Preparation of Amphiphilic Membranes Using Photosensitive Polymers and Their Applications on the Enzyme Electrode

JUI-HSIANG LIU,* YI-CHANG CHUNG, and MING-TER LIN

Department of Chemical Engineering, National Cheng Kung University, Tainan, Taiwan, 70101, Republic of China

SYNOPSIS

To decrease the mass transfer resistance of oxygen permeation and to improve the response of the enzyme electrode, a series of hydrophilic–hydrophobic polyvinylcinnamate (PVCin) membranes were developed. The PVCins were synthesized from polyvinyl alcohol using various amounts of cinnamoyl chlorides in the presence of alkali. The amphiphilic membranes were prepared by using photosensitive PVCin and/or sensitizers that were used instead of the traditional Teflon–hydrophilic two-layer membranes. The properties of photocrosslinking, water and oxygen permeability of the amphiphilic membranes, the calibration curves, and the characteristics of the enzyme electrode prepared by the amphiphilic membranes were investigated. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

A number of studies on enzyme immobilization have been carried out[1–5] and a number of immobilization techniques developed.[6–9] Photosensitive polymers have been particularly interesting in enzyme immobilization. With the advantages of the entrapment method, they could be taken as an application of immobilizing carriers; that is, there are no covalent bonds between the polymer matrix and enzyme, thereby minimizing harm to the higher order structures of the enzyme, and maintaining the characteristics of the native enzyme.

Previously, the designs of enzyme electrodes involved wrapping two-layer membranes, a hydrophobic layer of gas permeable Teflon membrane and a hydrophilic layer of enzyme-containing membrane,[10–12] around the terminal of a dissolved oxygen electrode. However, this introduces an interface between the two membranes, which hinders oxygen permeation and slows the rate of response.

To decrease the resistance of gas transfer and to increase the response rate, we present in this article the preparation of hydrophilic–hydrophobic membranes that not only immobilize the enzyme, but also display the characteristics of the Teflon membrane: water impervious and gas permeable. The amphiphilic membranes were prepared from polyvinylcinnamates (PVCins) and polyvinylalcohol (PVA) in the presence of diazoresin and sodium benzoate (SB) as photosensitizers. The hydrophilic–hydrophobic properties and the water and oxygen permeability of the amphiphilic membranes were investigated. The effect of UV light irradiation on the degree of crosslinking was also investigated. The combination of amphiphilic membranes were examined by the scanning electron microscope (SEM) technique. Characteristics of the amphiphilic membranes on the enzyme electrode were compared with those of traditional two-layer systems.

EXPERIMENTAL

Materials

Commercially available PVA of P = 72,000 (n = 1,637; degree of hydrolyzation = 98%) was used without further purification. Cinnamoyl chloride

* To whom correspondence should be addressed.

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purchased from Tokyo Chemical Industry Co., Ltd. was marked EP grad. Glucose oxidase (90 U/mg) and D-(+)-glucose monohydrate (biochemical grade) were from Merck Co.; SB was from Hayashi Co. (GR grade). Diazoresin was synthesized according to the literature.[13]

Measurement

The infrared (IR) spectra were recorded on a JASCO VALOR-III FTIR spectrophotometer. The nuclear magnetic resonance (NMR) spectra were recorded on a Brucker-100, high resolution NMR spectrometer. The UV spectra were recorded on a JASCO 7850 UV/VIS spectrophotometer. The concentration of dissolved oxygen was estimated by using a SUNTEX SD-70 D.O. (dissolved oxygen) meter. The contact angle was evaluated by a face contact anglemeter (Kyowa Kaimenkagaku CA-DT-A).

Synthesis of PVCin

Aqueous Alkali Solution Method

Cinnamoyl chloride dissolved in 24 mL of toluene and 116 mL of methylethylketone (MEK) was added dropwise to the mixture of PVA (1 mol/L, 100 mL) and sodium hydroxide (4 N, 100 mL) at 0–5°C and then stirred for 2 h. The reaction mixture was then dropped into methanol to precipitate the product PVCin.

Organic Base Catalysis

PVA (2.2 g) was suspended in 50 mL of pyridine and heated at 80°C in a steam bath for 24 h, then 50 mL of pyridine was added after the reaction mixture had been cooled to 50°C; cinnamoyl chloride was then added dropwise with shaking; the mixture was stirred at 50°C for 4 h. The reaction mixture was dropped into water to precipitate the PVCin. Hydrophilic HPVCin (7% cinnamoyl group content) and hydrophobic OPVCin (72% cinnamoyl group content) were synthesized in this study.

