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Development of Crack-Free Alumina Sol-gel/Poly(vinyl Alcohol) Membranes for Glucose Oxidase Immobilization

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A simple procedure to incorporate PVA into alumina sol-gel membrane was investigated as a suitable support material for glucose oxidase. The alumina sol was prepared using aluminum iso-propoxide via the sol-gel process. PVA was employed as the organic binder to enhance the mechanical strength of the fragile sol-gel membranes. The ability of the hybrid membrane to retain glucose oxidase and the apparent enzyme activities were studied. The resulting composite membranes were found to be crack-free, stable, and still very active after 60 days. However, the enzyme leakage period was observed to be quite long. The enzyme was still leaking from the membrane after more than 10 days albeit at a very low level.

Keywords: Glucose Oxidase (GOD), PVA, alumina sol-gel, composite membranes, immobilization, enzyme leakage

INTRODUCTION

The need to maintain blood glucose physiological level has led to the development of a series of glucose-sensing devices suitable for measuring glucose levels in physiological fluids both *in vivo* and *in vitro*. Amperometric peroxide-based glucose biosensors are the simplest and are the most widely used. The main concern of this paper is the sensing layer of the sensor where glucose oxidase (GOD) is immobilized. The performance of a glucose biosensor is strongly dependent on the sensing layer. It is important that the immobilized enzyme should be able to retain as much catalytic activity as possible. Different immobilization methods impose different limitations for optimum preservation of enzyme activity. Many conventional immobilization methods that have been investigated include alternately assembled polyion films (Onda, Ariga,

and Onitake 1999; Forzani and Solis 2000), electropolymerization of conductive polymer such as polypyrrole films (Brahim, Narinesingh, and Guiseppi-Ellie 2002), covalent binding (Yang 2003), physical entrapment of enzyme (Doretti *et al*., 1997), cross-linking with glutaraldehyde (Abdul-Aziz 2001), and sol-gel derived materials (Lillis et al. 2000, Chen, Hu, and Wilson 2002).

Recently, sol-gel technology has drawn much attention due to its special properties. It is a simple process that involves the hydrolysis and polycondensation of suitable precursors to form ceramic materials (Brinker and Scherer 1990). The biological compound is entrapped by adding to the sol prior to its gelation. The low temperature of the sol-gel process allows the entrapment of enzyme within the aqueous pores and provides a stable environment to the enzyme (Lev et al. 1995). While most of the studies were based on silicate precursors, a number of other precursors, such as early transition metals (Ti, V, Zr) and Group IIIB (B and Al), have also been of interest for enzyme immobilization. These nonsilicates precursors have greater reactivity towards water compared to silicates due to the lower electronegativity of the metal and its ability to exhibit several coordination states (Brinker and Scherer 1990). Glezer and Lev (1993) had coated platinum electrode with a highly conductive and porous sol-gel derived vanadia matrix for a glucose biosensor. GOD had been successfully entrapped within alumina sol-gel (Liu et al. 1999; Chen, Hu, and Wilson 2002). Alumina had also been employed to immobilize quince polyphenol oxidase by adsorption (Yağar and Sağiroğlu 2002).

However, the notable drawback of sol-gel derived material is the fragility and ease of cracking of the ensuing matrix (Dave et al. 1994, Lev *et al*., 1995). Cetyltrimethylammonium bromide (CTAB) had been used as a surfactant to prevent fracturing of sol-gel membranes (Li, Tan, and Ge 1996). Addition of organic hydrogel substance can limit the shrinkage effect and avoid cracking of gel upon drying. Typical advantages of organic polymers are flexibility, formability, and biocompatibility. Wang and coworkers (1998) have developed hybrid material based on silica sol and poly(vinyl alcohol) grafting 4-vinylpyridine (PVA-g-P(4-VP) copolymer for GOD immobilization**.** Miao and Tan (2001) have demonstrated that the addition of chitosan into silica sol-gel for the application of amperometric hydrogen peroxide sensor improved the sensor. For nonsilicates system, organic groups can act as the binder to provide sufficient strength and prevent cracking of the gel. PVA has been shown to be effective in the development of crack-free alumina membrane and has been used widely as the binder for applications in filtration and in the electronic industry (Lambert, and Gonzalez 1999, Venkatesh and Ramanan 2000, Ananthakumar, Manohar, and Warrier 2004). In this work, a simple method of incorporating GOD into PVA-modified alumina sol-gel has been shown to improve the performance of the support matrix for glucose oxidase immobilization.

