Urease Immobilized Polyvinyl Alcohol-*g*-Butyl Acrylate Membrane for Urea Sensor

G. H. HSIUE* and Z. S. CHOU, *Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan, Republic of China,* and N. YU and K. P. HSIUNG, *Union Chemical Laboratory, Industry Technology Research Institute, Hsinchu, Taiwan, Republic of China*

Synopsis

A copolymer with balanced hydrophilicity and hydrophobicity was synthesized by grafting butyl acrylate onto poly(vinyl alcohol) (PVA-*g*-BA). Films made from it had good urea permeability. After immobilization of the enzyme urease on the surface of it, the film was attached to the tip of an ammonia gas electrode to form an enzyme sensor. The sensor was able to detect urea in solution in the range of 6 to 600 mg/dL with a response time of about 5 min. It might be reused for over 100 times, and the reproducibility was very good. Therefore, it is very promising that this sensor may be commercialized for clinical use. The synthesis of PVA-*g*-BA was initiated with cerium ammonium nitrate, and the immobilization of urease was done by crosslinking with cyanuric chloride of glutaraldehyde. The former agent was proved to be more effective than the later. The amount of enzyme immobilized with the former were 26 and 13 times more than the one- and two-step methods with the latter, respectively.

INTRODUCTION

Many electrode-type sensors have long been commercialized for measuring and monitoring of chemical substances: for example, pH, carbon dioxide, oxygen, and ammonia electrodes, etc. They are faster, easier to operate, and sometimes more accurate than traditional wet-chemistry or spectroscopic methods. By combining these sensors to other automatic accessories, they may be used for continuous and long-term monitoring of the concentration changes of a certain chemical.

When a membrane with biological functionality is attached to the electrode, a biosensor is formed.[1] The acquirement of biological functionality may be done by immobilizing biomolecules (i.e., enzyme, bacteria, organelle or antigen/antibody, etc.) onto the membrane. When substrate molecules come into contact with their corresponding biomolecules (e.g., urea and urease), chemical reaction between them generate a certain change which can be transformed into electric signal and detected by the readout system. There are three major

*To whom correspondence should be addressed.

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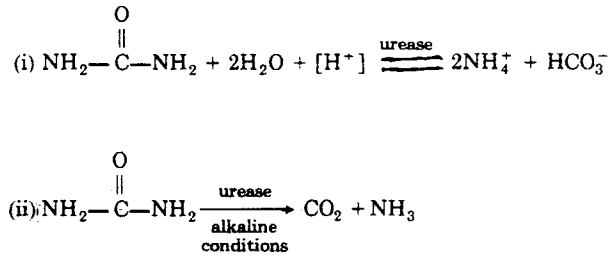


types of electrode for the detection of urea in blood. They are (1) NH_4^+ electrode,[2-4] (2) NH_3 gas electrode,[5-7] and (3) pH electrode.[8]

In the case of the urea–urease reaction, two different chemical equations have to be considered:[8]

When the first equation applies, the concentration of urea may be detected with an NH_4^+ or a pH electrode. An ammonia gas electrode, however, may be used in the second case.

Pioneer works on the urease-electrode-involved physical entrapment of the enzyme within a polyacrylamide gel membrane which was attached to a glass NH_4^+ electrode,[2] adding a layer of nonactive-silicone rubber membrane over the enzyme membrane to increase the selectivity of the NH_4^+ electrode,[3] and chemical crosslinking of urease and semipermeable membrane with glutaraldehyde and usage of an NH_3 electrode.[5-7] Research works had also been done on urea sensors[9,10] by combining various types of enzyme immobilized membranes with an ammonia gas electrode.[11-14] However, membranes used in these other studies were common, commercialized, nonspecific films with limited capability for urease immobilization. Therefore, the amount of enzyme immobilized was usually very low and response time was too long.

In this study, a new type of polymer membrane, PVA-*g*-BA, was introduced. After treatment with hydrazine, this membrane has many functional amino groups on its surface which are available for chemical bonding with urease. Different crosslinking reagents, glutaraldehyde and cyanuric chloride, were tested in order to find the optimal enzyme loading condition. In addition, properties of selective gas-permeable membrane were studied.

After combining the urease–PVA-*g*-BA membrane with an NH_3 electrode, this biosensor was able to detect urea in the range of 6–600 mg/dL, and serum samples were tested. Since it gives a fast response time (about 5 min) and highly reproducible results, it has a good potential to be commercialized for clinical use.

EXPERIMENTAL

Reagents

Poly(vinyl alcohol) was obtained from Chang Cheu Petrochemical Co. Ltd., Taipei, R. O. C. (identification code no. BF-17). It has a polymerization degree

of 1750 and is 98.3–99 mol % hydrolyzed. Butyl acrylate is of industrial grade. After washing with 5% NaOH, it was dehydrated with $CaCl_2$ and distilled under vacuum. Cerium ammonium nitrate [$Ce(NH_4)_2(NO_3)_6$], hydrazine 100%, and glutaraldehyde are all products of Merck Co. (Hohenbrunn, F.R.G.). Cyanuric acid, also a Merck reagent, was recrystallized with carbon tetrachloride before use. Urease, phenol nitroprusside, alkaline hypochlorite, urea nitrogen standard solution, and urease buffer reagent were all obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Urea is a product of Kanto Reagent Co. (Osaka, Japan). Other chemicals used are all of reagent grade. The porous polypropylene membrane is a product of Ceglard (no. 3510), thickness 2.5×10^{-2} mm, pore size 0.04 μm, and porosity 45%.

EXPERIMENTAL PROCEDURE

Graft Copolymerization of PVA-*g*-BA

A 5% (wt %) PVA solution was bubbled with nitrogen gas under stirring for 1 h to deprive oxygen. Definite amounts of BA and 0.1*N* cerium ammonium nitrate (Ce^{+4}: BA = 0.05 : 1, w/w) were added successively. The stirring was continued for 1.5 h until a milk-white copolymer was obtained.

