Comparative study of poly(vinyl alcohol)-based support materials for the immobilization of glucose oxidase

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Abstract

BACKGROUND: The performances of four types of glucose oxidase (GOD) immobilization materials based on poly(vinyl alcohol) (PVA) were compared. The matrices of interest were chemically-linked PVA, freeze-thawed PVA cryogel, tetramethoxysilane (TMOS) sol-gel-PVA hybrid material, and alumina sol-gel-PVA hybrid material.

RESULTS: Overall, the membranes showed good sensitivity except for the chemically cross-linked PVA. However, the main differences with the enzyme immobilization methods were enzyme leakage and values of K_m^{app}.

CONCLUSION: Freeze-thawed PVA-GOD membranes and TMOS-PVA, which showed satisfactory sensitivity and adequate value of K_m^{app}, were quite promising as support materials for immobilizing GOD.

Keywords: immobilization; glucose oxidase; poly(vinyl alcohol); sol-gel; hybrid

INTRODUCTION

For an amperometric hydrogen peroxide-based glucose biosensor, where interference has profound effects on sensor reliability, high current response to glucose is particularly important. The immobilization method and the host material used can influence the sensitivity of the biosensor. The porosity, pore size distribution, mechanical properties, and operational stability of a support material will affect the overall performance of the trapped enzyme. Poly(vinyl alcohol) (PVA), a hydrogel, is an ideal enzyme immobilization material. The abundance of hydroxyl groups provides a microenvironment similar to the enzyme's natural environment.[1] PVA has been widely used because of its inherent good biocompatibility and desirable physical properties, such as elastic nature, good film-forming property, high degree of swelling in aqueous solutions, and its water content matches that of biological tissue.[2] PVA hydrogel has been reported to have selective permeability for low molecular weight substances such as glucose.[3] In addition, PVA can stabilize the activity of enzymes, through the inhibition of the formation of non-functional conformations due to the extensive hydrogen bonding between the H atoms of alcohol groups in PVA and the O atoms of the carbohydrate groups in glucose oxidase (GOD).[4] Cross-linking of PVA can be achieved through different preparation approaches. Several authors have employed glutaraldehyde (GA) to cross-link PVA through its pendant hydroxyl groups.[5,6] Abdul-Aziz[4] investigated the optimum cross-linking ratio between PVA and GA that exhibited the highest apparent enzyme activity, while showing low enzyme leakage. Physical hydrogels can be prepared by photopolymerization[7] or a freeze-thawing method. The latter has been extensively studied by Peppas and coworkers.[8,9] Freeze-thawed PVA has been employed as the matrix for cell immobilization.[10] Immobilization of enzyme on PVA cryogels has also been performed.[11,12]

Successful encapsulation of enzymes in silicates by a sol–gel method in the early 1990s brought about a fast-growing interest in sol–gel processes for electrochemical biosensor applications.[13] Applications of sol–gel derived matrices for use in sensors has been reviewed by several authors.[14–17] The sol–gel process involves hydrolysis and condensation reactions at room temperature of an organic precursor to form inorganic polymeric materials. The sol–gel matrix provides an aqueous and stable environment that favors the entrapment of enzyme inside the pores.[18] The porous nature of the matrix offers a very large surface area and facilitates the interaction of immobilized enzyme with substrate.[16] Such a matrix traps large biomolecules while permitting the diffusion of small analytes through the sol–gel network.[16]

However, the disadvantage of sol–gel derived material is the fragility and susceptibility to cracking of the matrix.[14,15] Cracking occurs due to large internal pressure gradients generated by evaporation of water and solvent from the pores.[15] Recently,

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hybrid materials that take advantage of the merits of individual membrane components have been widely used.[19,20] Organic–inorganic hybrid materials can overcome the brittleness of sol–gel derived materials by limiting the shrinkage effect and reducing swelling of the hydrogel that may lead to the release of immobilized biomolecules. Typical advantages of organic polymers are flexibility, formability, and biocompatibility, while ceramics have rigidity and optical properties. Some of the organic polymers added to the sol–gel matrix are poly(ethylene oxide), polyhydroxyl, chitosan[20,21] and PVA. [22,23] These organic additives are introduced into the starting sol to form interpenetrating networks. Generally, hydrogen bond interactions form between the two components. Wang and coworkers[22] described the use of hybrid material based on silica sol and PVA grafting 4-vinylpyridine (PVA-g-P(4-VP) copolymer as a matrix for GOD immobilization.

