Application of UV-Curable Diazoresin. I. Immobilization of Glucose Oxidase into PVA in the Presence of UV-Sensitive Diazoresin and Sensitizers

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Synopsis

A study of the immobilization of glucose oxidase into poly(vinyl alcohol) membrane in the presence of UV light sensitive diazoresin and benzoic acid and/or sodium benzoate sensitizers was carried out. An effective enzyme electrode by using the immobilized glucose oxidase membrane was developed. The effect of the concentration of diazoresin and sensitizers in poly(vinyl alcohol), UV light irradiation on the degree of insolubility as well as the activity yield of the membrane were examined for the immobilized glucose oxidase using glucose as a substrate. An unstable phenomenon was found in the initial usage of the immobilized glucose oxidase membrane prepared from poly(vinyl alcohol)-sensitizer system. Activity yields of 39.98 and 35.30 for poly(vinyl alcohol)-diazoresin and poly(vinyl alcohol)-sensitizer system were obtained, respectively.

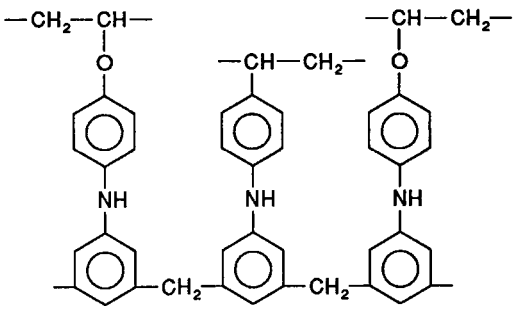
INTRODUCTION

Many studies on the enzyme immobilization have been carried out,[1-7] and a number of immobilizing techniques have been developed.[8-14] Photochemical gelation has been also employed in the biological application to immobilize bioactive materials.[15-17] The photochemical technique to entrap bioactive materials possesses merits due to the milder network formation of polymeric matrix where no change in pH or temperature is necessary.

There are many photosensitive polymers (polyvinyl cinnamate,[18] polyvinyl-p-azidobenzoate,[19] etc.) were synthesized from PVA and the photopolymers were applied in many industrial fields. The entrapment of enzyme within a membrane is favorable for enzyme reaction since the surface area of a membrane is large. It is well known that PVA is nontoxic and gives a hydrophilic tough membrane, these properties of PVA are favorable in its usage as a immobilizing carrier. PVA membrane can be easily cross-linked by means of ultraviolet (UV) light irradiation in the presence of photosensitizer. This immobilization method is useful because of its easy handling and a mild condition for the immobilization of bioactive materials including enzymes, organelles, and microbial cells.

In the present paper we report results of a study of the immobilization of glucose oxidase into PVA membrane in the presence of UV light sensitive diazoresin and benzoic acid and/or sodium benzoate sensitizers. We developed an effective enzyme electrode by using the immobilized glucose oxidase membrane. An unstable phenomenon was found in the initial usage of the immo-

Journal of Applied Polymer Science, Vol. 40, 2161–2171 (1990)
 CCC 0021-8995/90/11-122161-11$04.00



Scheme 1.

bilized glucose oxidase membrane, which was prepared from PVA containing a certain amount of benzoic acid and/or sodium benzoate. Activity yield of the glucose oxidase membrane in the photochemical immobilization was evaluated. Effects of irradiation time on degree of insolubility of the photosensitive membranes and on activity of glucose oxidase membrane were also investigated.

EXPERIMENTAL

Materials

Commercially available PVA of $\bar{P}$ = 72,000 ($\bar{n}$ = 637 degree of hydrolysation > 98%) was used without further purification. Glucose oxidase from Sigma Co. (3500 U/mg) and from Merck Co. (90 U/mg), glucose from Merck Co. (biochemical grade), benzoic acid from Merck Co. (GR grade), and sodium benzoate from Hayashi Co. (GR grade) were used without purification. Diazoresin was prepared according to the literature.[20]

Measurements

IR spectra were recorded on Jasco IR-810 grating IR spectrophotometer. NMR spectra were recorded on a Brucker-100 high resolution NMR spectrometer. UV spectra were recorded on Hitachi UV-200-20 spectrophotometer. Concentrations of oxygen dissolved in water were estimated by using Suntex SD-70 D.O. meter.