Photocrosslinking Reaction

When PVCin was irradiated by UV light (8 W, with main wavelength of 254 and 366 nm), the unsaturated cinnamoyl groups photodimerizated to form a cyclobutane ring as well as cross-links.

PVA-diazoresin membrane forms water-insoluble materials by irradiation with UV light (366 nm). PVA is not a photosensitive polymer and does not undergo direct photocrosslinking. If SB is added to PVA matrix[8] as a sensitizer, it will absorb energy and be raised from the ground state to the excited state on irradiation with UV light. The sensitizer then transmits energy to PVA to form active species that initiate crosslinking.

Preparation and Characterization of PVCin Membrane

Preparation of Amphiphilic Membrane

The membranes were made from various wt % PVA–diazoresin and PVA–SB solutions and OPVCin in MEK solution by means of a casting method.[14,15] A typical procedure is described as follows. Glucose oxidase and PVA–diazoresin/PVA–SB were dissolved in phosphate buffer solution; the mixture was poured into the Teflon plate and then dried at 20°C for about 7 h to prepare the first hydrophilic thin membrane. To fabricate an amphiphilic membrane, 3% OPVCin in MEK solution was added on the upper side of the hydrophilic thin film. After drying, the amphiphilic membrane was irradiated by UV light at 20°C and then stored in the freezer.

Identification

Two methods were employed to ensure the combination of amphiphilic membranes. One was to dip the amphiphilic membranes into water for a long time and observe whether the two layers separated due to swelling. The other was to see the SEM photographs of a cross section across the interface of the amphiphilic membranes.

Properties of PVCin Membrane

The properties of the PVCin membrane were evaluated as follows.

Evaluation of Gel Content of Membranes

Some pieces of hydrophilic membranes were irradiated 3.2 cm from the UV lamp for various periods. The membranes were then immersed into 30 mL water at 80°C for 20 min. The degree of insolubility of membranes (wt %) was estimated according to the following equation:

$$W/W_0 \times 100\% \quad (1)$$

where W = residual weight of the membrane after complete washing and W_0 = weight of dry membrane.

Tests of Contact Angle

The hydrophilicity of the membranes were evaluated by the contact-angle meter.

Tests of Oxygen Permeability

The PVCin membranes were affixed to the terminals of a dissolved oxygen electrode by O-ring. The oxygen permeability, the response time, and the change (ppm) or rate of change (ppm/min) of dissolved oxygen concentration were all estimated.

Calculation of Enzyme Activity

The enzyme activity was estimated by recording the rate of change (ppm/min) of dissolved oxygen concentration in buffer solution while the substrate (glucose) was added. The enzyme reaction is shown in eq. (2):

$$\text{glucose} + O_2 + H_2O \rightarrow H_2O_2 + \text{gluconic acid.} \quad (2)$$

Glucose oxidase, 20 mg, was dissolved in 10 mL of phosphate solution (0.03*M*, pH 7.05) to prepare the enzyme solution. Various amounts of the solution were then added to 50 mL of phosphate buffer solution. The D.O. value became stable within a few minutes, and various amounts of glucose dissolved in the buffer solution were added to react. The initial change rate of the D.O. value (ppm/min) in the system was then recorded.

The activity and the maximum reaction rate of native enzyme were evaluated by varying the amount of enzyme or substrate. The amphiphilic enzyme membranes were cut into very small pieces and then put into the 50-mL buffer solution. The method of evaluating the activity of the immobilized enzyme was the same as that used for the native enzyme.

The activity of the immobilized enzyme membranes was calculated at 25°C in 50 mL, pH 7.05 phosphate buffer solution. Glucose solution (2 mL) in various concentrations was added and the activity of the enzyme membranes was then estimated by measuring the change (ppm) of D.O. value in the buffer solution.

RESULTS AND DISCUSSION

Preparation of OPVCin and HPVCin Membranes

HPVCin and OPVCin were synthesized in this investigation; the content of cinnamoyl groups were evaluated by IR, UV, NMR, and elementary analysis (EA) analyses.

As can be seen in Figure 1, the hydrophilicity of the PVCin was measured by the contact angle technique. The contact angle was about 48 and 116° for the HPVCin (C.C./OH = 0.07) and the OPVCin (C.C./OH = 0.72), respectively. The result suggests that increasing the cinnamoyl group centent, decreased the hydrophilicity.

The cinnamoyl group content (C.C./OH) of HPVCin was about 7% and had properties similar to PVA. However, HPVCin was more difficult to dissolve in water than PVA, and it showed toughness, excellent transparency, high absorption of water, and swelling in membrane form.