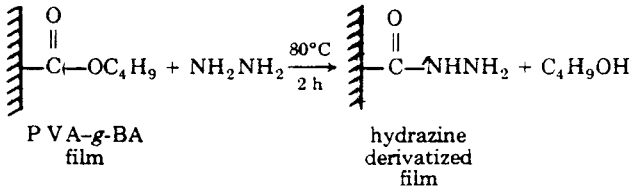
Membrane Casting

The milk-white copolymer was casted on a glass plate which had been first covered with a layer of PP film. The thickness of the membrane was controlled with a tape. The membrane was then air-dried at ambient temperature and stored in a beaker with distilled water. The beaker was then put inside a 80°C over for 2 days to drive out unreacted PVA. The water had to be changed every 12 h. The extracted membrane was again air-dried at room temperature.

The degree of grafting was examined by an IR spectrometer (Perkin-Elmer, Model 567) and an element analyzer (Perkin-Elmer 240C).

Preparation of Hydrazine Derivatized PVA-g-BA Membrane

The membrane was reacted for 2 h at 80°C with a 100% hydrazine solution. Hydrazine transformed the butyl groups on the membrane into amino groups. The membrane was washed with distilled water and stored in a cold room.



Determinations of the Water Content, Degree of Swelling, Wettability, Solute[15] and Water[16,17] Permeabilities, and Mechanical Properties of the Membrane

Water content and degree of swelling were calculated by the following equations:

$$\text{water content (\%)} = \frac{\text{wt of wet membrane} - \text{wt of dried membrane}}{\text{wt of wet membrane}} \times 100$$

$$\text{degree of swelling (\%)} = \frac{\text{diameter of wet membrane} - \text{diameter of dried membrane}}{\text{diameter of dried membrane}} \times 100$$

Wettability was determined with a contact-angle meter (ERMA, Model G-1). Solute permeabilities were tested with an apparatus described in previous paper[15] and calculated according to the following equation:[15]

$$P = \frac{-\Delta x}{(1/V_1 + 1/V_2)} \ln \frac{1 - C_2/C_1}{1 + (V_2/V_1 \cdot C_2/C_1)}$$

where P = permeability (cm^2/s), Δx = thickness of the wet membrane (cm), V_1, V_2 = volumes of the left and the right cells (cm^3), A = area of the membrane (cm^2), and C_1, C_2 = concentrations of solute in the left and the right cells, after time t (mg/mL). Water permeability was calculated by the following equation:[16,17]

$$K = \frac{VL\eta}{tA\Delta P}$$

where K = permeability coefficient of water (cm^2), V = volume of water permeated (mL), η = viscosity of water (P),[18] L = thickness of the membrane (cm), A = area of the membrane (cm^2), ΔP = pressure drop (dyn/cm^2), and t = time (s). In addition, the averaged pore radius r was calculated[17] as $r = \sqrt{8K/s}$, where s is the water content of the membrane.

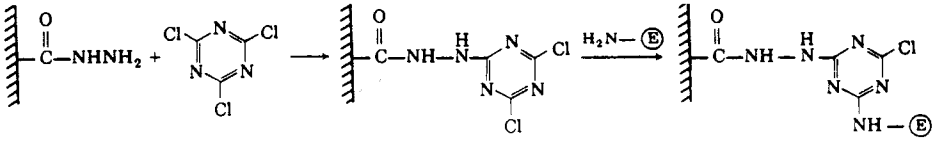
The mechanical properties, i.e., tensile strength and elongation at break, were tested with a Shimadzu Co. Universal Testing Machine, Model DCS-500.

Enzyme Immobilization

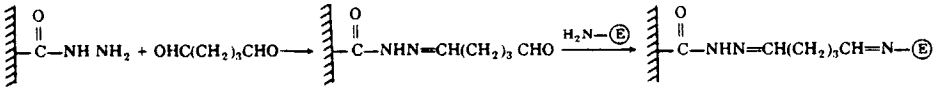
Urease was attached to the membrane through chemical bonding. Three different methods had been tested. They were:

Cyanuric Chloride Method (or Trichloro-s-Triazine (TST) Method.[19] Small disks of hydrazine derivatized membrane, with a diameter of 2 cm, were prepared with a punch. They were first immersed into a dioxane solution containing 5 wt % TST and stirred at 40°C for 2 h. They were then washed

rapidly with 0.01*M*, pH 2.0 HCl solution and immersed again into 0.1*M*, pH 6.0 phosphate buffers solution (KPB), which contained urease. The reaction was carried out at 4°C under shaking for 9 h. The membranes were washed with a 0.01*M* phosphate buffer and soaked in 0.1*M*, pH 8.5 phosphate buffer to hydrolyze the unreacted chloride on TST. They were stored in 0.1*M*, pH 8.0 phosphate buffer. The scheme for this method is shown below.



One-Step Glutaraldehyde Method.[20] The membrane was immersed into a PH 6.0, 0.1*M* phosphate buffer solution (KPB) containing urease. Glutaraldehyde was added to a final conc of 3.33%. They were reacted with shaking, at 40°C for 10 h. The membrane was washed with 0.01*M* phosphate buffer and stored in PH 8.0, 0.1*M* phosphate buffer. The scheme is shown below:



Two-Step Glutaraldehyde Method.[20] The membrane was first reacted with 5% glutaraldehyde for 10 min, and washed with distilled water. It was then soaked in pH 6.0, 0.1*M* phosphate buffer solution (KPB) containing urease. They were reacted, with shaking, at 4°C for 10 h. The membrane was washed and stored as described above.

