Glucose Oxidase Immobilization onto a Plasma-Induced Graft Copolymerized Polymeric Membrane Modified by Poly(ethylene oxide) as a Spacer

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SYNOPSIS

Plasma-induced graft copolymerization of acrylic acid, which was incorporated onto polyethylene (PE) film, was prepared. A bisamino poly(ethylene oxide) (PEO) was immobilized onto the poly(acrylic acid) (PAAc)-grafted PE membrane to modify the surface properties. The samples were characterized by ESCA. A respective chemical shift of Ar plasma-treated and control polymeric film was revealed by ESCA. The presence of the grafted PAAc and PEO was also verified. Glucose oxidase (GOD) was immobilized onto this novel grafted polymeric film with and without PEO being used as a spacer. The Michaelis constant, K_m, and the maximum reaction velocity, V_{max}, were estimated for the free and the immobilized GOD. GOD immobilized onto the polymeric films with and without a spacer obeyed Michaelis kinetics. The Michaelis constant, K_m, was larger for the immobilized GOD than for the free one, whereas V_{max} was smaller for the immobilized GOD. The bioactivity of PEO-modified PAAc-grafted PE membrane (PAAc–PEO–GOD) was higher than that of PAAc-grafted PE membrane (PAAc–GOD). The pH and thermal stabilities of the immobilized GOD without a spacer (PAAc–GOD) were higher than those of the immobilized GOD with a spacer (PAAc–PEO–GOD) and the free form. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

A large variety of natural and synthetic polymers have been previously used as solid supports for the attachment of an enzyme.[1-3] However, the application of graft copolymers as supporting materials remains relatively unexplored, where the number of reactive groups can be considerably increased and controlled and the microenvironment of the enzyme can also be altered. The unique value of using graft copolymers as supports has been reported.[4-7] In this examination, plasma-induced grafting procedures were adopted for producing poly(acrylic acid) (PAAc)-grafted polyethylene (PE). This support has also been used here for immobilizing glucose oxidase (GOD). Poly(ethylene oxide) (PEO) is used here for exploring the effect of spacer length on the activity of the immobilized GOD. The pH and thermal stabilities for the immobilized GOD are also given a detailed description.

EXPERIMENTAL

Materials

Glucose oxidase (GOD, E.C.1.1.3.4) from *Aspergillus niger*, type V, was purchased from Sigma Chemical Co. (St. Louis, MO). The acrylic acid monomer was purchased from Riedel-de Haën Co. The acrylic acid monomer was redistilled in a vacuum (bp_{10} 39.0°C) before usage. The bisamino PEO samples of molecular weight 4000 were kindly provided as a gift from the Nippon Oil and Fats Co. Ltd., Japan. The substrate polymer used for graft copolymerization was a linear low-density PE film (thickness 20 μm), which was kindly provided as a gift from USI Far East Co. The film was purified by Soxhlet extraction with methanol (Merck) for 24 h and stored in a desiccator before usage. The coupling agent, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide

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metho-*p*-toluene sulfonate (CMC), was obtained from Merck. The coupling agent, glutaraldehyde (25% aqueous solution), was obtained from Merck. All other chemicals used in this study were of reagent grade and used as obtained.

ESCA-Study

A spectrometer (Perkin-Elmer, PHI-560 AM SAM/1605) was employed for ESCA measurement of control, Ar plasma-treated, PAAc-grafted, and PEO-immobilized films at a pass energy of 1253.6 eV with a MgKα X ray source. The angle of incident X-rays to the film surface was fixed at 60°.

The Preparation of Substrate Polymer

Graft Copolymerization of Acrylic Acid onto PE Surface

The PAAc-grafted PE membrane was prepared according to the procedure of Wang and Hsiue.[8] A glow discharge reactor Model PD-2 plasma deposition was used with a bell jar-type reaction cell manufactured by Samco Corp., Kyoto, Japan. The frequency applied was 13.56 MHz and an impedance matching unit was required.