In the present work, PVA, whether on its own or as an organic additive, was studied as a host material for the immobilization of GOD. PVA-based support materials were prepared through chemical cross-linking, freeze-thawing, hybridization with tetramethoxysilane (TMOS) and hybridization with alumina. The performances of the enzyme immobilized in these different matrices were studied using the same biosensor configuration.

EXPERIMENTAL

Reagents

Glucose oxidase (GOD) (EC 1.1.3.4, type X-S, 190 000 units g^{-1} solid), peroxidase horseradish (HRP) (EC 1.11.1.7, type VI from horseradish, 330 purpurogallin units mg^{-1} solid), lysozyme (Grade 1 from chicken egg, 58 100 units mg^{-1} solid), PVA with average molecular weight 70 000–100 000, glutaraldehyde (GA) 50%w/v aqueous solution, O-dianisidine tablets (10 mg substrate/tablet), and D-(+)-glucose were obtained from Sigma (St Louis, MO, USA). Aluminium isopropoxide 98 + % was purchased from Aldrich (Steinheim, Germany). Tetramethoxysilane (TMOS) was obtained from Merck (Darmstadt, Germany). Other reagents used were potassium phosphate monobasic, potassium phosphate dibasic, acetic acid, methanol, sulfuric acid, and hydrochloric acid. All chemicals were used as received.

Apparatus

Electrochemical measurements were carried out using a conventional three-electrode potentiostat system (μAutolab Type III) obtained from Metrohm, Netherlands. The setup consisted of an Ag/AgCl reference electrode (RE) and a platinum sheet counter electrode (RE). A platinum disk electrode was employed as the working electrode (WE) throughout the work.

Immobilization of GOD

Preparation of chemically cross-linked PVA–GOD membrane (GAPVA)

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.[4] 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, lysozyme was added into 280 mg mL^{-1} GOD solution at a concentration ratio of 0.5:1. Then, the PVA casting solution was mixed with the enzyme solution at a volume ratio of 6:1. This volume ratio was fixed for the preparation of other types of membrane. An aliquot of the mixture was pipetted quickly onto a glass slide, air-dried for 10 min and covered with another glass slide. The two glass slides were clamped together, and left for 24 h at 25 °C. Membrane thickness was controlled with aluminium spacer tapes. The membranes were then swollen in phosphate buffer at 4 °C.

Preparation of freeze-thawed PVA–GOD membranes (FTPVA)

The preparation was based on the methods established by Hickey and Peppas[24] using 10% PVA. A mixture of PVA and GOD was pipetted onto glass slides and maintained at −20 °C for 6 h to induce crystallization. Following the freezing process, it was allowed to thaw at 25 °C for 6 hours. The freezing and thawing cycle, n, was repeated five times. The membranes were then swollen in phosphate buffer at 4 °C.

Preparation of TMOS–PVA–GOD membranes (TMOS-PVA)

A typical TMOS solution was prepared according to the method described by several authors.[18,25] A defined amount of TMOS, acidic water and methanol, were mixed, and stirred for 30 min on ice at 300 rpm. Then, an appropriate amount of HCl was added into the mixture, and stirred for 1 h to obtain a transparent sol solution. The molar ratio of TMOS:water:methanol:HCl was 1:3.7:3:0.0013. To cast the membrane, 80 µL of 10% PVA solution was mixed with 27.7 µL TMOS sol before GOD was added. Then, the membrane was left for 24 h to polymerize. The membranes were then swollen in phosphate buffer at 4 °C.

