Immobilization of Glucose Oxidase on Poly(vinyl alcohol): The Effect of Immobilization Temperature on Apparent Enzyme Activity

Wong Fui Ling*, and Azila Abdul Aziz

Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia.
*Corresponding author. Phone: +607-5535585, Fax: +607-5581463
Email: r-azila@utm.my

ABSTRACT

Amperometric enzymatic glucose biosensors based on glucose oxidase (GOD) are the most widely used devices for continuous regulation of diabetics' blood glucose levels. An effective glucose biosensor must contain an immobilized enzyme layer which is stable and is active for a sufficiently long time. For GOD immobilized on poly(vinyl alcohol) through chemical cross-linking, temperature plays an important role on the effectiveness of the cross-linking process. The higher the temperature, the faster the cross-linking process. In an attempt to minimize the possible denaturation of enzyme due to the exposure to the cross-linking temperature, the possibility of immobilizing GOD onto PVA at a lower temperature was investigated. For a hydrogel, membrane water content is generally a good indicator of the cross-linking density of a particular membrane. Thus, the performances of PVA-GOD membranes immobilized at either 4°C or 25°C but having similar water content were studied. However, no significant differences in the enzyme activity of both types of membranes were observed.

Keywords: Glucose Oxidase; Poly(vinyl alcohol); Temperature; Water content

1. INTRODUCTION

Amperometric enzymatic electrodes based on glucose oxidase (GOD), which generates hydrogen peroxide in the presence of oxygen and glucose are the most widely used for measurement of blood glucose concentration. One problem with enzymes is that they are progressively denatured and will lose their tertiary structure as temperature rises. With the attempt to minimize the inactivation of GOD during the cross-linking process, the possibility of potentially immobilizing the enzyme on poly(vinyl alcohol) (PVA) at low temperature was investigated. With this effort, the exposure of GOD to high temperature would be reduced. However, the reaction time must be increased to allow for the cross-linking reaction to reach completion. In this work, the effects of immobilization temperature on enzyme activity and efficiency of immobilization are of concern.

Water content of a membrane indicates the mesh size or the cross-linking density of a particular membrane. In order to compare the apparent enzyme activity of membranes immobilized at different temperatures, one condition that can be fixed is the equivalent cross-linking density of the membranes immobilized at the respective temperatures. Different duration of the cross-linking process would affect the water content of a membrane and thus the cross-linking density. As the efficiency of the cross-linking process depends on temperature, a membrane immobilized at a higher temperature will have a higher cross-linking density relative to a membrane immobilized at a lower temperature if the same reaction time is employed. Thus, in this work, in order to obtain equivalent cross-linking density, the water contents of PVA-GOD membranes immobilized at 4°C at different durations of cross-linking time have been compared to the water content PVA-GOD membranes immobilized at 25°C. A duration range of 1-8 days of cross-linking process was investigated.

2. MATERIALS AND METHODS

Glucose Oxidase (EC1.1.3.4, type X-S, 157,500units/g solid), peroxidase horseradish (EC 1.11.1.7, type VI from Horseradish, 330 purpurogallin units/mg solid), glutaraldehyde 50%w/v aqueous solution, D-glucose, O-dianisidine tablets (10mg substrate/tablet), Poly(vinyl alcohol) with average molecular weight of 70,000-100,000 were obtained from

Sigma Chemical Co. Other reagents used were acetic acid from R& M Co., methanol from AnaPure™ , sulfuric acid from Merck & Co. Inc., and hydrochloric acid from Merck & Co. Inc. All chemicals were used as received.

2.1. ENZYME IMMOBILIZATION

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. Next, this solution was mixed with 245mg/mL GOD solution in a volume ratio of 6:1. An aliquot of the mixture was pipetted quickly on a glass slide, air-dried for 10 minutes and covered with another glass slide. The two glass slides were clamped together, and left for 1-8 days at 4°C and 24 hours at 25°C. Membrane thickness was controlled with aluminium spacer tape. The membranes obtained were swollen in phosphate buffer at 4°C.

2.2. DETERMINATION OF WATER CONTENT

The PVA-GOD membranes were swollen in phosphate buffer. After the PVA-GOD membranes reach equilibrium hydration, the weights of the swollen membranes were recorded. The membranes were then left to dry at 25°C and the membranes' weights were recorded each day. The water content, H, was determined according to the following equation:

$$H = (W_w - W_d)/ W_w \times 100 \quad (1)$$

H ~ Water content
W_w ~ Fully hydrated weight of the membrane
W_d ~ Dehydrated weight of the membrane

2.3. DETERMINATION OF APPARENT ENZYME ACTIVITY

Enzyme activity was determined by measuring the amount of hydrogen peroxide produced. The enzyme activity was determined colourimetrically using GOD-HRP coupling method.

3. RESULTS AND DISCUSSION

3.1. DETERMINATION OF WATER CONTENT

A comparison of the water contents of membranes immobilized at 4°C with different durations of dehydration process and the water content of membranes immobilized at 25°C is shown in Table 1.