In a typical reaction, the films exposed to oxygen after Ar plasma treatment (90 W, 60 s, 300 mTorr) were placed in glass ampules containing a 50% acrylic acid monomer and Mohr's salt (1×10^{-3} *M*) aqueous solution. The ampules were sealed after degassing three times and kept at 80°C for 24 h. The reaction scheme is illustrated in Figure 1. PAAc-grafted films were removed from the monomer solution and washed with deionized water in an ultrasonic water bath for 2 h, which was followed by soaking in deionized water overnight. These films were then stored in deionized water. The amount of grafted PAAc was determined as follows: Each PAAc-grafted PE membrane was reacted for 2 h, at 60°C, with 10 mL of 0.01*N* NaOH; then, 5 mL of the supernatant was back-titrated with 0.01*N* HCl using a titrator (Mettler DL21). Various degrees of grafting were obtained. The PAAc-grafted PE films with a higher degree of grafting have been known to possess poor mechanical properties.[7] A 60 $\mu g/cm^2$ grafted sample was selected in this study for the coupling process.

Coupling of PEO Spacer onto PAAc-grafted PE

In a typical reaction, the PAAc-grafted PE films were added to an acetate buffer solution (0.05*M*, pH 3.5) containing bisamino PEO (200 mg/mL). The PEO solutions also contained 4 mg/mL of coupling agent CMC. The reaction was carried out for 16 h at 4°C with constant shaking. The overall reaction scheme of the preparation of substrate polymer is shown in Figure 1.

Enzyme Immobilization

Coupling of GOD onto PAAc-grafted PE

The GOD is shown in Figure 2(A) to have been covalently immobilized onto the PAAc-modified PE. A typical immobilization procedure without a spacer is as follows: one piece of 60 $\mu g/cm^2$ PAAc-grafted PE was added to 4 mL of 0.05*M* acetate buffer, pH 3.5. Twenty milligrams of CMC and 1 mL (5 mg/mL) of GOD solution (in 0.05*M* buffer, pH 3.5) were then added. The mixture was shaken at 4°C for 16 h. The membrane was then filtered and washed with deionized water three times. The enzyme membrane (PAAc–GOD) was soaked further in acetate buffer, pH 5.6, and was used for determining the activity of GOD-immobilized membrane.[6,9]

Coupling of GOD onto PEO Spacer-Modified PAAc-grafted PE

The immobilization of GOD onto the PAAc-modified PE membrane through PEO chains being used as a spacer is shown in Figure 2(B). A typical immobilization procedure with a spacer is as follows: One piece of PEO-modified PAAc-grafted PE (PAAc–PEO) was added to 4 mL of 0.05*M* acetate buffer, pH 5.6. Glutaraldehyde, 0.5 mL (25% aqueous solution) and 1 mL (5 mg/mL) of GOD solution (in 0.05*M* acetate buffer, pH 5.6) were then added. The mixture was shaken at 4°C for 16 h. The membrane was then filtered and washed with deionized water three times. The enzyme membrane (PAAc–PEO–GOD) was soaked further in acetate buffer, pH 5.6, and was used for determining the activity of GOD-immobilized membrane.

Assay of Enzyme Activity

Throughout all the experiments, the PAAc-grafted PE membrane without immobilized GOD or the PAAc-grafted PE membrane coupled only with the PEO spaces were used as a "control" in the determination of the amount of immobilized GOD. The activity of the immobilized enzymes was expressed as the relative activity in percent based on that of free enzymes.