Preparation of alumina–PVA–GOD membranes (Al-PVA)

Alumina sols were prepared according to the method established by Yoldas.[26] An appropriate amount of Al(*i*-PrO)$_3$ was added to deionized water at 80 °C

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and stirred for 1 h. Then, 1 mol L^{-1} 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 24 h. The resulting clear sol was decanted and stored at 4 °C. Prior to membrane casting, the sol was dried at 100 °C for 5 h to evaporate part of the water and alcohol. Then, PVA solution was mixed with the ensuing alumina sol (1:2 v/v) to prepare the casting solution. After addition of GOD, the solution was cast on a polystyrene petri dish and left for 24 h to polymerize. The membranes were then swollen in phosphate buffer at 4 °C.

Determination of enzyme leakage

Buffer solutions that were used to store the membranes were changed every 6 h for the first day, every 12 h for the second day, and every 24 h thereafter. These washing solutions were collected and analyzed for the amount of enzyme released from the membrane into the solutions. The free enzyme activity assay was performed using a GOD–HRP coupling colourimetric method.

Determination of apparent enzyme activity in the membrane

The apparent enzyme activity determination was carried out amperometrically using the three-electrode amperometric system. 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 dipping into a cell containing 10 mL of 0.1 mol L^{-1} phosphate buffer, pH 6.0 at room temperature under constant stirring. A constant potential of +700 mV versus Ag/AgCl, was applied to the electrode. Background current was allowed to stabilize prior to glucose addition. All measurements were done with stirring. The current response based on the oxidation of enzymatically liberated H_2O_2 at +700 mV was recorded until steady state was reached.

RESULTS AND DISCUSSION

The resulting GAPVA and FTPVA matrixes were physically robust and can be handled easily. Lysozyme was incorporated into GAPVA to improve its sensitivity[27] as some GA-containing sensing layers tend to exhibit low current response. For sol–gel membranes, incorporation of PVA into the networks improved the mechanical strength of the membranes and cracking was avoided. The performance of each type of membrane is detailed in Table 1.

Enzyme leakage

The ability of the membranes to retain the enzyme within the matrices was investigated. An example of a GOD leakage profile is shown in Fig. 1. Of the four types of membrane with immobilized GOD, chemically cross-linked PVA membranes were excellent for retaining the enzyme within the matrix. They stopped leaking within 1 day. TMOS-PVA sol–gel required 1 week while alumina-PVA and freeze-thawed PVA took about 18 days to achieve negligible leaking. In terms of amount, GAPVA membranes leaked a very small quantity with the aid of a bifunctional agent. Both sol–gel membranes showed overall better retention of enzyme than FTPVA. The amount of leakage was 45–58% less

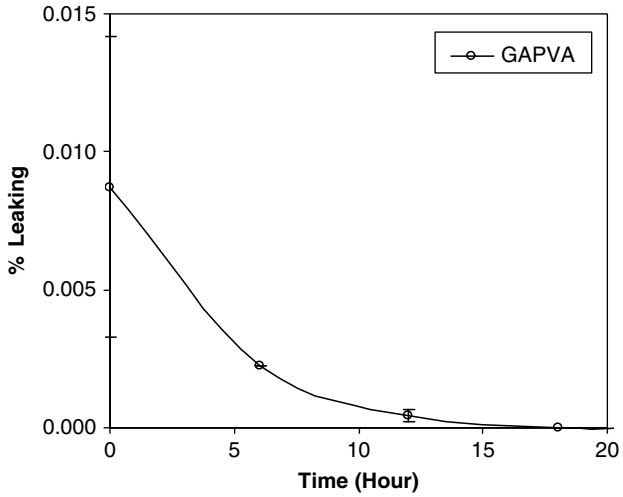

Figure 1. Enzyme leakage profile of GOD immobilized in GAPVA membrane. Enzyme activity was determined using a GOD-HRP coupling colourimetric method.