By comparison OPVCin with 72% cinnamoyl group content (C.C./OH) was examined and showed mechanical brittleness, poor transparency (with slight yellowing), and hydrophobic properties.

The hydrophilic–hydrophobic membranes were prepared by the steps described in Experimental.

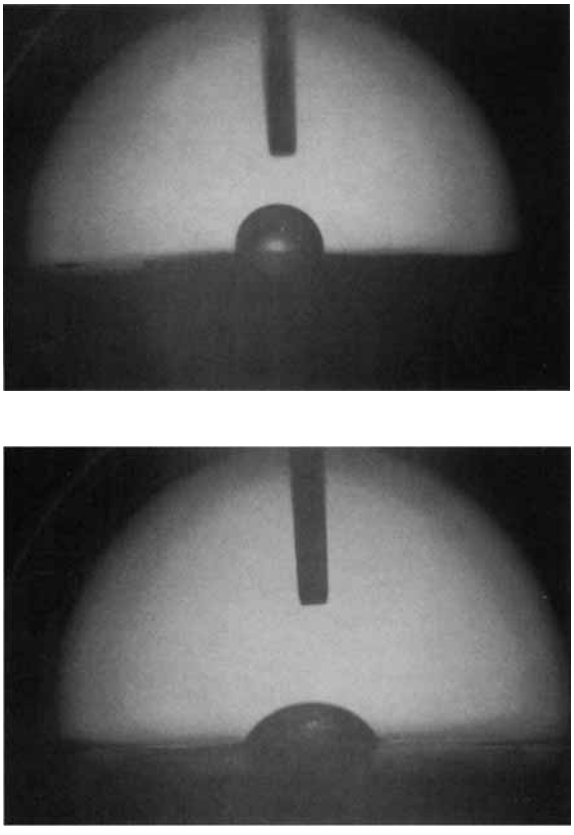

Figure 1 Schematic diagram for contacting angle test: (a) diagram of OPVCin contacted with water drop; (b) diagram of HPVCin contacted with water drop.

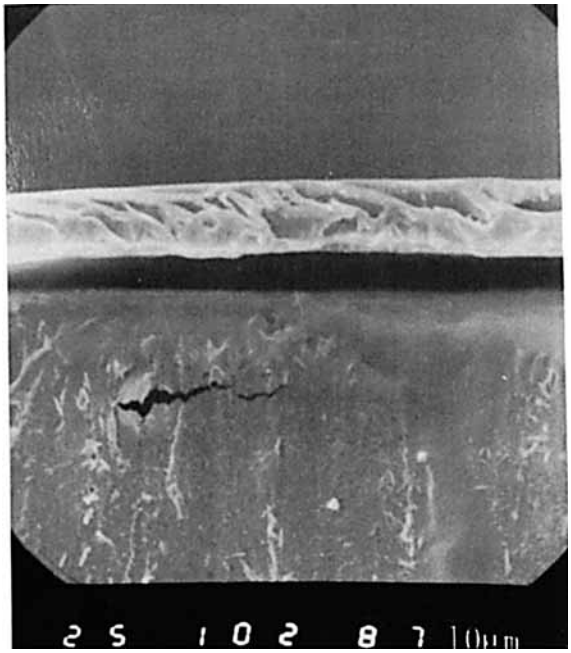

Figure 2 SEM photographs of PVCin amphiphilic membranes: (a) separated HPVCin–OPVCin membrane, ×1,000. (b) Combined HPVCin–OPVCin membrane, ×1,800.

The SEM photographs of the double-layer amphiphilic membranes are shown in Figure 2. The membranes were immersed in water for 1 day at room temperature. Figure 2(a) shows the separation of the amphiphilic membrane that was not irradiated with UV light. This suggests that no chemical bonding existed between the HPVCin and OPVCin layers. When the amphiphilic membrane was exposed to UV light, however, the HPVCin-OPVCin layers were bound together tightly, as can be seen in Figure 2(b). The results suggest that some photosensitive cinnamoyl groups distributed in the HPVCin and OPVCin formed cross-linked spots, preventing the two layers from separating. The extent of swelling was described for HPVCin. But on raising the temperature of immersed membrane to 70°C for 6 h and holding the immersed membrane at room temperature for 7 days, a part of the HPVCin was dissolved. This may be due to the lower content of cinnamoyl groups in the HPVCin leading to fewer crosslinks.