EXPERIMENTALS

REAGENTS

Glucose oxidase (GOD) (EC1.1.3.4, type X-S, 190 000 U/g); poly(vinyl alcohol) (PVA) with average molecular weight of 70,000-100,000; horseradish peroxidase (HRP) (EC 1.11.1.7, type VI from Horseradish); o-dianisidine tablets; and, D-glucose were obtained from Sigma. Aluminum iso-propoxide (Al(*i*-PrO)$_3$) 98+% was purchased from Aldrich. Hydrochloric acid (HCl), potassium phosphate monobasic, and potassium phosphate dibasic were from Merck. All chemicals were used as received.

Apparatus

Amperometric measurements were performed using a three-electrode system potentiostat, μAutolab (Metrohm, the Netherlands), consisting of a platinum working electrode, a platinum sheet counter electrode, and a Ag/AgCl reference electrode.

Preparation of Alumina Sol-Gel Solution

Alumina sols were prepared according to the method established by Yoldas (1975). The appropriate amount of Al(*i*-PrO)$_3$ was added to the deionized water at 80°C and was stirred for 1h. Then, 1M of peptization agent, 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.

Casting of Alumina-GOD Membranes

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. For enzyme immobilization, an appropriate amount of the casting solution was mixed with 280mg/mL GOD in phosphate buffer in a volume ratio of 6:1. An aliquot of the mixture was pipetted onto a polystyrene petri dish, and air-dried for 10mins. Then, it was covered with a lid and left for 24h at 25°C. The membranes obtained were swollen in phosphate buffer at 4°C.

Determination of Enzyme Activity

Enzyme activity was determined by measuring the amount of hydrogen peroxide produced. The enzyme activity was determined colorimetrically using GOD-HRP coupling method and it was also determined amperometrically.

For the electrochemical measurements, the alumina-GOD membrane was attached to the platinum working electrode tip. The enzyme electrode was then dipped into a cell containing 10mL of phosphate buffer at room temperature and a potential of 700 mV vs Ag/AgCl was applied. The background current was allowed to stabilize prior to glucose addition. All measurements were done with stirring.

RESULTS AND DISCUSSION

Addition of PVA

As the alumina membranes did not show much mechanical strength, a binder was required to strengthen the matrix. Since both alumina sol and PVA possess hydroxyl group, PVA appears to be a good choice as the binder (Yang et al. 1996). Addition of PVA into the casting solution reduced the surface tension of the membranes and thus avoided cracking. Lambert and Gonzalez (1999) had observed that alumina membranes with 2wt% PVA added following the peptization step cracked slightly upon drying at room temperature. In this work, free-standing and crack-free alumina-GOD membranes were obtained with 3w/v% of PVA. This allowed the membranes to be peeled off from the support.

Retention of Immobilized Enzyme

It is very important that the alumina-PVA-GOD membranes possess the ability to retain the immobilized enzyme. Washing solutions were collected at certain periods for enzyme leakage determination using colorimetric method.