TABLE I
Dependence of the Degree of Insolubility on the Diazoresin Concentration in Poly(Vinyl Alcohol)

Diazoresin added (wt %)	1	2	3	4	5	6
Appearance	Dissolved	Rent	Rent	No change*	No change	No change

* No change on external appearance.

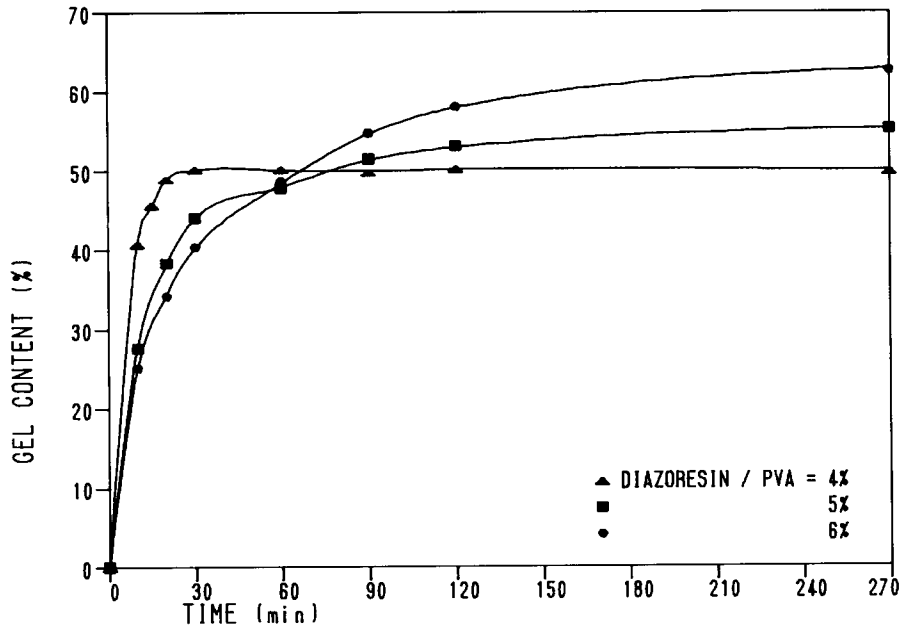


Fig. 1. Effect of irradiation time on degree of insolubility of the membrane for PVA-diazoresin system.

Gelation of Poly(Vinyl Alcohol)

The PVA was gelled as follows. Membranes ca. 60 μm in thickness were obtained by casting 10 wt-% PVA solution containing diazoresin or benzoic acid and/or sodium benzoate sensitizers on polytetrafluoroethylene plate dried at room temperature. Various amounts of sensitizer were used to evaluate the effects of sensitizer concentration on gel content. The membrane was irradiated with 8 W Camag UV lamps (main wave-length, 254 nm and 365 nm) at a distance of 3 cm at room temperature. Effects of irradiation time on degree of insolubility of the membrane for PVA-diazoresin (irradiated with λ_m = 365 nm lamp) and PVA-sensitizer (irradiated with λ_m = 254 nm lamp) systems were investigated.

Gel content was estimated by heating W_0 g UV light irradiated PVA membrane in 30 mL water at 80°C for 1 h, and then evaluated the residual membrane weight W g. The gel content (%) was defined by

$$\frac{W}{W_0} \times 100\%$$

Diazoresin is sensitive to UV light, and decompose photochemically according to the two primary processes represented by eq. (1) and (2).

$$R{-}C_6H_4{-}N_2^+X^- \xrightarrow{h\nu} R{-}C_6H_4^+ + N_2 + X^- \quad (1)$$

$$R{-}C_6H_4{-}N_2^+X^- \xrightarrow{h\nu} R{-}C_6H_4^{\bullet} + N_2 + X^{\bullet} \quad (2)$$