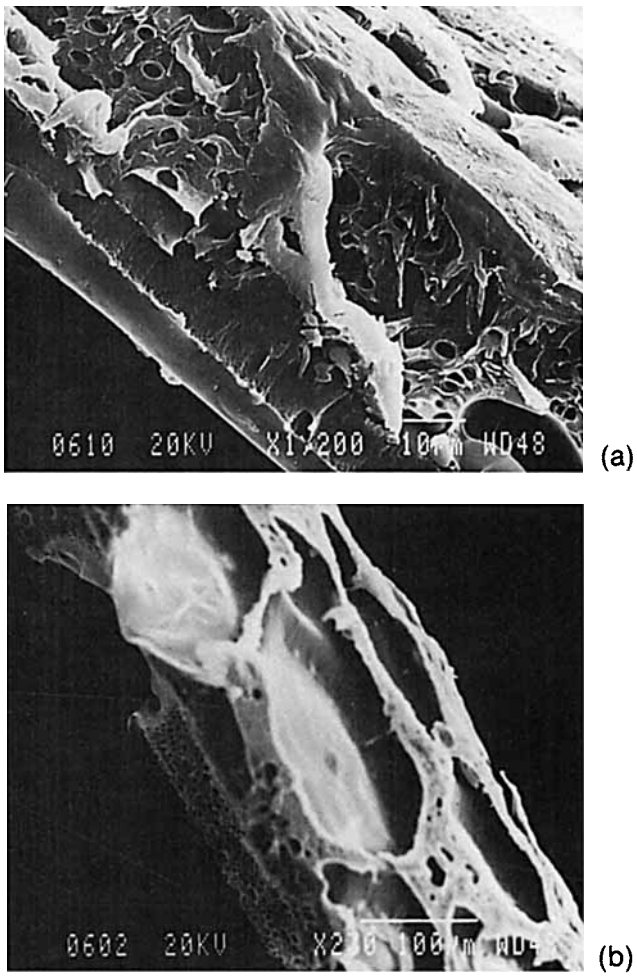

Figure 3 SEM photographs of cross section of the hydrophilic–hydrophobic membranes prepared from PVA and diazoresin: (a) without soaking; (b) after a soaking in water.

The effect of the addition of the UV-curable agent, diazoresin, and the sensitizer, SB, to the amphiphilic membranes was investigated.

Preparation of Amphiphilic Membranes by Diazoresin

The degree of crosslinking depends on the content of diazoresin in the membrane. Increasing the content of diazoresin in the membrane decreased the enzyme loss from the carrier and the toughness of the membrane increased.

To fabricate a 30-μm thick PVA membrane, PVA (0.81 g) dissolved in water was poured into a Teflon cell (7.5-cm diameter). With less PVA, the membrane seemed too thin and brittle; with more PVA, the membrane was distorted and not uniform due to shrinkage. A thicker film might affect the transmission of UV light and increase the resistance to mass transfer.

Alone, the OPVCin membrane was brittle and had poor permeability to oxygen. OPVCin (0.09 g) in MEK was poured into the cell to form a 10-μm OPVCin membrane on top of the PVA membrane. The thickness of the membranes were evaluated by the SEM technique.

It was also found that the ions in buffer solution affects the stability of diazoresin. A critical value of

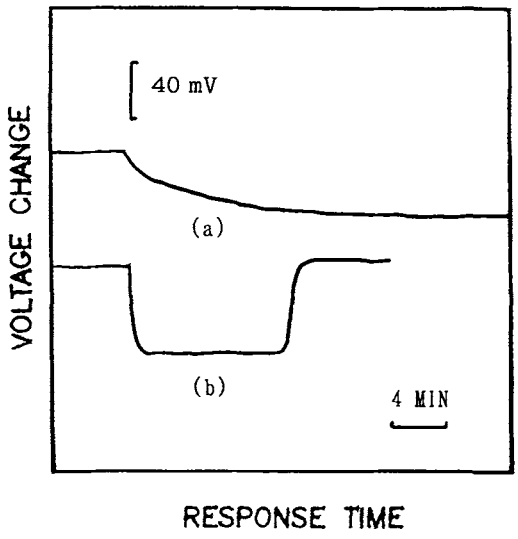

Figure 4 Oxygen permeability of two-layer membrane (PVA layer containing diazoresin with Teflon layer) and amphiphilic membrane (PVA layer containing diazoresin combined OPVCin layer): (a) two-layer membrane; (b) amphiphilic membrane.