Table 1. Characteristics of GOD immobilized in different types of membranes

	Type of enzymatic layer			
	GAPVA	FTPVA	TMOS-PVA	Al-PVA
PVA content (vol %)	100	100	74	33
Total leakage (%)	0.01 in 18 h	58.9 in 15 days	24.5 in 8 days	32.3 in 17 days
Sensitivity (nA mmolL^{-1} mm^{-2})	0.35 ± 0.08	128.48 ± 17.8	136.6 ± 29.0	74.7 ± 1.4
Stability	>80% for 40 days	>80% for 60 days	>80% for 60 days	>80% for 80 days
K_m^{app} (mmolL^{-1})	52.7 mmol L^{-1}	7.00	4.75	2.47
I_{max} (μA)	0.06 μA	5.00	4.88	1.78
Membrane thickness (μm)	21 ± 2	160 ± 16	107 ± 3	108 ± 10
Response time (s)	~250	~300	~100	~60

than for FTPVA. This might be attributed to the interconnected 'bottleneck-like' pores in the structure of the sol–gel network, which locked the enzyme within the matrices.[16] The long leakage time observed in FTPVA may be attributable to the swelling of the hydrogel, which allowed the enzyme to leak out slowly over time.[1] The rigidity of TMOS was able to limit the swelling of PVA contained in its pores. A lengthy, slow leaking process was also observed in alumina–PVA membrane, similar to FTPVA. This is most probably due to the rather large/open pores size formed by alumina (three bonds) compared to TMOS (four bonds), even though the alumina–PVA membranes contained less PVA. Another possible explanation is that the higher amount of PVA contained in the TMOS matrix had packed the enzyme better.

For membranes other than GAPVA, about 80–90% of total leakage was observed during the unclamping process. This indicated that a substantial amount of enzyme was not entrapped within the matrix. The explanation for this inadequate immobilization might be insufficient capacity of the matrix to effectively retain the enzyme loaded. The enzyme is only trapped within the matrices by physical means, so that once the membranes become swollen upon immersion in phosphate buffer, 'loose' enzymes at the membrane surface dissolve back into the solution. These enzymes might not be trapped within the matrix during the gelation process.

Apparent enzyme activity and stability

The stability of 'repeated-use' PVA-GOD membranes was examined since a decay of apparent enzyme activity over time and limited lifetime of the enzyme layer in a biosensor have been reported. The apparent enzyme activity of the membranes was tested after enzyme leakage stabilized. A comparison of the current response for different types of membrane is shown in Fig. 2.

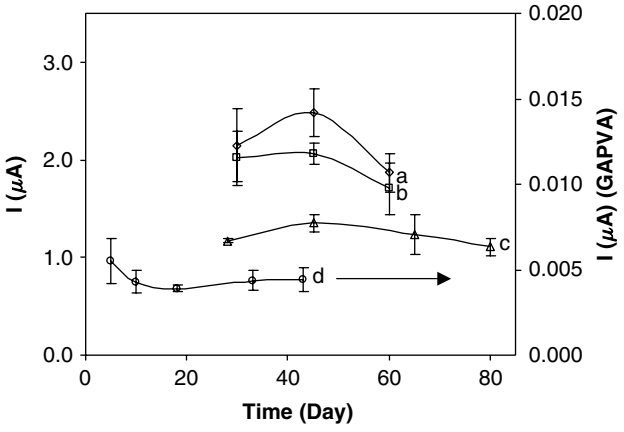

Figure 2. Stability of GOD immobilized in different types of membrane: (a) TMOS-PVA hybrid membrane; (b) FTPVA membrane; (c) Al-PVA hybrid membrane; (d) GAPVA membrane. Current response was measured at +0.7 V versus Ag/AgCl upon 5 mmol^{-1} glucose addition in phosphate buffer, pH 6.0. The curve for GAPVA is shown at a different scale from the rest.