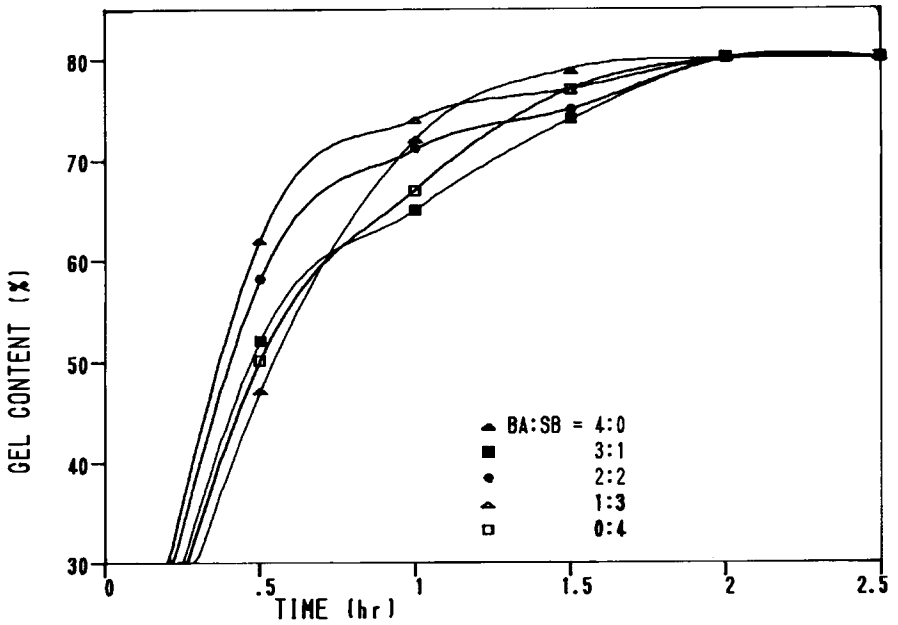


Fig. 2. Effect of irradiation time on degree of insolubility of the membrane for PVA-sensitizer system.

As shown in scheme 1, diazoresin cross-linked the chain molecules of PVA on irradiation with UV light. Covalent bonds were formed between PVA and diazoresin in the photochemical reaction.

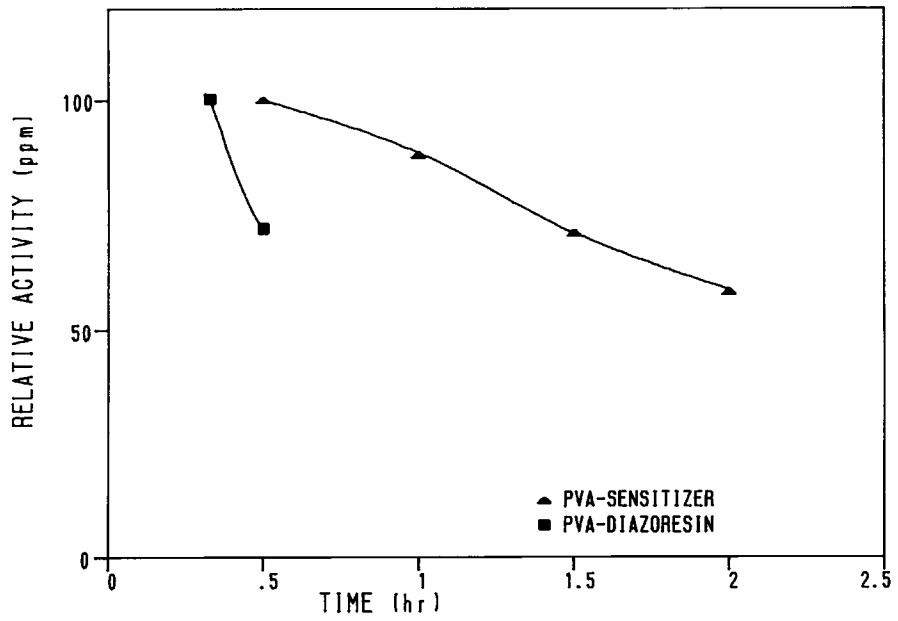


Fig. 3. Irradiation time dependence of the relative activity of immobilized enzyme membranes.

TABLE II
Activity Yields of Enzyme Membranes in pH 7.05 Phosphate Buffer Solution at 25°C

System	PVA-Diazoresin		PVA-Sensitizer	
	No. 1	No. 2	No. 1	No. 2
Wt. of membrane (g)	0.1051	0.1105	0.1090	0.1007
Real amount of enzyme in these membranes (mg)	3.713	3.900	3.847	3.554
Activity of immobilized enzyme (ppm/min)	0.291	0.318	0.266	0.256
Activity of free enzyme (ppm/min)	0.742	0.780	0.769	0.711
AY (%)	39.19	40.77	34.59	36.00
Average AY (%)	39.98		35.30	

In the PVA-sensitizer system, PVA was activated by the UV light excited sensitizers (benzoic acid and/or sodium benzoate). The mechanism of the photochemically reaction can be expressed as following equations.

$$D_0 \xrightarrow{h\nu} D^* \tag{3}$$

$$D^* \rightarrow D_0 + \text{hv (or heat)} \tag{4}$$

$$D^* + \text{PVA} \rightarrow D_0 + \text{PVA}^* \tag{5}$$

$$\text{PVA}^* \rightarrow \text{crosslinking} \tag{6}$$

D_0 denotes the ground state, D^* denotes the excited state of the sensitizers. Equation (4) shows the deactivation of the excited sensitizers.