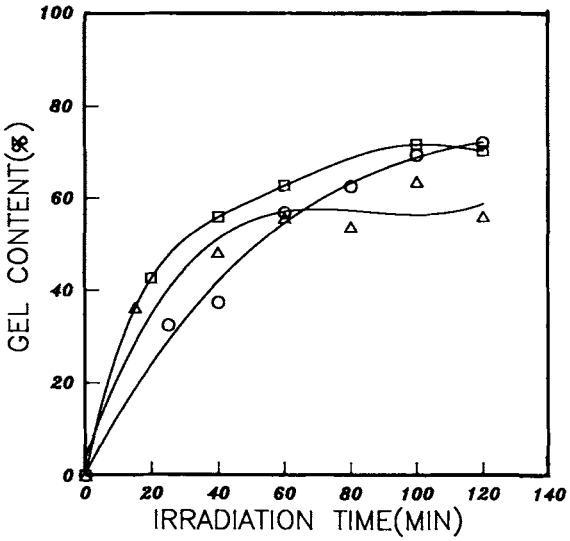

Figure 5 Effect of UV irradiation on degree of insolubility of the membranes for the PVA–SB system: (●) 2% SB; (□) 3% SB; (△) 4% SB.

25 for the diazoresin (g)/buffer (mmol) ratio was determined. When the proportion of the resin was higher than that, the diazoresin precipitated and formed a number of brown particles. When the proportion was lower than the value, however, the precipitation disappeared. A value of 6 wt % of diazoresin in buffer solution was used in this investigation. Gel content was estimated as 80% for the amphiphilic membrane by 6 wt % of diazoresin at an irradiation time of 1 h.

Figure 3 shows the SEM photographs of a combination amphiphilic membrane before and after immersion in water. As shown in Figure 4, the response time of the two-layer film is up to 25 min; however, it takes only 2 min for the amphiphilic membrane. This suggests that the amphiphilic membrane has the advantage over the two-layer system in that it has a short response time and high oxygen permeability.

Preparation of Amphiphilic Membranes by SB

The SB was used as a sensitizer and it can be activated by UV irradiation. As can be seen in the Figure 5, the gel content increased with increasing irradiation time. SB at 3% in PVA should be optimal for the amphiphilic membrane at an irradiation time of 2 h.

For comparison with the PVA–diazoresin system, PVA–SB membranes were prepared under the same

conditions as those described in the PVA–diazoresin system.

Figure 6 shows SEM photographs of the combination amphiphilic membranes made by the PVA–SB system. As shown in Figure 6(a, c), the two layers permeated and crosslinked each other at the interface. In Figure 6(d), the membrane swelled and shrank on immersion in water for 24 days causing the OPVCin to break down. Figure 6(b) shows similar results for a membrane immersed only 8 days. The structure of the membrane was destroyed; the crosslinked spots between the two layers were maintained but some cracks at the juncture of the two components were found.

Application of Amphiphilic Membranes to Enzyme Immobilization

The effect of UV light on the activity of enzymes was investigated by UV spectrophotometer, and the results are shown in the Figure 7. As shown by the Figure 7(a, b), the relative enzyme activity after irradiation at a 366-nm wavelength was much higher than that when irradiated at 254 nm. When irradiated for 90 min, for example, the relative enzyme activity of the enzyme solution decreased to 74 and 4% for 366 and 254 nm, respectively. The sensitizer and crosslinking agent absorbed some of the irradiated UV light and stabilized the enzyme activity.