In terms of biosensor response and sensitivity, the lowest response was observed in chemically cross-linked PVA membrane (Table 1). The dense structure obtained may have blocked the enzyme and made it less permeable to glucose.[28] The current response to 5 mmol^{-1} glucose was approximately 4 nA, while the responses of the other membranes were greater than 1 μA. The sensitivities of the other membranes were 200–400 times higher than the GAPVA membrane. Such poor apparent enzyme activity agrees with the results obtained by Lillis *et al.*[18], who compared BSA-GA membranes with sol–gel membranes in a lactate oxidase immobilization network. Jaffrezic-Renault *et al.*[29] also reported lower sensitivity of a urea sensor when urease was immobilized in a BSA-GA matrix compared to a nafion and photocross-linked PVA/SbQ membrane. Even though GA cross-linked PVA-GOD membranes suppressed enzyme leakage earlier than the other methods, which suggested better enzyme retention and very high cross-link densities, the tight network might have adverse effects on membrane permeability and the conformational configuration of the immobilized enzymes.

Although FTPVA lost a considerable amount of enzyme through leakage (Table 1), FTPVA showed a comparable sensitivity to that of TMOS-PVA membrane, and a higher sensitivity than alumina-PVA membrane. PVA has good water-retaining ability that offers a biocompatible microenvironment for the enzyme to maintain its natural configuration and hence preserve its sensitivity.[1] This is in line with the findings in this work, where sensitivities increase in ascending order from alumina-PVA, TMOS-PVA to FTPVA membranes where PVA contents were increased. In general, the sensitivities of GOD-membranes obtained in this work (except GAPVA) were comparable to those obtained by others.[6,30] However, the sensitivities were lower than those obtained by Wang *et al.*[22] and Malitesta *et al.*[31] The grafting copolymer and electropolymerized material used by these researchers may have helped in transferring electrons more effectively to the sensor surface.[19]

Table 1 shows that the response times observed in this work were long compared to others.[22,30,31] This can be attributed to the thicker films that were formed in this work. Thick polymer membrane can create a diffusion barrier to penetration of the substrate through the matrix and hence result in a long response time for the sensor.[32] In this work the same type of cast was used for casting all the membranes. Thinner membranes were obtained from sol–gel derived organic–inorganic materials compared to FTPVA. These membranes also showed a faster response. Glutaraldehyde in the GAPVA matrix caused the formation of a very tight network that resulted in a thin membrane, but the response time was still longer than others.[22,30,34] Response time can be improved by improving the casting method to obtain thinner membranes.

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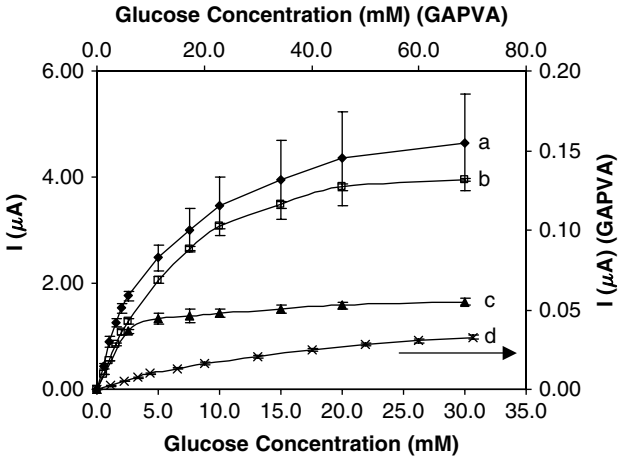

Figure 3. Calibration curves of GOD immobilized in different types of enzymatic layer: (a) TMOS-PVA hybrid membrane; (b) FTPVA membrane; (c) Al-PVA hybrid membrane; (d) GAPVA membrane. Current response was measured at +0.7 V versus Ag/AgCl upon 5 mmol L^{-1} glucose addition in phosphate buffer, pH 6.0. The curve for GAPVA is shown at a different scale from the rest.