Measurement of the Enzyme Activity[21]

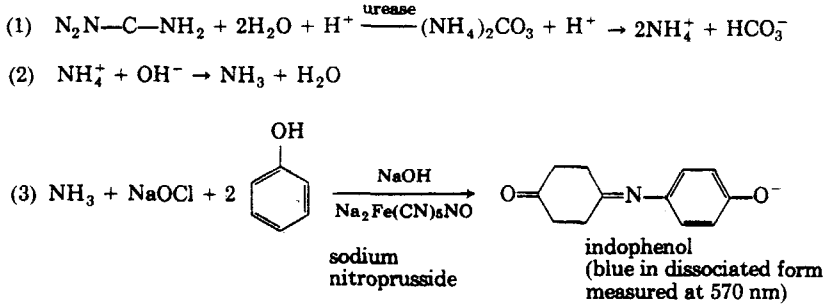
Immobilized Urease. One unit is defined as that amount of urease, immobilized on a membrane which is 2 cm in diameter and 0.05–0.01 mm in thickness, which liberates one μmol of NH_3 in 1 min at 25°C and pH 7.0 using the Berthelot method[21,22] of measurement described below:

$$\text{immobilized urease unit } (\text{Unit}_{\text{immob}}) = \frac{\mu\text{mol } NH_3}{\text{min} \times \text{film area} \times 2}$$

Soluble Urease. One unit is defined as that amount of urease which liberates 1 μmol of NH_3 in 1 min at 25°C, pH 7.0 using the Berthelot method:

$$\text{soluble urease unit } (\text{Unit}_{\text{sol}}) = \frac{\mu\text{mol } NH_3}{\text{min} \times \text{mL enzyme solution}}$$

Berthelot Method. The reactions involved are:



Ammonia Gas Electrode

The ammonia gas electrode was made of pH combination electrode (Ingold, made in Swiss) and a porous, hydrophilic polypropylene membrane (Celgard, thickness 2.5×10^{-2} mm, pore size 0.04 μm). The inner electrolyte solution was made of 0.5 mL, 0.05 *M* KCl, 1.5 mL, 0.005 *M* NH_4Cl and 5 mL, pH 6.0, 0.1*M* phosphate buffer. The electrode was connected to a pH meter (Orion Research Digital 611) and a recorder (Linear Instrument Co., Model 885) and tested for its performance.

Urea Sensor

The urea sensor was made by covering the tip of the ammonia gas electrode with a piece of membrane which had the enzyme urease immobilized on it.

RESULTS AND DISCUSSION

Synthesis and Characterization of the PVA-*g*-BA Membrane

The composition and the synthesis conditions of the PVA-*g*-BA membrane are shown in Table I. The weight percents of PVA and BA were determined by the elementary and IR analyses. PVA has a characteristic —OH group absorption peak at 3300 cm^{-1} whereas BA has a C=O peak at 1725 cm^{-1}. The ratio of PVA to BA is linearly correlated with the ratio of these two peaks. Figure 1 shows the IR spectra of PVA, PBA, and samples nos. 1–3.

TABLE I
Composition of PVA-*g*-BA Graft Latex Membranes[a] after Hot Water Extraction[b]

Sample no.	PVA (g)	H_2O (g)	BA (g)	Monomer conversion (%)	PVA content (wt %)	BA content (wt %)	BA graft percent (%)
1	5	95	3	88.6	71.8	28.2	39.4
2	5	95	6	88.2	65.4	34.6	52.9
3	5	95	9	85.2	60.2	39.8	66
4	5	95	12	88.4	53.6	46.4	86.5
5	5	95	15	90.5	52	48	92.2

[a] $Ce^{+4} = 0.025 \times$ BA (w/w).
[b] Extracted with water at 80°C for 12 h.