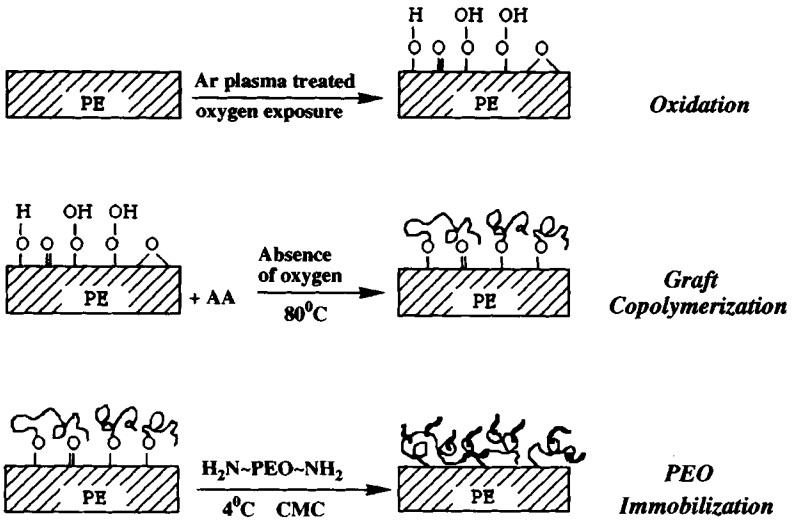
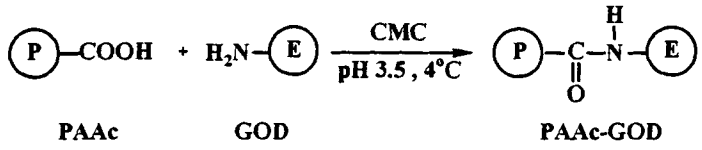
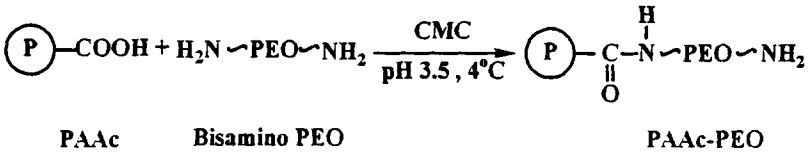


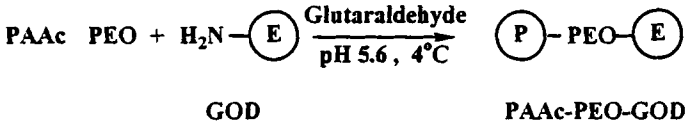
Figure 1 Schematic diagram of the bisamino PEO immobilization onto PE film functionalized with Ar plasma-induced graft copolymerization of acrylic acid.

The amount of immobilized GOD was estimated by subtracting the amount of GOD determined in the supernatant after immobilization from the total amount of GOD used for immobilization. The amount of GOD in the supernatant was determined at 455 nm with a Hitachi U2000 spectrophotometer. The activity of the free and immobilized GOD was assayed using the electrochemical procedure.[6]

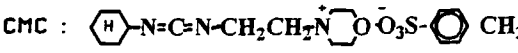


(A) immobilizing GOD onto PAAc grafted PE





(B) immobilizing GOD onto PAAc grafted PE with PEO spacer



1 - Cyclohexyl - 3 - (2 - morpholinoethyl) Carbodiimide metho - p - toluene sulfonate

Figure 2 Schematic diagram of immobilizing GOD onto (A) PAAc-grafted PE and (B) PAAc-grafted PE with a PEO spacer.