Immobilization of Glucose Oxidase

PVA (0.6 g) in 8 mL phosphate buffer solution (0.03 M, pH = 7.05) was heated to 60°C with stirring. After PVA was desolved completely, the PVA

TABLE III
Enzyme Loss from the Immobilized Membrane. Reaction was Carried Out at 25°C in pH 7.05 Phosphate Buffer Solution

System	PVA–Diazoresin		PVA–Sensitizer	
	No. 1	No. 2	No. 1	No. 2
Wt. of membrane (g)	0.1048	0.1057	0.1068	0.1032
Activity of solution (ppm/min)	0.011	0.014	0	0
B: Apparent amount of enzyme in solution (mg)	0.055	0.057	0	0
A: Amount of enzyme used in immobilization (mg)	3.699	3.731	3.769	3.642
B/A (%)	1.49	1.53	0	0
Average B/A (%)	1.51		0	

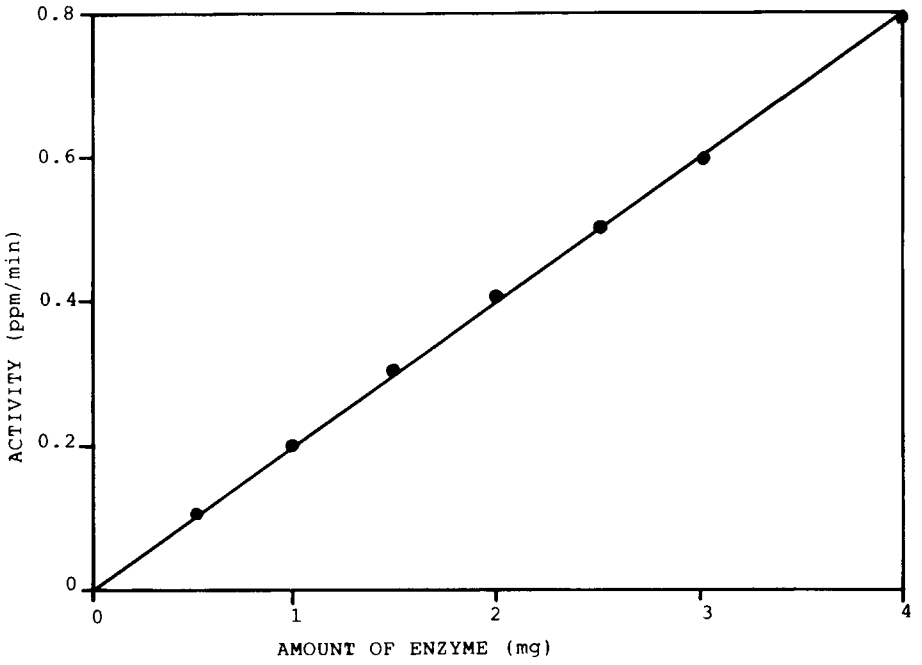


Fig. 4. Calibration plot of activity versus free enzyme concentration.