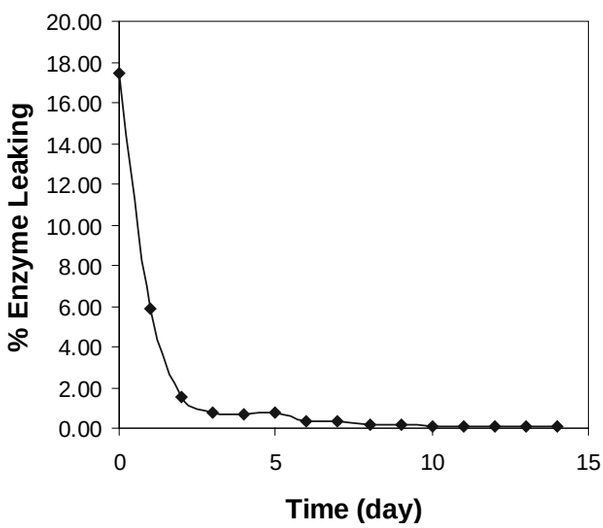

Figure 1. GOD Leaking Profile of Alumina-PVA Hybrid Membranes

Figure 1 shows that the enzyme activities of the washing solutions for the alumina-PVA-GOD membranes decreased with time. The

highest amount of enzyme activity in the washing solution was observed right after the membranes were detached from the petri dish. Based on the original enzyme loading, enzyme leakage was less than 1% during the first 3 days and 0.1% within two weeks.

However, a very small amount of enzyme activity could still be observed at day 25. The amount of enzyme leakage and the leaking period were higher compared to chemically cross-linked PVA-GOD membranes with the same concentration of GOD (Wong and Azila 2004). It is possible that the concentration of enzyme introduced during membrane casting might have exceeded the immobilization capacity of the membranes. Without a cross-linker, such as glutaraldehyde, the enzymes were merely entrapped within the matrix. Although this resulted in a conducive environment for the preservation of apparent enzyme activities, it resulted in poor enzyme retention. Lower concentration of enzyme might be employed to investigate the immobilization ability of the matrix. Besides, addition of an appropriate coupling agent, such as (3-aminopropyl)trimethoxysilane, might be able to enhance matrix formation as well as increase enzyme immobilization capacity (Yang et al. 2003).

Stability of membranes

The long-term stability of the alumina-PVA-GOD membranes was investigated to determine the shelf life of the sensor since a limited lifetime for the enzyme layer of the biosensor had been reported (Doretti et al. 1997, Abdul-Aziz 2001). The colorimetrical enzyme assay based on the oxidation of o-dianisidine through peroxidase-coupled system had been performed as preliminary apparent enzyme activities determination. Next, the amperometric method was employed. The current was generated from the oxidation of hydrogen peroxide at the surface of the platinum working electrode at 700mV vs Ag/AgCl. The apparent enzyme activities of the membranes were tested for around 2 months.

The apparent enzyme activities of the alumina-PVA-GOD membranes are shown in figures 2 and 3.

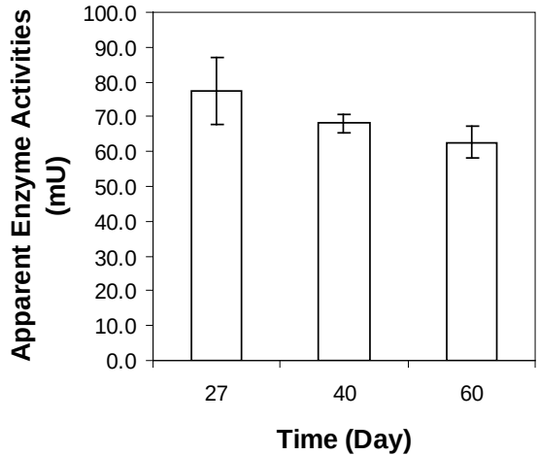

Figure 2. Apparent Enzyme Activities of Alumina-PVA-GOD Hybrid Membranes Determined by Colourimetrical Assay

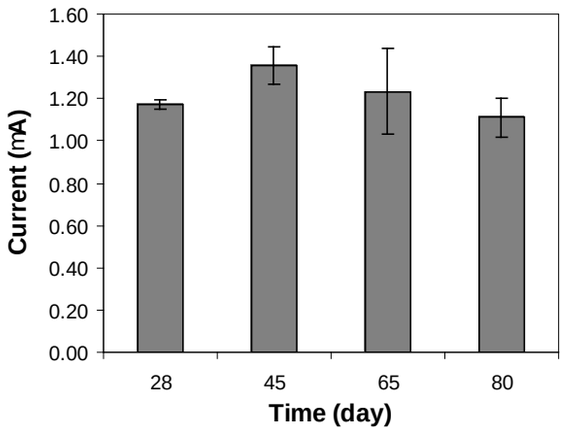

Figure 3. Apparent Enzyme Activities of Alumina-PVA-GOD Hybrid Membranes Determined by Amperometric Method. Current outputs were based on response to 5mM glucose.

