the retained enzyme activity was approximately 67% and 66%, respectively. Freeze-thawed PVA-GOD membranes with 15% PVA were more stable with 70% of the initial activity remained at day 55.

3.2 GOD Immobilization in PVA/TMOS Matrix

GOD was immobilized in PVA/TMOS matrix by utilizing 3-glycidoxypropyldimethylethoxy)silane (3GPDES) to cross-link the TMOS based matrix and to improve the retention of enzyme in the matrix. Figure 4 shows the total protein concentrations of the washing solution for the PVA/TMOS-GOD membranes with different TMOS to 3GPDES volumetric ratios. Within 72 hours, the protein concentrations in the washing solutions for all types of membranes have reached zero, indicating that the PVA/TMOS matrix was effective enough to retain GOD. The membrane with the higher concentration of the 3GPDES was expected to show better enzyme retention. However, Figure 4 shows that the leakage profiles for the three membranes were not much different, indicating that the method was effective for all membranes.

To determine the stability of the PVA/TMOS-GOD membrane, current response of the membranes to 5 mM glucose over time was recorded. Figure 5 shows the activities of all three types of membranes against time. The apparent enzyme activities of the membranes decayed over time as expected [13]. As explained earlier loss of activity of glucose sensors was usually strongly influenced by loss of enzyme through denaturation or detachment [15].

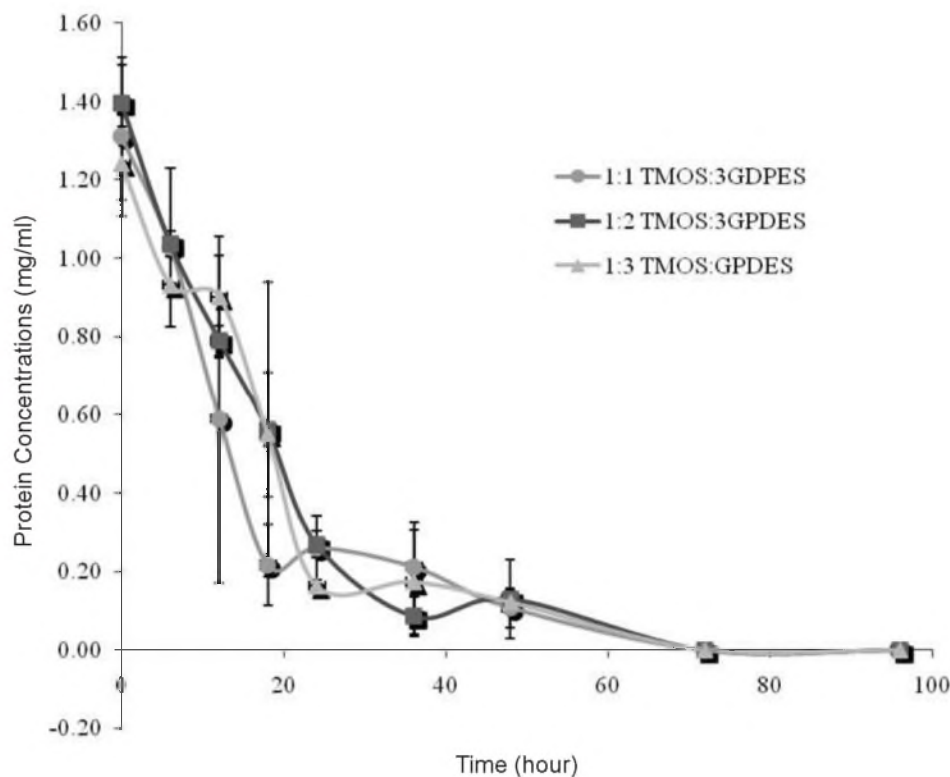

Figure 4 Total protein concentration (mg/ml) in the washing solutions for PVA/TMOS-GOD membranes

Until day 25, membranes with 1:1 (TMOS:3GPDES) showed higher activities than the other membranes. The activities then decreased rather rapidly. At day 40, only 51% of the initial activity remained. Membranes with 1:2 TMOS:3GPDES were quite stable with 69% of the initial activity remained at day 40. For membranes with 1:3 TMOS:3GPDES, 58% of the initial activity remained at day 40. Thus, membranes with 1:2 TMOS:3GPDES was most effective in term of stability.

4.0 CONCLUSIONS

In this work, GOD was immobilized in freeze-thawed PVA and PVA/TMOS matrix. The immobilization through the freeze-thaw method was milder compared to chemical cross-linker method. Better enzyme retention was achieved with higher PVA concentration used for immobilization. However, 10% freeze-thawed PVA-GOD