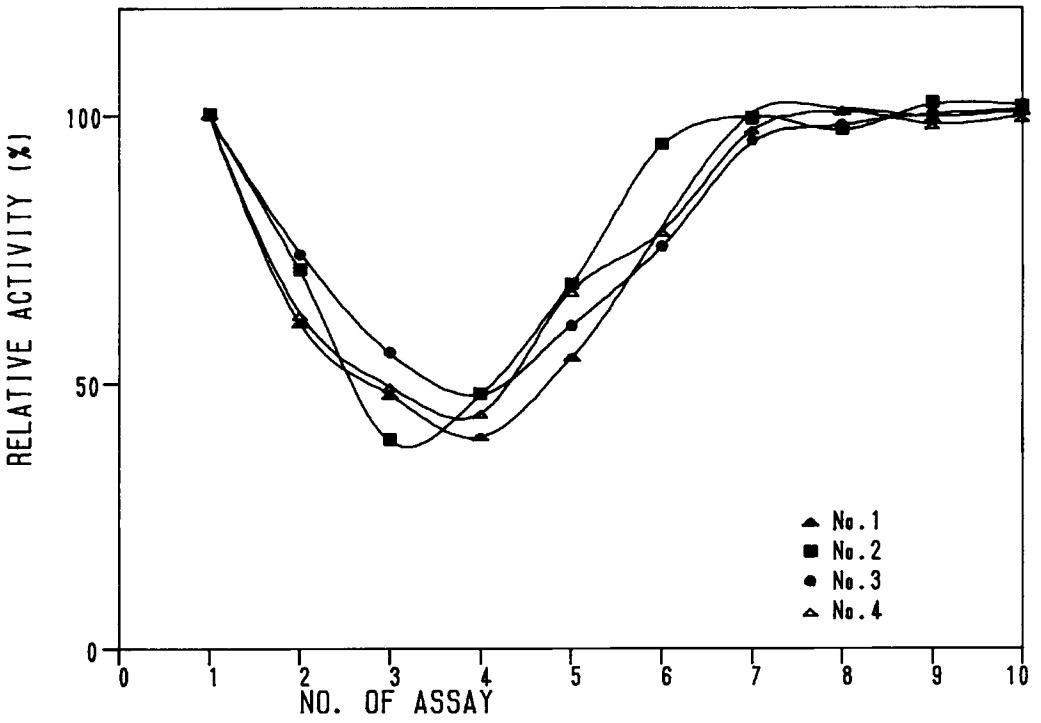


Fig. 5. Activity in repeating usage for PVA-sensitizer system.

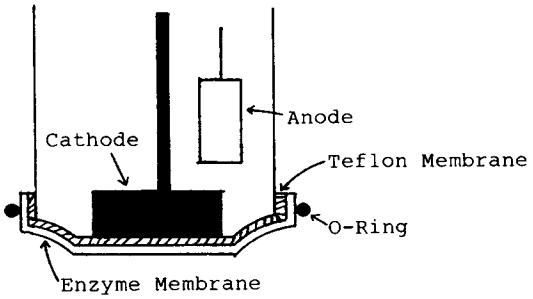


Fig. 6. Enzyme electrode.

buffer solution was cooling at room temperature. To the PVA buffer solution was added 2 mL 0.03 M (pH = 7.05) phosphate buffer solution containing a given amount of glucose oxidase. Membranes were prepared by casting the PVA buffer solution on a polytetrafluoroethylene plate and dried at room temperature. The enzyme-entrapping membrane was cut into 1.7 × 1.7 cm squares and then irradiated with a 8 W Camag UV lamp (main wavelength of 254 nm and 365 nm were used) at a distance of 3 cm at room temperature. The enzyme-entrapping membranes thus obtained were stored in refrigerator to be subjected to the series.

Estimation of Enzyme Activity

Enzyme activity was estimated by measuring the change (ppm) or change rate (ppm/min) of dissolved oxygen concentration in buffer solution while the substrate (glucose) was added. Enzyme membrane was fixed at the surface of cathode with a O-ring as shown in Figure 6. A schematic diagram for enzyme analysis is shown in Figure 7. Enzyme reaction occurred as the following eq. (7) in the enzyme membrane, and the principle of the glucose electrode is shown in Figure 8. Enzyme reaction:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\textit{glucose oxidase}} H_2O_2 + \text{Gluconic Acid} \qquad (7)$$

Glucose oxidase 20 mg was dissolved in 10 mL phosphate solution (0.03 M, pH = 7.05) to prepare an enzyme solution. Enzyme solution (0.25 mL) thus obtained was added to 50 mL phosphate buffer solution, the D.O. (dissolved oxygen) value will appear a stable data within a certain time. Glucose solution (0.5 mL, 10%) was then added and recorded the initial change rate of the D.O. value (ppm/min) in the system.