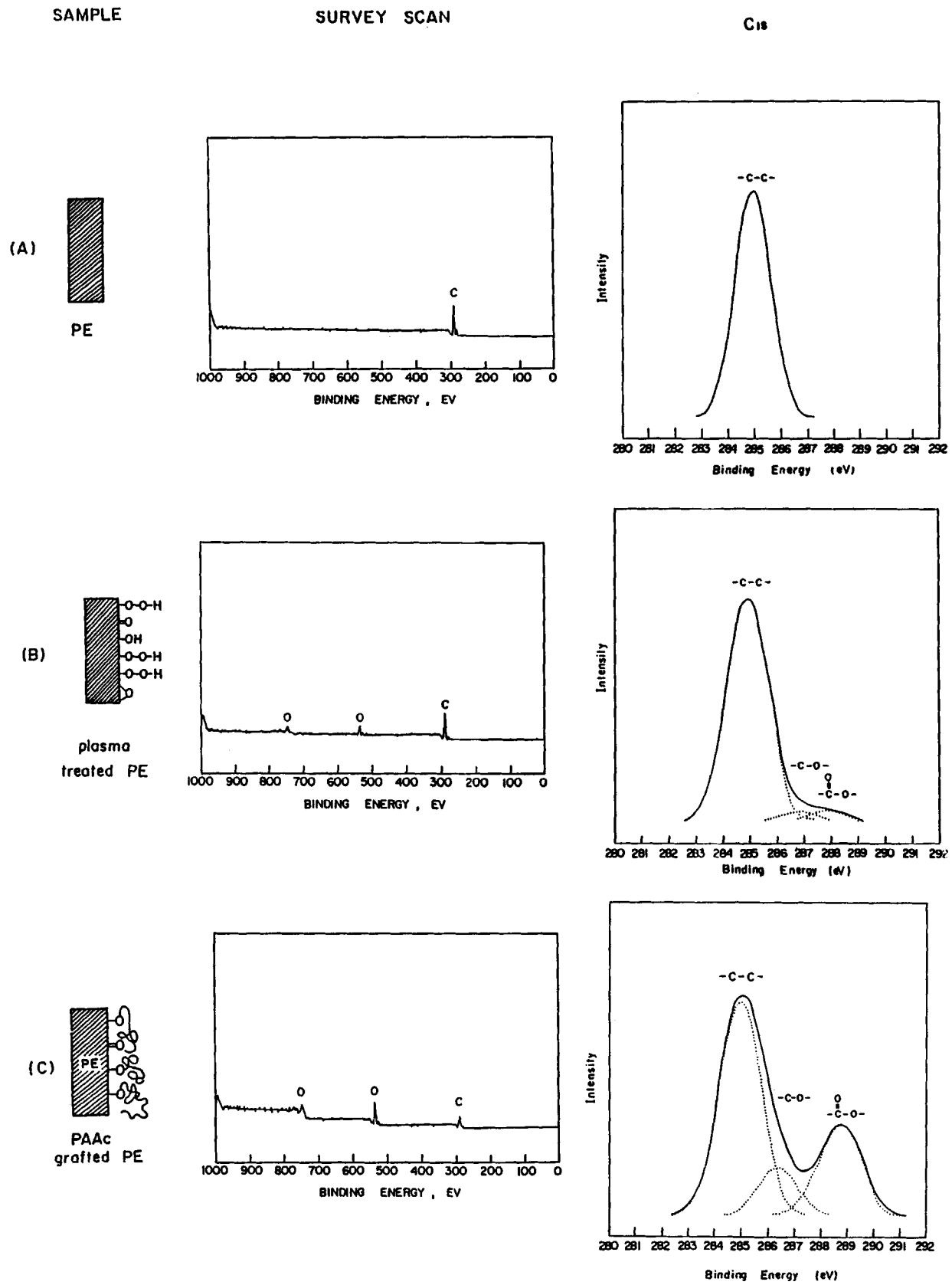


Figure 3 ESCA survey scan and C1s spectra during each stage of the surface modification of PE.