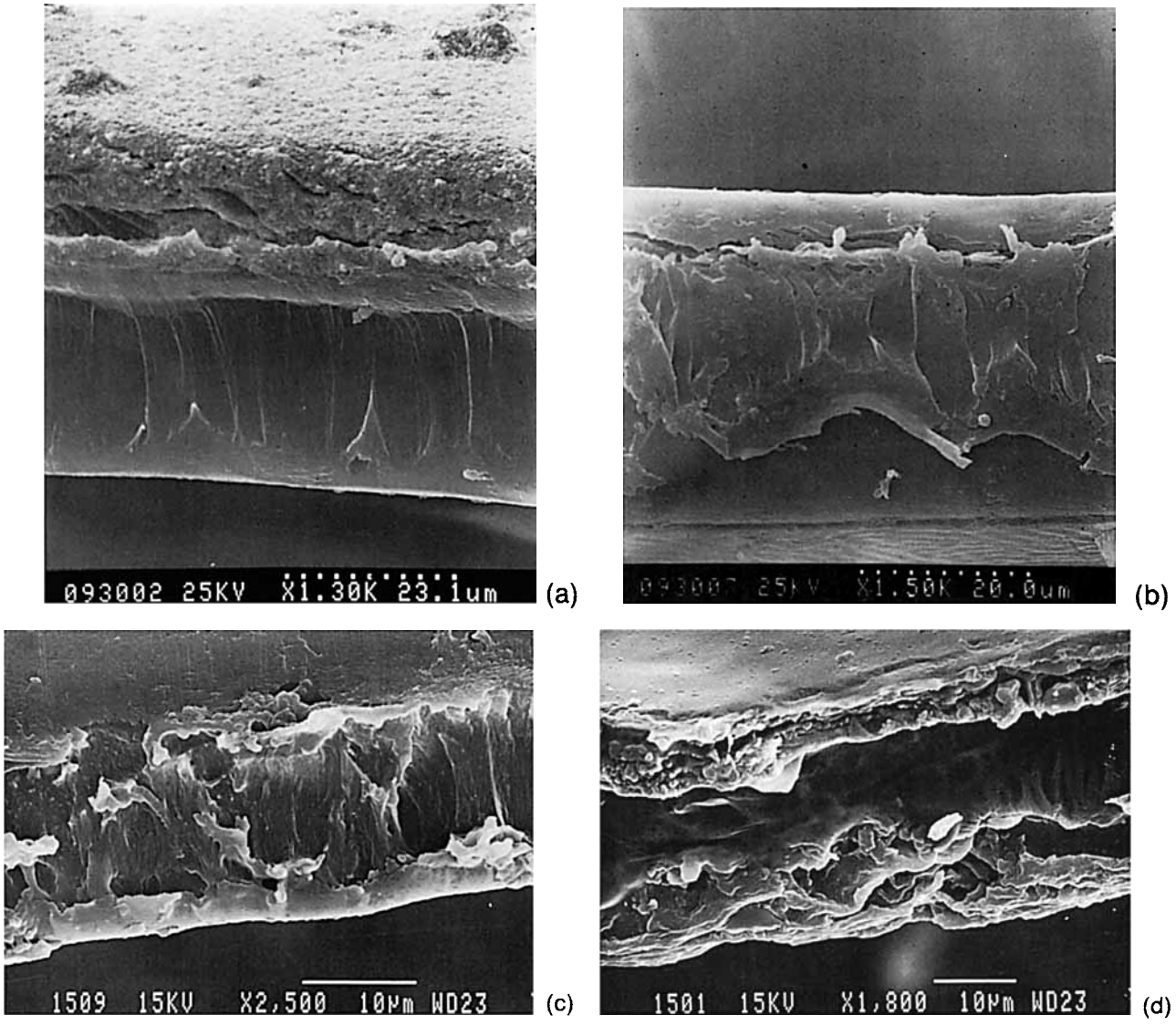

Figure 6 SEM photographs of cross section of the hydrophilic–hydrophobic membrane prepared from PVA and SB: (a), (c) unsoaked; (b) after a soaking for 8 days; (d) after a soaking for 24 days.

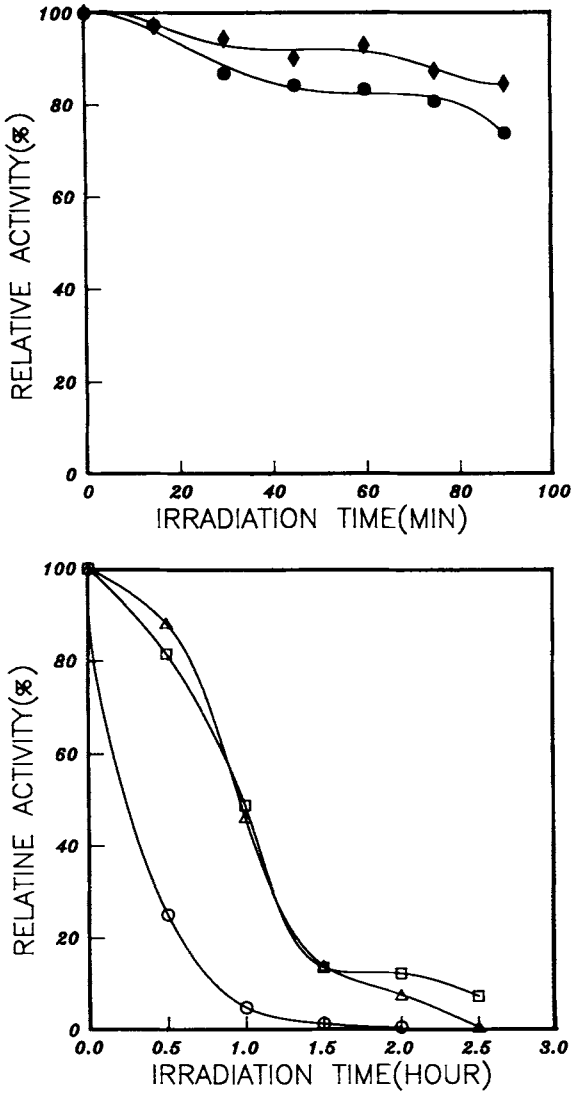

Figure 7 Effects of UV irradiation on enzyme activity. (a) Irradiated at 366 nm: (●) enzyme solution irradiated at 366 nm; (♦) immobilized enzyme in PVA–diazoresin membrane irradiated at 366 nm. (b) Irradiated at 254 nm: (○) enzyme solution irradiated at 254 nm; (□) enzyme solution with SB irradiated at 254 nm; (△) immobilized enzyme in PVA–SB irradiated at 254 nm.