Immobilized Enzyme Activity

Enzyme membrane was cut into very small pieces and then put into 50 mL buffer solution. The immobilized enzyme activity was then estimated by the method as described in free enzyme activity. The release of immobilized enzyme was evaluated by washing the enzyme membrane in 10 mL phosphate buffer solution. The release amount of the immobilized enzyme was estimated by the

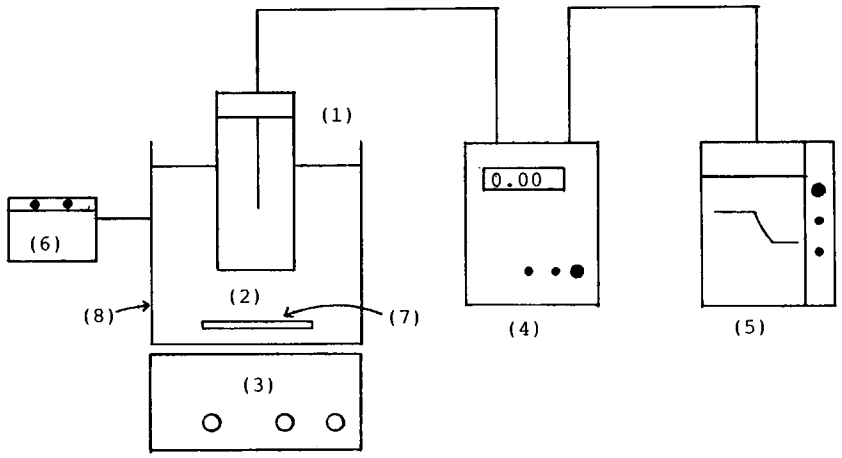


Fig. 7. Schematic diagram for enzymatic analysis. (1) Oxygen Electrode; (2) Bulk Solution; (3) Magnetic Stirrer; (4) D.O. Meter, (5) Recorder; (6) Temperature Controller; (7) Stirring Bar; (8) Reaction Cell.

calibration curve of the activity versus free enzyme concentration as shown in Figure 4.

RESULTS AND DISCUSSION

Gelation of Poly(Vinyl Alcohol)

(a) PVA-Diazoresin System

The photochemical reaction of the UV light sensitive PVA-diazoresin system was examined by IR spectra. IR absorption of the photosensitive polymer at 2240 cm^{-1} ($-N\equiv N$ absorption) and 1595 cm^{-1} (C—N absorption) disappeared while the polymer was irradiated by UV light for a certain period. This result shows that N_2 was released during the UV light irradiation. Diazoresin crosslinked the chain molecules of PVA on irradiation with UV light as shown in scheme 1.

Table I shows the dependence of the degree of insolubility on the diazoresin concentration in the PVA membrane. As can be seen in the table, the degree of insolubility increased with increasing the diazoresin concentration added. Figure 1 shows the effect of irradiation time on the degree of insolubility of the membrane in various sensitizer concentrations for PVA-diazoresin system. Although the gel content increased with increasing the diazoresin concentration after a sufficient UV light irradiation, as shown in Fig. 1, the initiating rate of the crosslinking decreased.

(b) PVA-Sensitizer System

Benzoic acid and sodium benzoate were used as UV light sensitizers in this photocurable system. Figure 2 shows the dependence of the gel content on the irradiation time in various sensitizer concentrations added in PVA membrane.

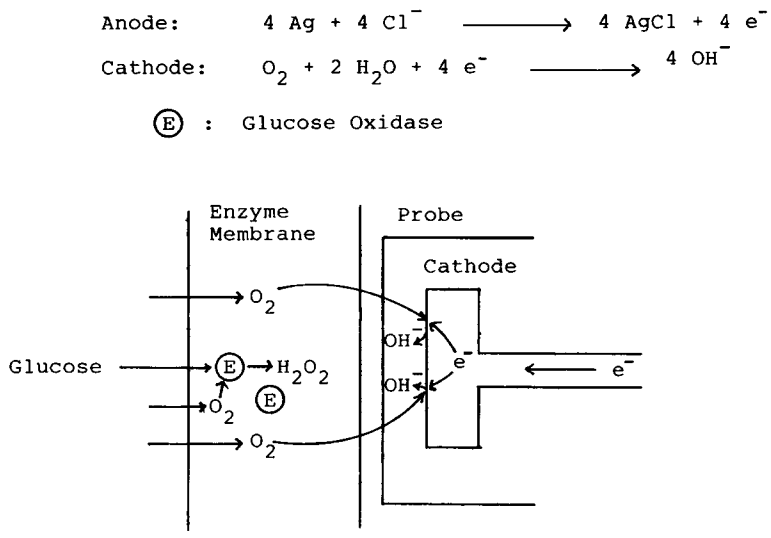


Fig. 8. The principle of glucose electrode.