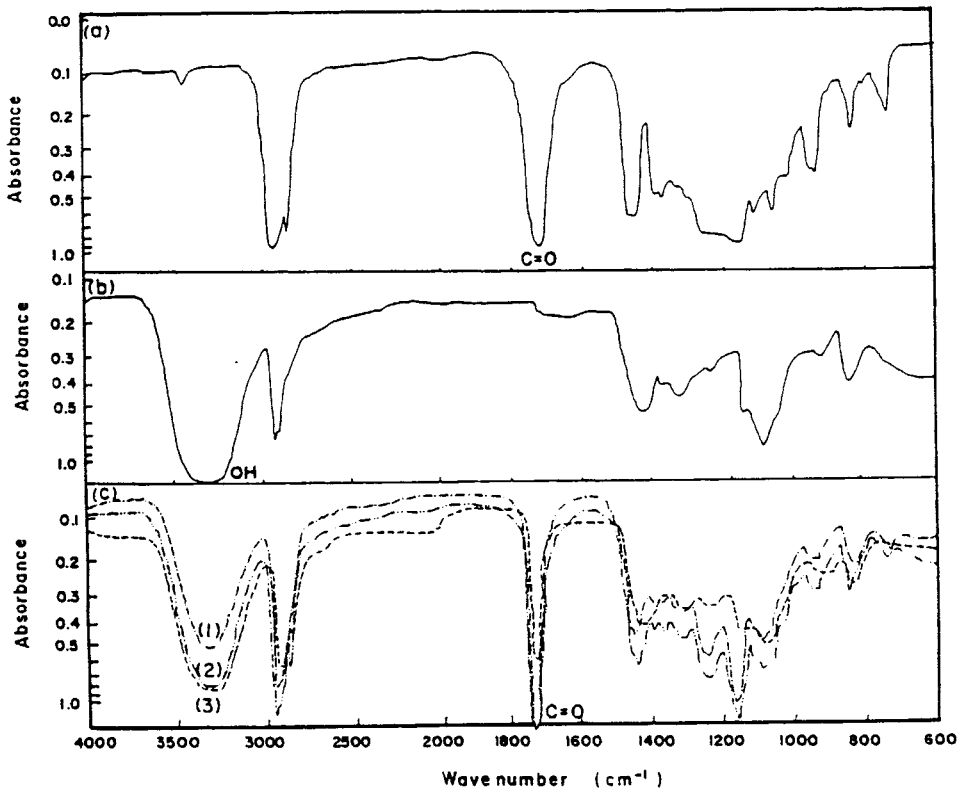


Fig. 1. Infrared spectra of: (a) polybutyl acrylate (PBA), (b) poly(vinyl alcohol) (PVA), and (c) PVA-*g*-BA copolymer: (1, ---) PVA 71.8 wt %, BA 28.2 wt %, (2, · · · ·) PVA 65.4 wt %, BA 34.6 wt %, (3, ---) PVA 60.2 wt %, BA 39.8 wt %.

TABLE II
Properties of the PVA-*g*-BA Graft Latex Membrane

Sample no.	Water content (%)	swelling (%)	permeability coefficient (cm^2)	Average pore radius (nm)	Permeability for urea (cm^2/s)	Contact angle (°)
1	14.8	7.5	4.18×10^{-15}	3.9	1.27×10^{-6}	43
2	11.2	5	3.5×10^{-16}	1.58	1.01×10^{-6}	75
3	7.95	4	3.6×10^{-16}	1.9	6.36×10^{-7}	81
4	4	1.5	3.7×10^{-16}	2.75	1.79×10^{-7}	85
5	2	0.5	2.1×10^{-16}	9.16	—	88

When the amount of BA in the PVA-*g*-BA membrane increases, the peak intensity at 1725 cm^{-1} increases while that at 3300 cm^{-1} decrease.

The Effect of the Amount of BA Grafted on the Properties of the Membrane

The properties of the PVA-*g*-BA graft latex membrane are shown in Table II. PVA is a crystalline polymer with high tensile strength. PBA, being amorphous in nature, has a glass transition temperature (T_g) at −50°C and is a rubber at room temperature. The mechanical properties of a dry PVA-*g*-BA

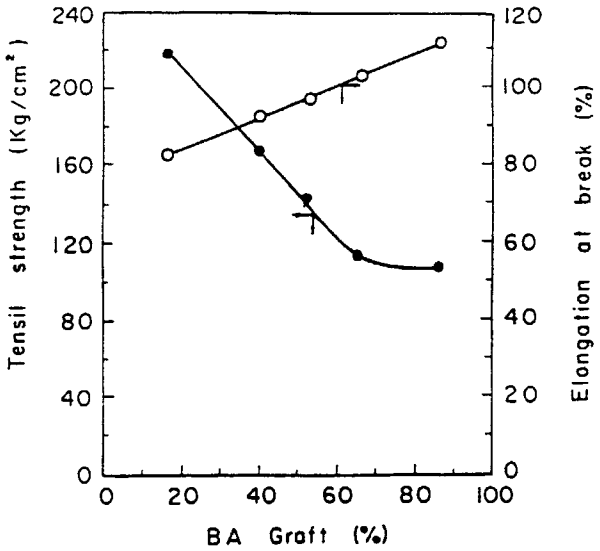


Fig. 2. Effect of the amount of grafted BA on tensile strength and elongation at break of PVA-*g*-BA latex membrane.

membrane are shown in Figure 2. The tensile strength decreases with the increase of the BA content whereas the elongation increases with the increase of BA. Since the butyrate group of PBA is hydrophobic in nature, when the amount of PBA increases, the hydrophobicity increases. The water content and the wettability, however, decrease. On the other hand, since urea is hydrophilic, its permeability through the membrane decreases as the hydrophobicity of the membrane increases. The no. 2 composition in Table I was chosen to synthesize a membrane with high urea permeability and good mechanical properties.

The Optimum Conditions for Enzyme Immobilization

The effect of temperature on the percentage of hrdrazine derivatization is shown in Figure 3. The wt % of the $-NHNH_2$ groups are calculated from elementary analysis.

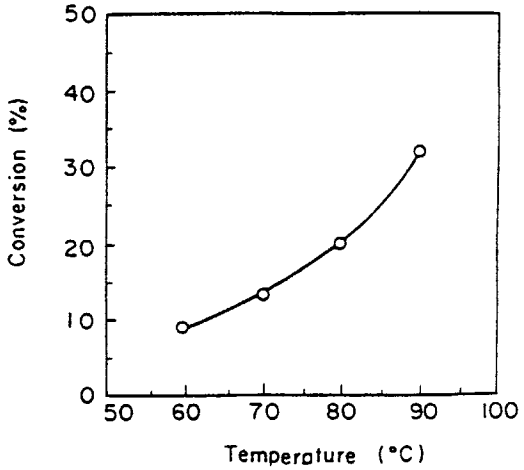


Fig. 3. Effect of temperature on the hydrazine conversion of PVA-*g*-BA latex membrane.

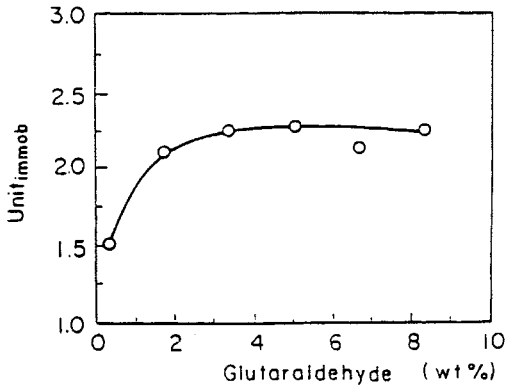
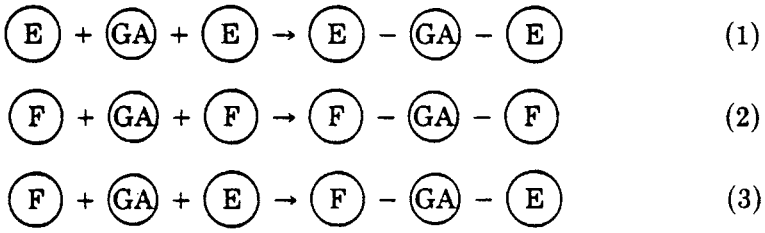


Fig. 4. Effect of glutaraldehyde concentration on the urease immobilization, one-step method (pH 8.0, 0.1*M* KPB, 303 urease unit/mL, immobilization time 6 h, 4°C).