The results suggest that 366 nm is more suitable than 254 nm for the enzyme immobilization. Accordingly, the activity of native enzyme was measured. At a concentration of glucose of 1.5 g/L, a maximum velocity of reaction, V_{max}, from the curve of V_{max} vs. glucose content was obtained. A 1.5-mL sample of 10 wt % (about 3 g/L) glucose solution was added to the reactor to ensure that the V_{max} could be obtained.

Figure 8 shows the enzyme leakage from the amphiphilic membranes. To obtain stable enzyme activity, the enzyme membranes should be immersed in buffer solution for a long period.

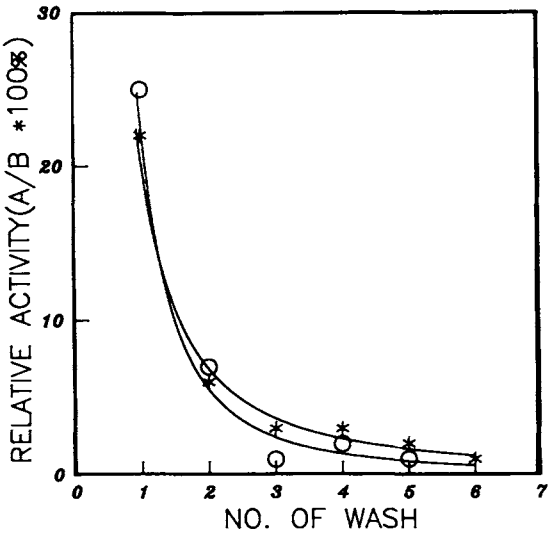

Figure 8 Enzyme leakage of PVA–diazoresin and PVA–SB enzyme membranes: (*) PVA–diazoresin system; (○) PVA–SB system.

Calibration curves on glucose standard solutions obtained with the amphiphilic enzyme membranes of the PVA–SB system are shown in Figures 9 and 10. As shown in Figure 9, when the concentration of glucose exceeded 250 ppm, the plots were no longer linear; on the other hand, for concentrations ranging from 0 to 250 ppm, the electrode could be used as a biosensor. Figure 10 shows the results of the PVA–diazoresin system; the same linear range

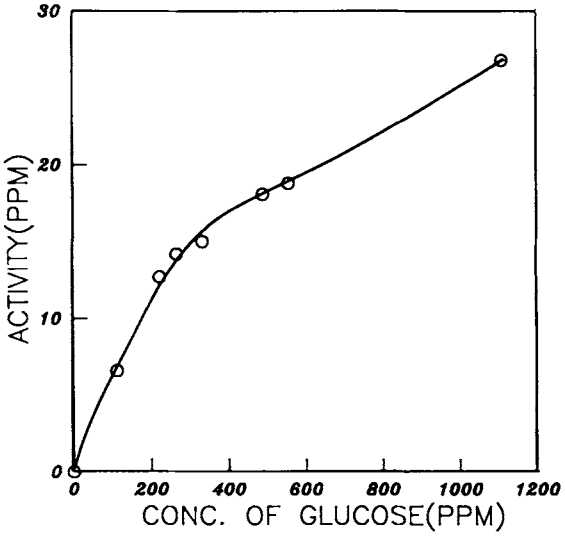

Figure 9 Calibration plot of activity vs. glucose concentration for PVA–SB system.

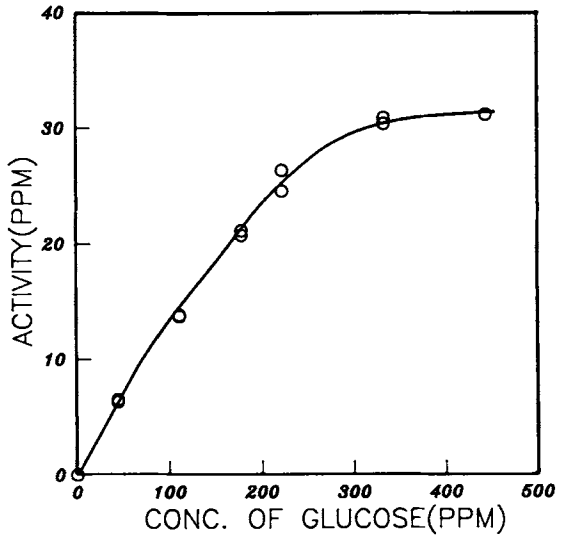

Figure 10 Calibration plot of activity vs. glucose concentration for PVA–diazoresin system.