In Fig. 2, BA : SB expressed the molar ratio of the benzoic acid to sodium benzoate. The condition of 1/3 (BA/SB) molar ratio and 1 h irradiation was choiced in our investigation, from the considering of the membrane strength and the enzyme activity decay on UV light irradiation. As shown in Fig. 2, the gel content increases to about 80% in the early stage but remains constant after 2 h UV light irradiation. The optimal initiating rate of the crosslinking was obtained in the 1/3 (BA/SB) molar ratio system.

Immobilization Conditions

Figure 3 shows the effect of irradiation time on immobilized enzyme activities. The reaction was carried out at 25°C in pH 7.05 phosphate buffer solution. As can be seen in the figure, the relative activity decreased rapidly with increasing the irradiation time for PVA-diazoresin system. This may be caused by the UV light (λ_{max} = 365 nm) degradation on the immobilized enzyme in PVA membrane and/or difficulty in diffusion of the substrate due to crosslinking of the membrane.

Relative activity of the immobilized enzyme in PVA-sensitizer system also decreased with increasing the irradiation time, however, the immobilized enzyme activity decreased slower than that of free enzyme while exposed in the UV-light. From Figs. 1–3, conditions of 4% diazoresin and 20 min irradiation for PVA-diazoresin system, 1/3 (BA/SB) molar ratio, and 1 h irradiation for PVA-sensitizer system show the optimal results. These two conditions were choiced in this investigation to estimate the characteristics of immobilized glucose oxidase.

Characteristics of Immobilized Enzymes

The effect of the concentration of diazoresin and sensitizers (benzoic acid and/or sodium benzoate) in PVA, and UV light irradiation on the degree of

insolubility as well as the activity of the membrane were examined for the immobilized glucose oxidase using glucose as a substrate. The degree of insolubility (gel content) was given by the percentage ratio of the weight of the membrane after and before immersion of the membrane in water for 1 h at 80°C. The activity yield (%) was defined by

$$\text{AY}\ (\%) = \frac{\text{activity of immobilized enzyme}}{\text{activity of native enzyme}} \times 100$$

where the amount of the native enzyme employed was the same as that employed in immobilization.

Activity yields of the PVA-diazoresin system and PVA-sensitizer system were summarized in Table II. Activity yields of 39.98 and 35.30 for PVA-diazoresin and PVA-sensitizer systems were obtained, respectively. The reaction was carried out at 25°C in pH 7.05 phosphate buffer solution, 4% diazoresin and, 20 min irradiation, BA/SB = 1/3 sensitizers and 1 h irradiation were choiced. Figure 4 shows the calibration plot of the activity of the immobilized enzyme membrane versus free enzyme concentration.

Table III shows the amount of enzyme loss from the immobilized membrane. As can be seen in the table, these photochemically immobilized membranes can provide a suitable environment to entrap the glucose oxidase effectively.

It is well known that, response time of the enzyme electrode is always affected by the concentration of the substrate, stirring speed, pH value of the buffer solution, and reaction temperature. Response time of the two photocurable system was also estimated.

The response time of 2–5 min and 1–3 min for PVA-diazoresin and PVA-sensitizer were obtained, respectively. This result suggests that structure of the polymer matrix after UV irradiation may affect the enzyme reaction though they have the same polymer backbone, i.e., steric hinderance of the polymer matrix should be an important factor on the enzyme reaction. A hydrophilic and soft polymer matrix provide less disturbance environment for the enzyme reaction and/or for easy diffusion of the substrate. Steric structure of the two UV curable PVA after UV irradiation are quite different since one is initiated by UV excited sensitizer and the other is cross-linked by UV-curable diazoresin.

An unstable phenomenon was found in the initial usage of the immobilized glucose oxidase membrane in PVA-sensitizer (benzoic acid and/or sodium benzoate) system as shown in Fig. 5. We believe that a conformational change and/or gel hindrance existed in polymer matrix will affect the relative activity of the enzyme membrane.[21] Detail studies on the unstable phenomenon and the characteristics (temperature and pH dependences of the relative activity, storage stability, and stability in repeating usage, etc.) of these two photochemically immobilized glucose oxidase are now in progress.

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Received February 1, 1989
Accepted August 23, 1989