Table 1: Water contents of membranes left to react for different period of times

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

As expected, the water content of PVA-GOD membranes immobilized at 4°C is observed to be inversely proportional to the period of dehydration of the PVA-GOD membranes. Membranes reacted for 7-8 days contain lower water content than membranes reacted for 1-6 days. A little discrepancy in the readings of the water content of PVA-GOD membranes reacted for 1-2 days compared to 3-6 days, might be because the

membranes obtained were partially gelled and very watery. This might have resulted in inaccurate water content determination.

From the percent difference in water content of GOD-PVA membranes immobilized at 4°C, it is clearly shown that GOD-PVA membranes dehydrated for 7 days had identical water content with the GOD-PVA membranes immobilized at 25°C. Thus, it is concluded that PVA-GOD membranes immobilized at 4°C have to be dehydrated for 7 days in order to have comparative cross-linking density with PVA-GOD membranes immobilized at 25°C and left dehydrated for 1 day.

3.2. ENZYME LEAKAGE

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.

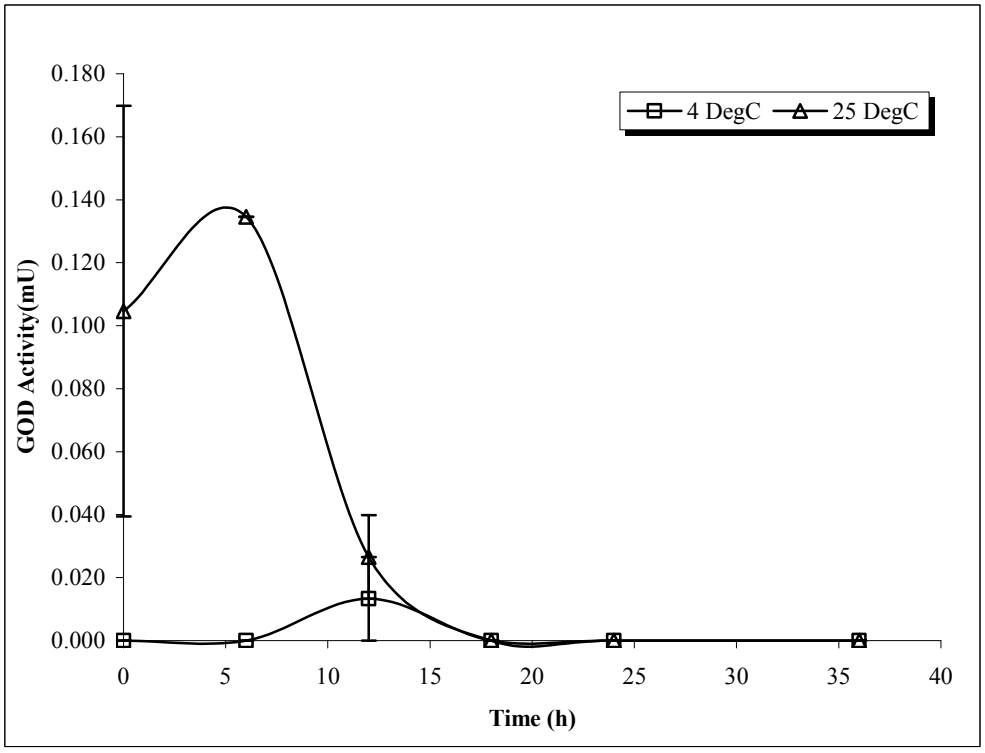

Figure 1: Comparison of the leaking profile of membranes immobilized at different temperatures

Figure 1 shows that the enzyme activities of the washing solutions for the PVA-GOD membranes immobilized at 4°C and 25°C decreased with time. PVA-GOD membranes prepared at both 4°C and 25°C stopped leaking enzyme within 18 hours. This indicated that the immobilization temperature did not affect the effectiveness of the membranes in immobilizing GOD on cross-linked PVA membranes. The results indicated that comparative amount of GOD had been retained in the PVA membranes at both temperatures.