However, with high percentage of derivatization, the mechanical properties of the membrane in water are bad. Therefore, a temperature of 80°C was chosen for the hydrazine reaction.

Figures 4–6 show the effects of glutaraldehyde concentration, the pH value, and the immobilization time on the amount of urease immobilized on the membrane (one-step method). The crosslinking reactions occur between enzyme–enzyme, enzyme–membrane, and membrane–membrane, as shown in the following diagram:



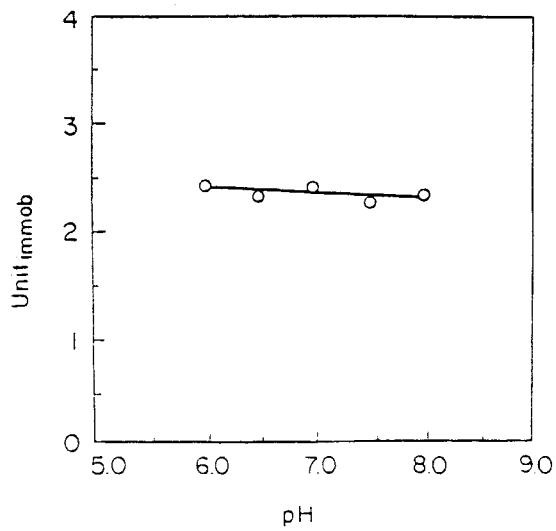


Fig. 5. Effect of pH on the urease immobilization, one-step method (0.1*M* KPB, 303 urease unit/mL, GA 3.33%, immobilization time 6 h, 4°C).

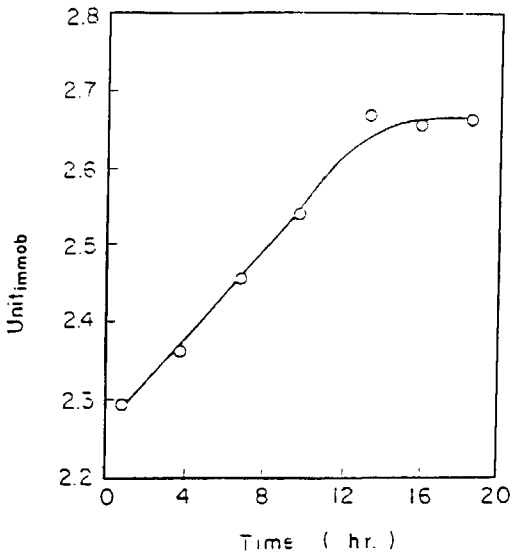


Fig. 6. Effect of immobilization time, one-step glutaraldehyde method. (pH 8.0, 0.1*M* KPB, GA 3.33%, 303 urease unit/mL, 4°C).

Where (F) = amino groups on the membrane, (E) = amino groups on urease, and, (GA) = glutaraldehyde. The probabilities of these three reactions are almost equal, and therefore the result is not satisfactory.

Figure 4 indicates that when the concentration of glutaraldehyde increases, reactions (1) and (2) have surpassed reaction (3) and result in a reduced amount of immobilized urease.

Figure 7 shows that when the concentration of urease increases, reaction (1) and (3) increase. But, reaction (3) has a lower probability than reaction (1) at high urease concentration. Thus, the amount of immobilized urease reaches a plateau and decreases.

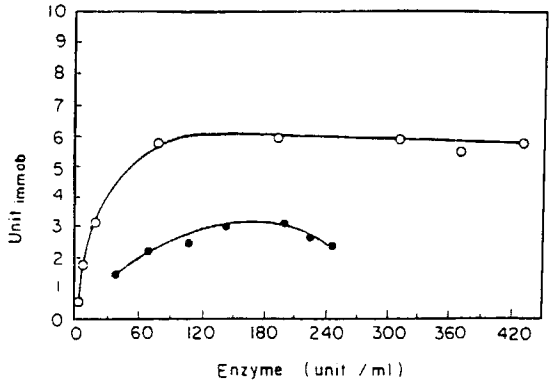


Fig. 7. Enzyme loading effects, one-step and two-step glutaraldehyde methods: (●) one-step (pH 6.0, 0.1*M* KPB, 5% GA, immobilization time 10 h, 4°C); (○) two-step (5% GA + Film reaction time: 10 min, pH 6.0, 0.1*M* KPB, immobilization time 7 h, 4°C).