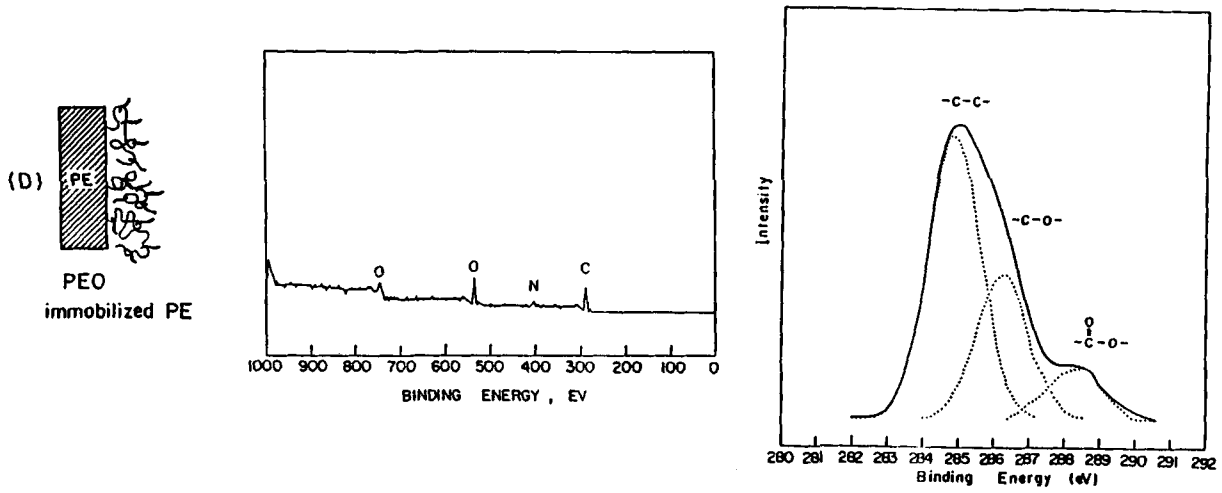


Figure 3 *(Continued from the previous page)*

Stability Measurements of Immobilized GOD

The thermal stability of the immobilized GOD was evaluated by measuring the residual activity of GOD exposed to various temperatures in a 0.05*M* acetate buffer solution of pH 5.6 for 10 min. Following heating, the samples were immediately cooled and assayed for enzymatic activity at 30°C. The remaining activities were expressed as being relative to the original activities assayed at 30°C without heating.

The pH stability of the free and immobilized GOD was studied by incubating them in 0.05*M* buffer solution at 30°C and various pH regions for 20 min. The activity was also assayed using the above-mentioned techniques.

RESULTS AND DISCUSSION

ESCA Analysis of Preliminary Screening Studies of Reaction System

Preliminary screening studies were carried out on PE surfaces treated by Ar plasma and then exposed to oxygen and allowed to polymerize with acrylic acid monomer and to react with bisamino-terminated PEO molecules. This screening process allowed for the selection of the best reaction system for further study. Figure 3 shows the ESCA survey scan and C1*s* spectra during each stage of the PEO immobilization reaction onto a PE film that was modified by a plasma-induced graft copolymerization of acrylic acid. Bisamino PEO-4000 was used in this reaction at a concentration of 200 mg/mL. The survey scan of the control PE demonstrated only carbon to be present on the film surface, as expected [Fig. 3(A)].

The C1*s* spectrum of the PE film exposed to Ar plasma is different from that of control PE. After the PE is treated with Ar plasma and then exposed to oxygen, the oxygen peaks become evident in the survey spectrum [Fig. 3(B)]. The oxygen came from the postreaction of free radicals on the PE film that was treated with Ar plasma and then exposed to oxygen. The C1*s* spectrum of Ar plasma-treated PE can be resolved into three distinct peaks that correspond to three different carbon atomic environments. The C1*s* spectrum of the PAAc-grafted PE is quite different from that of Ar plasma-treated PE [Fig. 3(C)].

Following the film reaction with bisamino PEO-4000, the C1*s* spectrum also changed [Fig. 3(D)]. The presence of oxygen and nitrogen on the film surface is demonstrated by the survey scan of the bisamino PEO-immobilized PE. The C1*s* spectrum of the —C—O—C— peak increases in intensity relative to that of PAAc-modified PE. Thus, bisamino PEO can be immobilized onto PE films exposed to Ar plasma and polymerized with acrylic acid. The binding energies assigned to these peaks are based on values previously determined by Clark.[10]