15 Azila.indd 190 5/28/10 11:52:52 AM

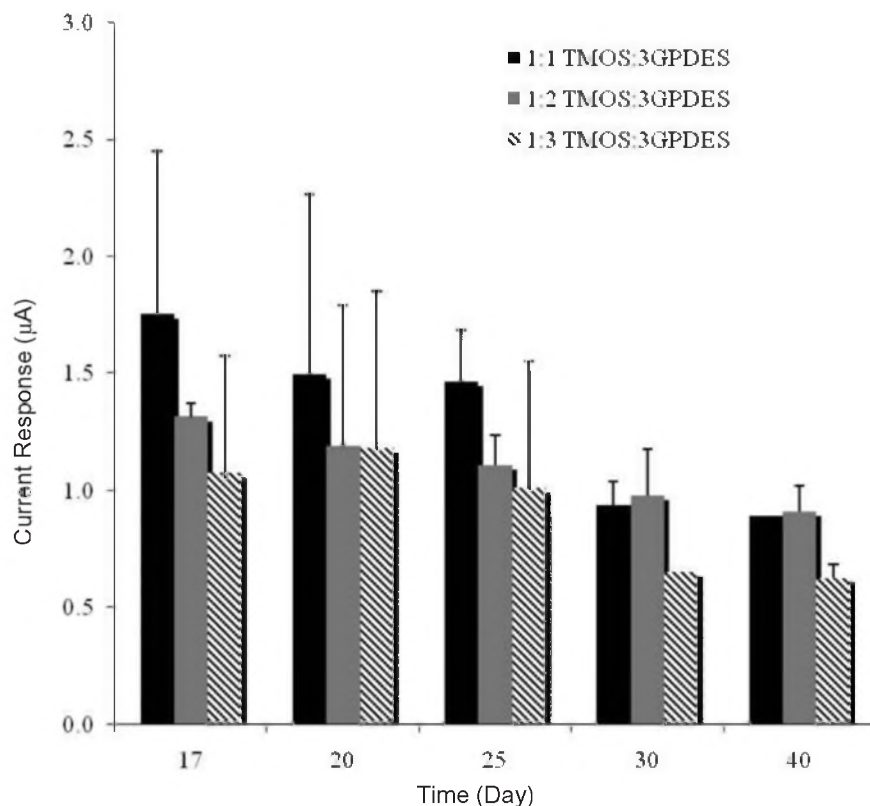

Figure 5 Comparison of current response (μA) towards 5mM glucose for PVA/TMOS-GOD membranes

membranes demonstrated the highest sensitivity to glucose. This might be due to the moderate cross-link density and also adequate permeability that effectively entrapped the GOD and maintained the appropriate micro-environment for the enzymes.

The effect of cross-linker concentration on enzymatic membranes was also investigated for PVA/TMOS matrix. PVA/TMOS matrix was effective enough to retain GOD. The leakage profiles for the three membranes with different cross-linker ratio were not much different. Even though initially PVA/TMOS matrix prepared with 1:1(TMOS:3GPDES) showed better sensitivity to glucose, the response decreased rapidly after day 25. PVA/TMOS matrix prepared with 1:1, and 1:3(TMOS:3GPDES) showed comparable sensitivity with 51% and 58% of enzyme activity remaining after 40 days, respectively. The stability of PVA/TMOS matrix prepared with 1:2 (TMOS:3GPDES) was better with 69% enzyme activity remaining after 40 days.

ACKNOWLEDGEMENTS

We would like to express our gratitude to the UTM's Research Management Centre for financing this study through a grant (Vot 71956).

REFERENCES

[1] Updike, S. J. and G. P. Hicks. 1967. The Enzyme Electrode. *Nature*. 214: 968–988.

[2] Gough, D. A., J. K. Leypoldt, and J. C. Armour, 1982. Progress Towards A Potentially Implantable Enzyme-based Glucose Sensor. *Diabetes Care*. 5: 190–198.

[3] Schichiri, M., N. Askawa, Y. Yamasaki, R. Kawamari, and H. Abe. 1986. Telemetry Glucose Monitoring Device with Needle-type Glucose Sensor: A Useful Tool for Blood Glucose Monitoring in Diabetic Individuals. *Diabetics Care.* 9: 298–301.

[4] Turner, R. F. B., D. J. Harrison, R. V. Rajotte, and H. P. Baltes. 1990. A Biocompatible Enzyme Electrode for Continuous In-vivo Monitoring in Whole Blood. *Sensors and Actuators B*. 1: 561–564.

[5] Moatti-Sirat, D., F. Capron, V. Poitout, G. Reach, O. S. Bindra, Y Zhang, G. S. Wilson, and D. R. Thevenot. 1992. Towards Continuous Glucose Monitoring: In-vivo Evaluation of A Miniaturized Glucose Sensor Implanted for Several Days in Rat Subcutaneous Tissue. *Diabetologia*. 35: 225–230.

[6] Eggins, B. R. 1996. *Bionsensors: An Introduction.* UK: John Wiley & Sons.

[7] Barker, S. A. 1987. In: Turner, A.P.F., Karube, I. and Wilson, G.S. eds. *Biosensors: Fundamentals and Applications*. Oxford: Oxford University Press.

[8] Liu, Z., B. Liu, M. Zhang, J. Kong, and J. Deng. 1999. Al_2O_3 Sol-Gel Derived Amperometric Biosensor for Glucose. Analytica Chimica Acta. 392: 135–141.

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

[10] Abdul-Aziz, A. 2001. *Amperometric Glucose Biosensors: Systematic Material Selection and Qualitative Analysis of Performance.* Doctor of Philosophy. The John Hopkins University, Baltimore, Maryland.

[11] Dai, W. S. and T. A. Barbari. 1999. Hydrogel Membranes with Mesh Size Asymmetry Based On The Gradient Crosslinking of Poly(vinyl alcohol). *Journal of Membrane Science.* 156: 67–69.

[12] Yeom, C. K. and K. H. Lee. 1996. Pervaporation separation of water-acetic acid mixtures through poly(vinyl alcohol) membranes crosslinked with glutaraldehyde. *Journal of Membrane Science*. 10: 257–265.1

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

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

[15] Guilbault, G. G., J. M. Kauffmann, and J. Patriarche. 1991. Immobilized Enzyme Electrode as Biosensors. In: Taylor, R.F. (Ed.) *Protein Immobilization: Fundamentals and Application*. New York, USA: Marcell Dekker, Inc. 209–262.