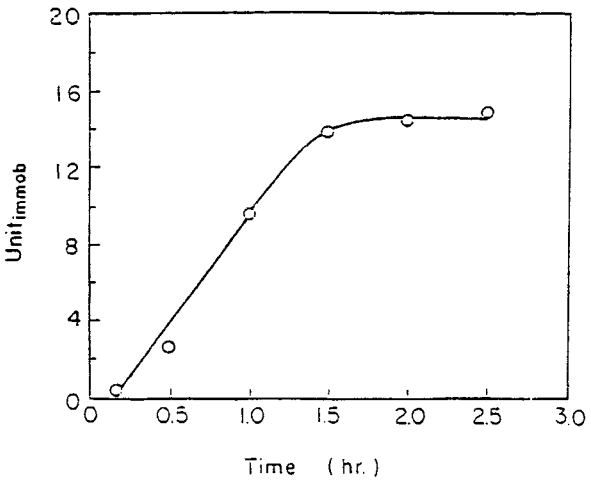
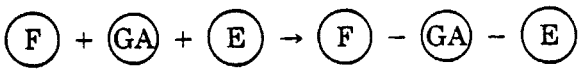


Fig. 8. Effect of the activation time on the amount urease immobilized on PVA-*g*-BA latex membrane by the trichloro-*s*-triazine method (pH 7.0, 0.1*M* KPB, 303 urease unit/mL, immobilization time 3 h, 4°C).

In order to increase the enzyme immobilized, two-step glutaraldehyde method was performed, and the result is also shown in Figure 7. The mechanism is shown as:

(4)



(5)

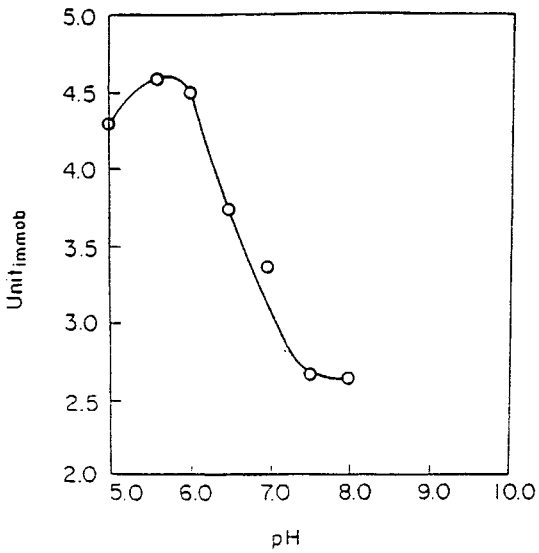


Fig. 9. Effect of pH on the urease immobilization, trichloro-*s*-triazine method ($KHC_8H_4O_2$–NaOH buffer solution: pH 5.0–6.0, KH_2PO_4–NaOH buffer solution (KPB): pH 6.0–8.0, urease 303 unit/mL, activation time 0.5 h, immobilization time 3 h, 4°C).

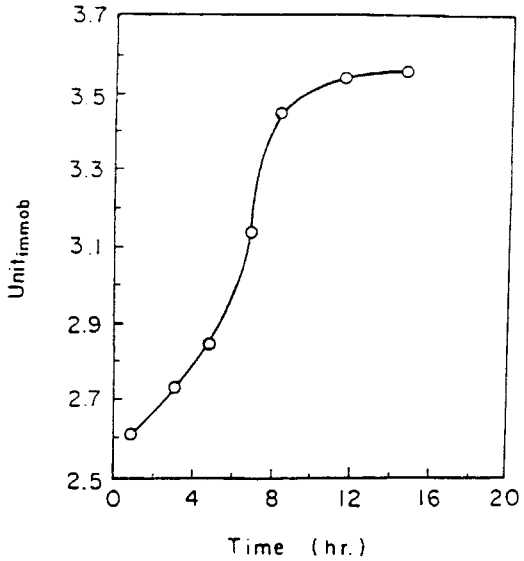


Fig. 10. Effect of urease immobilization time of PVA-*g*-BA latex membranes by trichloro-*s*-striazine method (conditions: pH 8.0, 0.1*M* KPB, urease 303 unit/mL, activation time 0.5 h, 4°C).

The amount of urease immobilized by the two-step method is twice of that by the one-step method.

The optimum conditions, i.e., activation time, pH value, and immobilization time, for urease immobilization using cyanuric acid as the crosslinking reagent are shown in Figures 8–10.

It is obvious from Figure 8 that the longer the activation time, the more the number of cyanuric acid molecules bound to the membrane and the more the number of urease molecules attached to the membrane.

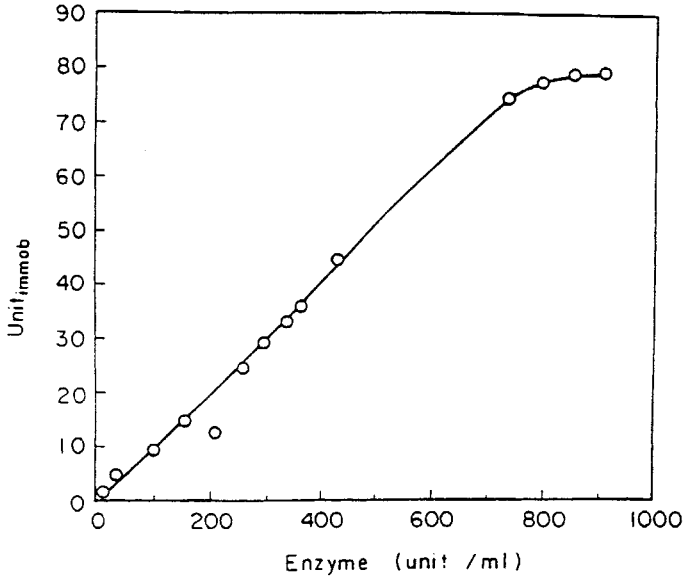


Fig. 11. Enzyme loading effect, trichoro-*s*-triazine method (pH 6.0, 0.1*M* KPB, activation time 2.5 h, immobilization time 9 h, 4°C).