3.3. APPARENT ENZYME ACTIVITY

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

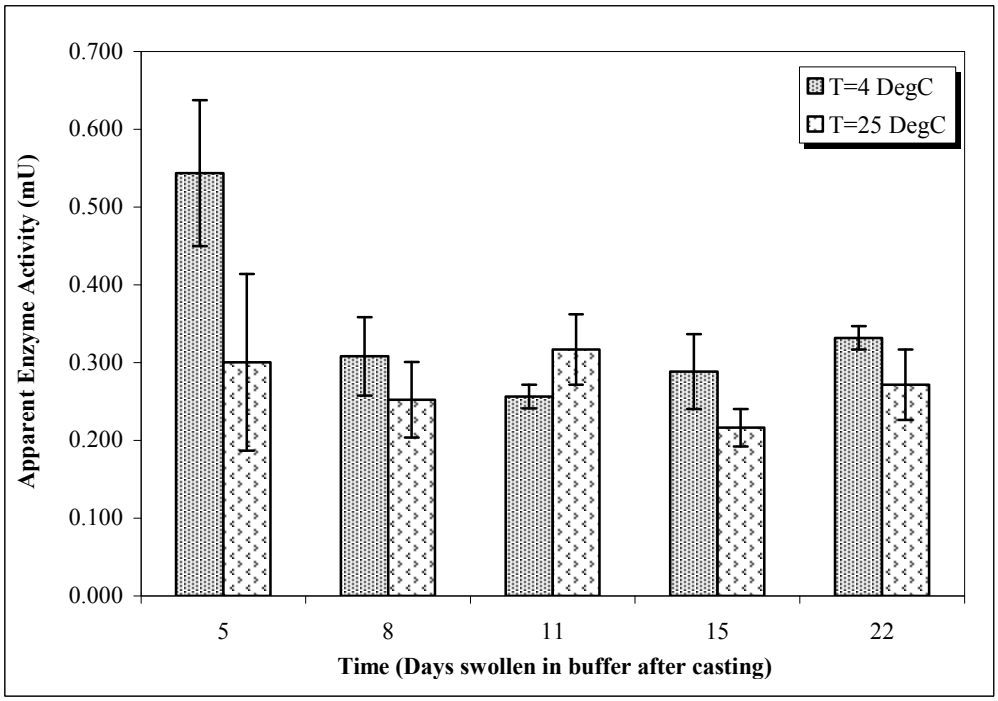

Figure 2: Apparent enzyme activities for membranes immobilized at different temperatures

The comparison of apparent enzyme activity for membranes immobilized at both temperatures is shown in Figure 2. For PVA-GOD membranes fabricated at 25°C the apparent enzyme activities initially decrease, followed by stabilization until day 22. This suggested that the immobilized GOD in the chemically cross-linked PVA membrane initially deteriorate. Hydrogen peroxide generated during the enzymatic reaction had been observed to be retained on the membranes surfaces, which can result in poisoning of the membranes (Hall *et. al.*, 1993). The retention of hydrogen peroxide could be a result of high mass transfer resistance to substrates and products imposed by the extensive cross-linking. The diffusional resistance encountered by the product molecules might have resulted in its accumulation near the center of the gel to an undesirably high level, leading to product inhibition for the enzyme. The stabilization that followed the initial decline might suggest that the trapped hydrogen peroxide diffused out after some time.

However, compared to PVA-GOD membranes formed at 25°C, the activities of the membranes formed at 4°C seemed to be stable throughout the 22 days. Membranes formed at 4°C did not show the same initial declining profile in apparent enzyme activity such as that demonstrated by PVA-GOD membranes casted at 25°C. This might suggest that the microenvironment of the two types of membranes were slightly different.

For a period of 22 days, it was observed that the enzymatic activity of the membranes immobilized at both temperatures were similarly quite low compared to the initial amount of enzyme immobilized and taking into account the amount of enzyme that had leaked out. This could be a result of the unpredictable conformational and microenvironment changes, as well as non-uniform distribution of cross-linked molecules by cross-linking. Lack of control of microenvironmental conditions is one of the main reasons that can result in reduced immobilized enzyme activity and stability (Shuler *et. al.*, 1992).

4. CONCLUSION

In conclusion, membranes dehydrated for 7 days at 4°C contained equivalent water content with membranes dehydrated for 1 day at 25°C. Overall, no significant differences in apparent enzyme activity for membranes immobilized at 4°C and 25°C with

comparative cross-linking density have been observed. However, membranes fabricated at 4°C seems to be more stable than the membranes formed at 25°C.

ACKNOWLEDGEMENT

This work was supported financially by Intensification of Research in Priority Areas (IRPA), project no: 03-02-06-0092 EA001.

REFERENCES

Azila Abdul Aziz (2001). Amperometric Glucose Biosensors: Systematic Material Selection and Quantitative Analysis of Performance. The Johns Hopkins University, Baltimore, Maryland: Dissertation Ph.D.

C. E. Hall and E. A. H. Hall (1993). Covalent Immobilisation of Glucose Oxidase on Methacrylate Copolymers for Use in an Amperometric Glucose Sensor, Analytica Chimica Acta. **281**. 645-653.

D. Maotti-Sirat, G. Velho, G. Reach (1992). Evaluating in Vitro and in Vivo the Interference of Ascorbate and Acetaminophen on Glucose Detection by a Needle-Type Glucose Sensor, Biosensors and Bioelectronics. **7**: 345-352.

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

K. Imai, T. Shiomi, K. Uchida, and M. Miya (1986). Immobilization of Enzyme into Poly(vinyl alcohol) Membrane, Biotechnology and Bioengineering. **28**: 1721-1726.

Lucio Doretti, Daniela Ferrara, Paola Gattolin, and Silvano Lora (1996). Covalently Immobilized Enzymes on Biocompatible Polymers for Amperometric Sensor Applications, Biosensors and Bioelectronics. **11**: 365-373.

Lucio Doretti, Daniela Ferrara, Paola Gattolin, and Silvano Lora (1997). Amperometric Biosensor with Physically Immobilized Glucose Oxidase on a PVA Cryogel Membrane, Talanta. **44:** 859-866.

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

Michael L. Shuler, and Fikret Kargi (1992). Bioprocess Engineering-Basic Concept. United State: Prentice Hall. 78-89.

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