Overall, good stabilities were observed during the investigations. The immobilized enzymes retained more than 80% of its initial activity at the end of the investigation period (Table 1). This may be attributable to high enzyme loading[33] and a stable microenvironment.

Kinetic parameters

Calibration curves of the enzymatic layers with glucose substrate are presented in Fig. 3. It can be seen that the current response increased linearly with glucose concentration at low concentrations, followed by a slower, non-linear increase. Such results show a Michaelis–Menten dynamic characteristic.[29]

To evaluate the kinetic parameters of enzyme immobilized in the membranes, the electrochemical form of the Hanes–Woolf regression was employed, in which s/i versus s graphs were constructed. S denotes glucose concentration and I the steady-state current. K_m^{app} and I_{max} were calculated as follows.

$$\frac{S}{I} = \frac{1}{i_{max}}\mathrm{S} + \frac{k_m^{app}}{i_{max}} \quad (1a)$$

$$I_{max} = \frac{1}{\text{Slope}} \quad (1b)$$

$$K_m^{app} = \text{y-intercept} \times I_{max} \quad (1c)$$

The apparent Michaelis–Menten constant, K_m^{app}, of chemically cross-linked PVA was the highest (52.7 mmol L^{-1}) (Table 1). This can be attributed to its highly cross-linked network, which imposed diffusional constraints on the substrate. K_m^{app} of the other materials that were formed through physical immobilization were generally low. The relatively looser meshes that were formed permitted higher accessibility of the substrate to the enzyme-active sites. FTPVA showed slightly higher K_m^{app} (7 mmol L^{-1}) than TMOS-PVA (4.75 mmol L^{-1}), while alumina-PVA gave the lowest K_m^{app} value (2.47 mmol L^{-1}). Low values of K_m^{app} have been reported by other researchers. A K_m^{app} value of 4.1–4.7mmol L^{-1} was reported by Poyard *et al*.[34] when immobilizing GOD in laponite–GA matrix and 0.8–2 mmol L^{-1} by Mousty *et al*.[35] in a trienzyme system. The depletion of oxygen in the host matrix as it is consumed in the oxidation process can drastically decrease the K_m^{app} value.[35] Poyard *et al*.[34] demonstrated an increase in the linear range by carrying out the reaction in an oxygen-saturated buffer solution. The addition of an outer membrane, which imposes a diffusional barrier to the flowing of substrates, especially oxygen, can also extend the linearity of the sensor.[30,36].

CONCLUSIONS

The immobilization approaches discussed in this work were simple, however, stable GOD-immobilized membranes were produced. The GAPVA membrane gave the shortest leakage period, which indicated that the ability to retain enzyme within the matrix was high. The apparent Michaelis–Menten constant K_m^{app} obtained was also the highest. However, its current response, and sensitivity were very much lower than that of the others, even when lysozyme was incorporated as a stabilizing agent. On the other hand, although FTPVA leaked for a considerably longer period, it showed satisfactory current response. It also exhibited an adequate level of K_m^{app}. Both types of sol–gel derived material, TMOS and alumina membranes, were brittle, and PVA was added during membrane fabrication to increase the mechanical strength. Both TMOS-PVA and Al-PVA showed lower enzyme leakage than FTPVA, however, the K_m^{app} obtained was lower than that for GAPVA and FTPVA. The advantage of the sol–gel hybrid membranes was that both of them had a shorter response time than GAPVA and FTPVA. In general, the membranes were stable for a period of more than 60 days. With the exception of GAPVA, the K_m^{app} of the membranes was quite low. However, K_m^{app} can be improved with use of a suitable outer membrane. Taking into account all considerations, FTPVA-GOD and TMOS-PVA membranes were quite promising as support materials for immobilizing GOD.

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