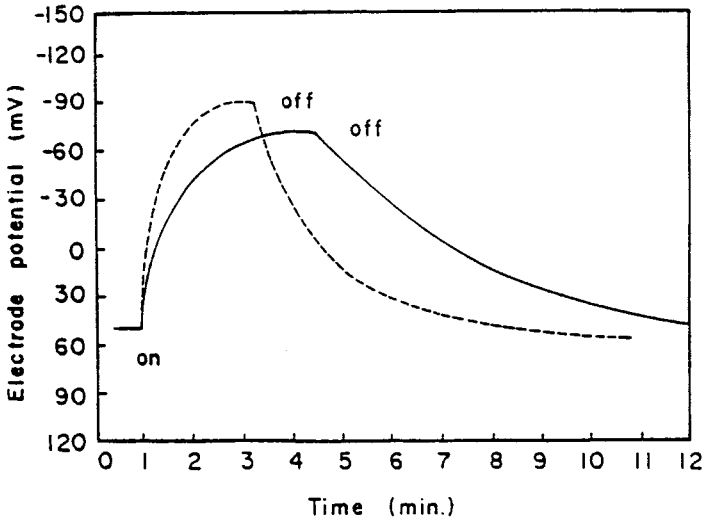


Fig. 12. Dynamic response curves of two different membranes toward 10 mL 0.01*M* NH_4OH was used: (—) hydrazine derivative of PVA-*g*-BA membrane; (---) porous PP membrane.

Since the first chloride ion on cyanuric acid dissociates at pH 1.0–2.0, the second one at pH 5.0–6.0, and the third one at alkaline pH, and since the optimum pH for urease immobilization is 5.6 (Fig. 9), it may be concluded that the urease molecule attaches when the second chloride ion dissociates from the cyanuric acid molecule.

The amount of urease attached to the membrane reached a value of 80 enzyme unit under the optimum conditions, as shown in Figure 11. When Figure 11 is compared with Figure 7, it is clear that the cyanuric acid method is much better than the glutaraldehyde method. The amount of urease immobilized by the former method is 13 times of that by the two-step glutaraldehyde method and 26 times of that by the one-step method.

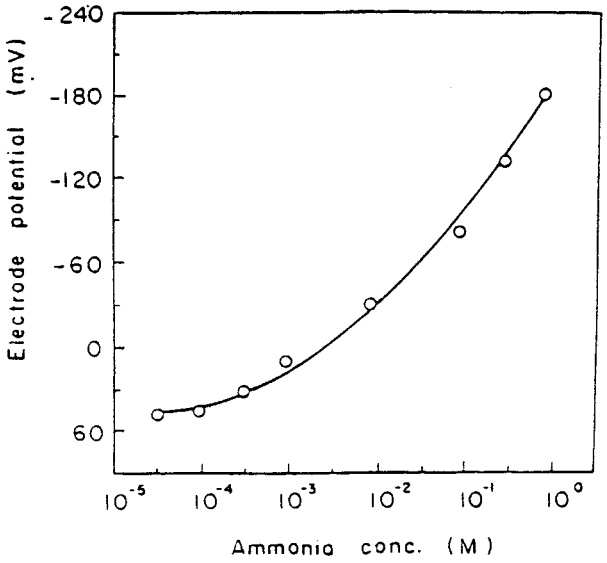


Fig. 13. Working curve for an ammonia-gas-sensing electrode.

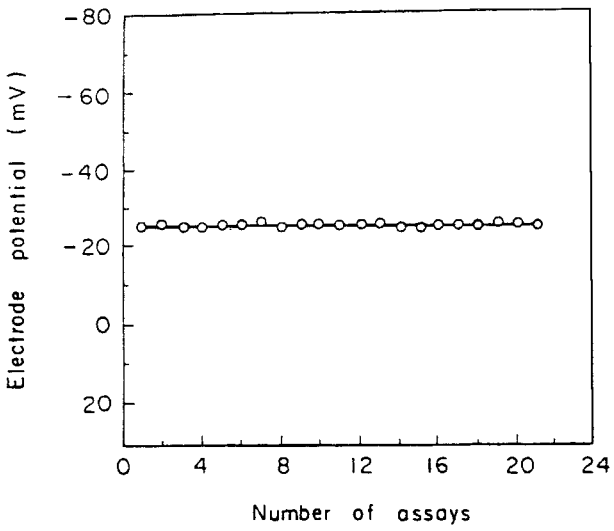


Fig. 14. Reproducibility of an ammonia-gas-sensing electrode.

Preparation of an Ammonia Gas Electrode

Figure 12 shows that both the response and the recovery times of the ammonia gas electrode prepared with a PVA-*g*-BA membrane are longer than an electrode prepared with polypropylene membrane. Since the porosity of the PP member is better than the PVA-*g*-BA membrane, the above-described phenomenon can be attributed to the faster diffusion velocity of ammonia through the former membrane, than the velocity of the latter.

The characteristics of the ammonia electrode are shown in Figures 13 and 14. The lowest concentrations of ammonia detectable by this electrode is $10^{-4}M$. The reproducibility is also very good, since the electropotential does not change even after 22 continuous determinations.

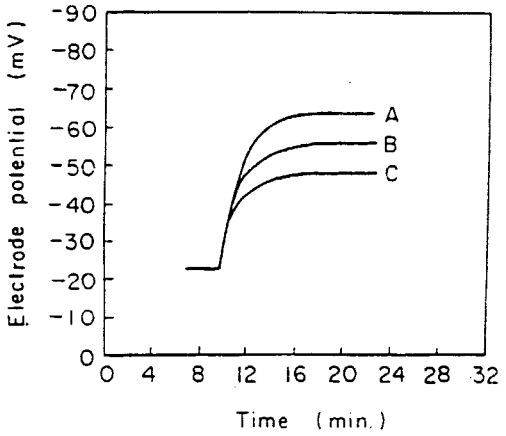


Fig. 15. Response time of urea electrode as a function of the amount of enzyme immobilized on the surface: (A) 310 urease unit/film area $\times 2$; (B) 244 urease unit/film area $\times 2$; (C) 143 urease unit/film area $\times 2$. Electrode was exposed to $10^{-2}M$ urea, and then to buffer (pH 8.0, $0.01M$ KPB). Film area 1.14 cm^2, thickness 0.01 mm.