Enzyme Immobilization

The amount of immobilized GOD is markedly influenced by the initial GOD concentration in the low concentration level below about 4.0 mg/mL, but then levels off (Fig. 4). However, the surface concentration of GOD immobilized onto a PAAc-grafted PE membrane with a PEO spacer is less than that without a spacer. This occurs as a result of the spacer

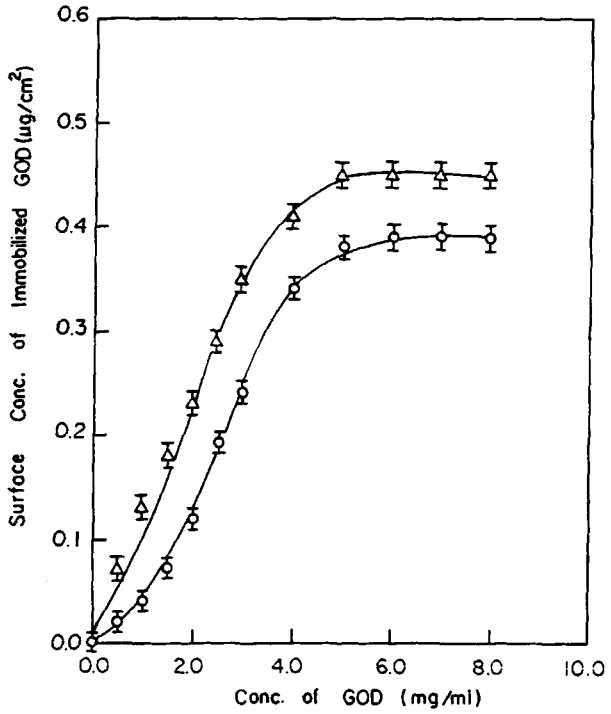


Figure 4 Effect of the GOD concentration upon the amount of GOD immobilized onto (△) PAAc-grafted PE and (○) PEO spacer-modified PAAc-grafted PE, 4°C and 16 h.

having fewer functional groups to react with GOD. The mechanism of immobilizing GOD onto PAAc-grafted PE membrane is that in which coupling occurs between the carboxylic groups of the PAAc chain and is mediated by carbodiimide, CMC, and the amino group of GOD. But the mechanism of immobilizing GOD onto bisamino PEO-modified PAAc-grafted PE membrane (PAAc-PEO) is illustrated in Figure 2(B) to be that in which coupling occurs for both the amino groups of PEO and GOD with glutaraldehyde. The initial GOD concentration was kept at 5 mg/mL throughout the following experiments. The maximum amounts of immobilized GOD were 0.45 $\mu g/cm^2$ for PAAc-grafted PE (PAAc-GOD) and 0.39 $\mu g/cm^2$ for PEO-modified PAAc-grafted PE (PAAc-PEO-GOD).

The effects of the surface concentration of GOD immobilized onto PAAc and PAAc-PEO are illustrated in Figure 5. The relative activity of the immobilized GOD without a spacer (PAAc-GOD) is clearly seen in this figure to decrease gradually with the decreasing surface concentration of the immobilized GOD, whereas the immobilized GOD with a PEO spacer (PAAc-PEO-GOD) gave an almost constant relative activity even at low surface concentrations; PAAc-PEO-GOD is markedly higher than is PAAc-GOD. This result may be accounted for in terms of structural deformation of the immobilized GOD molecules as previously reported by Hayashi and Ikada.[11,12]

The immobilized enzyme should undergo strong deformation, especially in the lower surface concentration region without a spacer [Fig. 6(A)], whereas the immobilized GOD molecule with a spacer [Fig. 6(B)] must be protected from the structure deformation even in the lower surface concentration region owing to the spacer effect.