Figures 2 and 3 show that the alumina-PVA-GOD membranes remained stable during the investigation period. This agrees with the study done by Chen, Hu, and Wilson (2002). The stability may be due to high enzyme loading (Pfeifer, 1997) and favorable micro-environment.

The apparent enzymatic activities of the GOD immobilized in alumina-PVA were quite high compared to those immobilized in chemically cross-linked PVA (Wong and Azila 2004). This can be attributed to the relatively mild immobilization condition for alumina sol-gel. Although 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 a higher response.

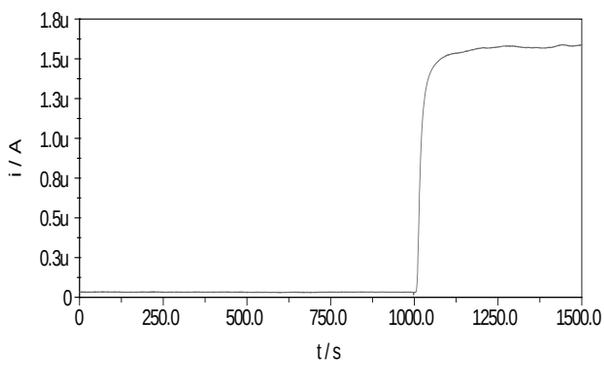

Figure 4. Current Response of Alumina-PVA-GOD Membranes Attached to Platinum Working Electrode to 5mM Glucose

Figure 4 shows a typical current response of alumina-PVA-GOD membranes upon addition of 5mM glucose. The sensitivity of alumina-PVA-GOD membranes corresponding to 5mM of glucose was 74.7±1.4 nA mM^{-1} mm^{-2}.

Kinetic Characteristics

By employing modified Hanes-Woolf method, kinetic properties of the alumina-PVA-GOD membranes were evaluated. A sample Hanes-Woolf plot for alumina-PVA-GOD membranes is shown in Figure 5. The apparent Michaelis constant, K_m^{app}, obtained was 2.47mM with maximum current of 1.78 μA. Such low K_m^{app} agrees with that showed by Chen, (2002) before adding an outer layer. The relatively low value of K_m^{app} obtained shows a typical thin layer of very active enzyme (Chen, 2002). The addition of an outer membrane which imposes diffusional barrier to the flowing of substrates, especially oxygen, will be able to extend the linearity of the sensor (Andrew, David, and Pankaj 1996; Chen, Hu, and Wilson 2002).

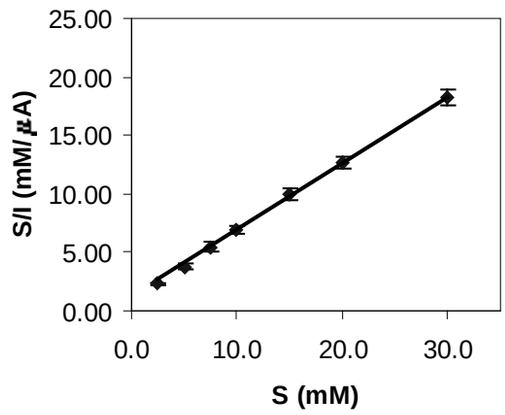

Figure 5. Modified Electrochemical Hanes-Woolf Plot of Alumina-PVA-GOD Membrane with $y = 0.5608x + 1.385$ ($R^2 = 0.9976$)

CONCLUSION

In this work, glucose oxidase has been successfully immobilized in unsupported crack-free alumina-PVA-GOD membranes. The addition of PVA has increased the mechanical strength of the membranes. Long stability period and relatively high apparent enzyme activities of the resulting membranes were observed. However, extensive enzyme leakage and low K_m^{app} need to be improved.

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