(0–250 ppm) was observed. The results suggest that the poor oxygen permeability of the amphiphilic enzyme membranes leads to the limitation of the measured range.

Figure 11 illustrates the response of PVA–diazoresin and PVA–SB amphiphilic membranes. Response times of 4–5 and 6–7 min for PVA–diazoresin and PVA–SB systems, respectively, were obtained. The response time of amphiphilic membranes was shorter than that of the traditional two-layer membrane system.

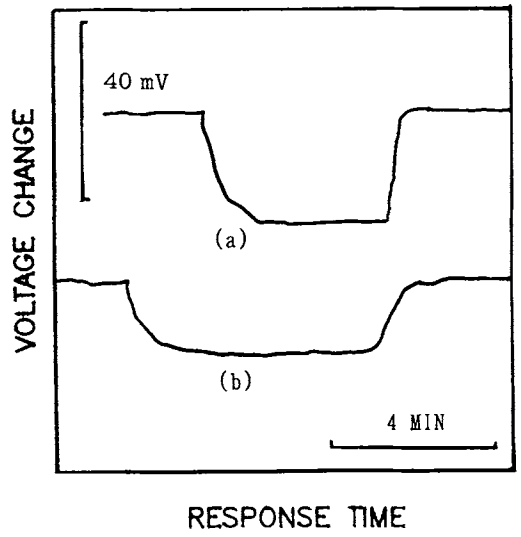

Figure 11 Responses of PVA–diazoresin and PVA–SB amphiphilic enzyme membranes in biosensor: (a) PVA–diazoresin amphiphilic membrane; (b) PVA–SB amphiphilic membrane.

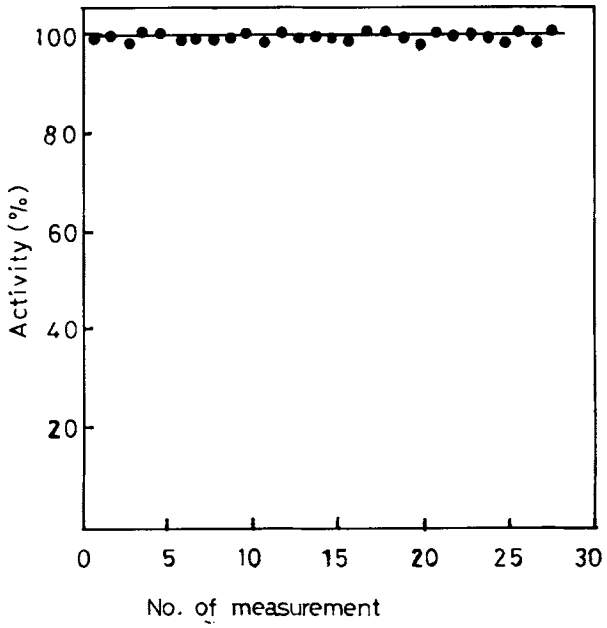

Figure 12 Activity in repeating usage for enzyme immobilized amphiphilic membrane by using diazoresin.

It was found that the activity of the amphiphilic enzyme membranes renews quickly. After the terminal of the enzyme electrode was wiped dry, a steady value was obtained in 3–5 min. As can be seen in Figure 12, the enzyme electrode shows a good service life of continuously working.

CONCLUSION

The hydrophilic HPVCin, PVA–diazoresin, and PVA–SB membranes can be used in combination with OPVCin membrane to fabricate hydrophilic–hydrophobic membranes.

By means of immersed tests, spots on the interface of amphiphilic membrane were shown to restrain the swelling of the hydrophilic layer and to prevent separation.

The advantages of the amphiphilic membrane conferred to the enzyme electrode were faster response and quick renewal of activity. On the other hand, the disadvantages that must be overcome were as follows:

1. When used for a long time, the amphiphilic membrane was destroyed due to the differing properties of the two layers.
2. Due to the lower permeability to oxygen, the measurable range for glucose concentration was narrow.

As mentioned above the linear range of the calibration curves could be applied to the measurement of the concentration of glucose in solution. Furthermore, if the disadvantages of the amphiphilic membrane could be overcome by choosing new materials or by changing the method of manufacture of the membrane, the studies of the amphiphilic membranes, obtained by means of photosensitive polymers, could be employed to develop a miniaturized biosensor.

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