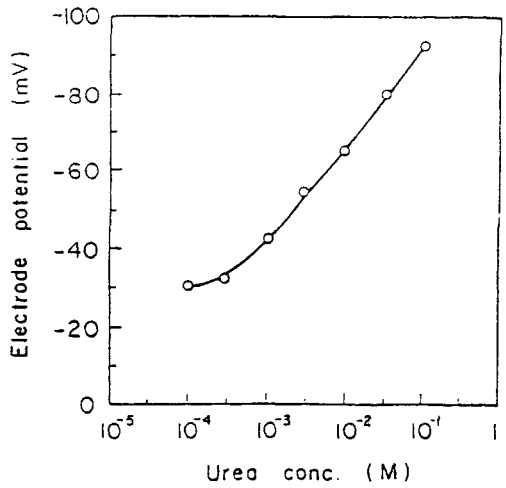


Fig. 16. Calibration curve for urea (pH 8.0, 0.01*M* KPB, 310 urease unit/film area ×2).

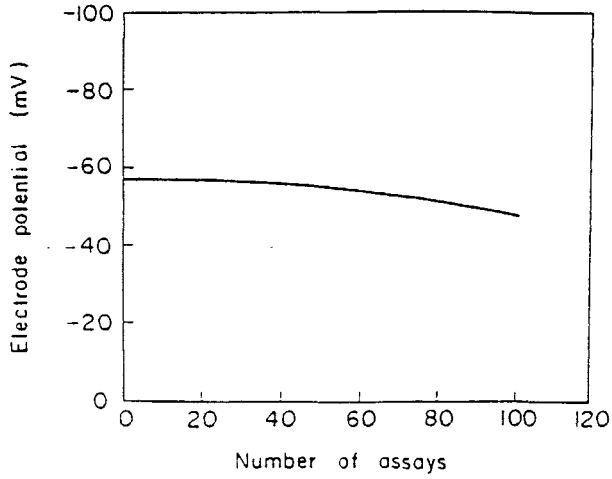


Fig. 17. Stability of the urea electrode ($10^{-2}M$ urea, urease 244 unit/film area ×2, pH 8.0, 0.01*M* KPB).

Preparation of an Urea Sensor

Figure 15 shows that at low immobilized urease concentration, the amount of ammonia generated is low which gives a small response. However, at high urease concentration, the formation of a thick enzyme layer on the surface of the membrane reduces the diffusion velocity of ammonia, which in turn results in a longer response time.

The detection range of urea of this urea sensor is 6-600 mg/dL, shown in Figures 16–19. Since the concentration of urea in the blood of a healthy human being is 7–18 mg/dL, this sensor is suitable for clinical use. The reproducibility of this sensor is also very good, since the electrode potential drops only slightly after 100 determinations.

There is a linear relationship between the electrode potential and the conc of urea regardless of the amount of urease immobilized on the membrane.

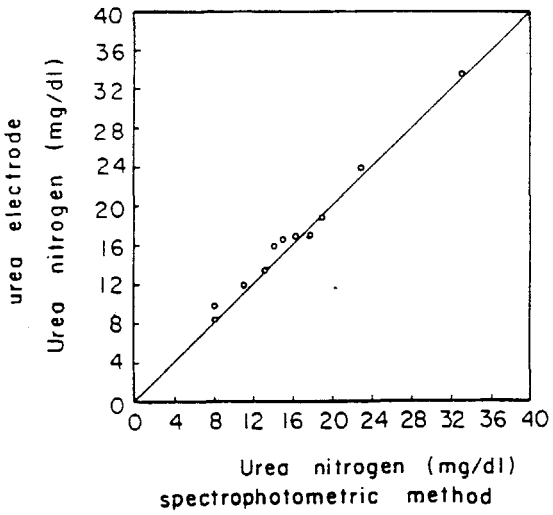


Fig. 18. Analysis of serum samples by spectrophotometric and urea electrode methods.

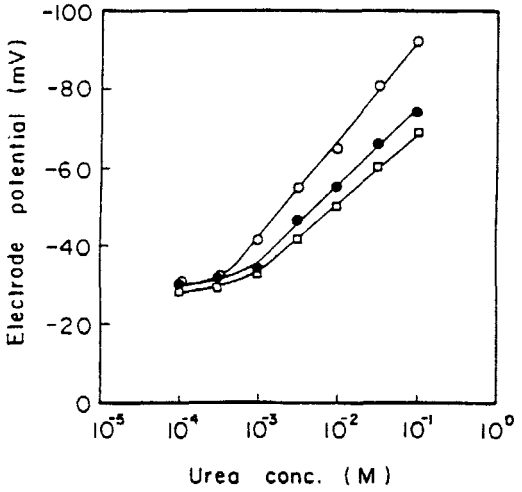


Fig. 19. Calibration curve for urea (○) 310 units/film area ×2 urease; (●) 210 units/film area ×2 urease; (□) 165 units/film area ×2 urease; pH 8.0, 0.01*M* KPB.

With serum samples, the data obtained with a urea sensor correlate well with those obtained with ordinary spectrophotometric method.

CONCLUSION

The PVA-*g*-BA membrane synthesized by the method described in this paper is a good enzyme support since it provides enough functional groups on its surface as crosslinking points to the enzyme. A high amount of immobilized urease may be obtained by controlling the pH of the cyanuric acid reaction. By combining this urease membrane with an ammonia gas electrode, a urea sensor is constructed which shows high reproducibility, accuracy, and longitivity. Therefore, the potential of commercialization of this sensor is great.

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