Determination of Michaelis Constant, K_m and Maximum Reaction Velocity, V_{max}

The permeability of GOD immobilized onto a PAAc-grafted PE membrane was studied by adding standard concentrations of the substrate, glucose, from 25 to 1000 ppm, and measuring the dissolved oxygen obtained at the oxygen sensor. The enzymatic reaction is catalytically controlled since these results provide straight lines in the Lineweaver-Burk plot (Fig. 7). The Michaelis constant, K_m, and the maximum reaction velocity, V_{max}, for the native GOD,

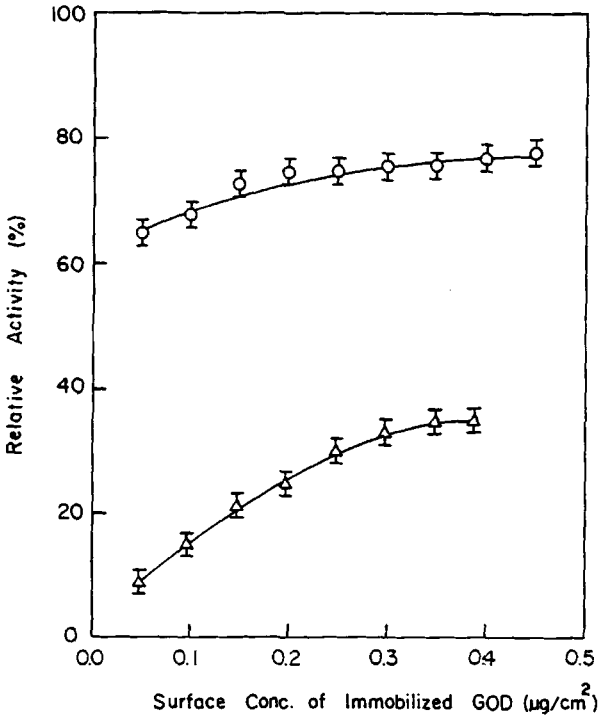


Figure 5 Effect of the surface concentration (SC) of the immobilized GOD upon the relative activity (RA). Glucose 500 ppm, pH 5.6, and 30°C: (△) PAAc-GOD; (○) PAAc-PEO-GOD.

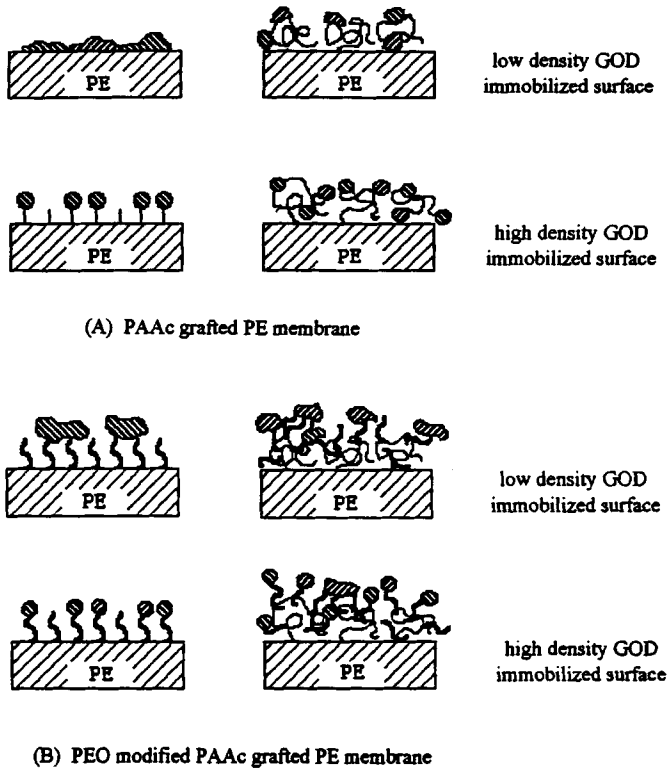


Figure 6 Schematic representation for molecular state of enzyme immobilized onto the surface of PAAc-grafted PE: (A) GOD immobilization without a spacer; (B) GOD immobilization with a spacer.

PAAc–GOD, and PAAc–PEO–GOD, estimated from Figure 7, are presented in Table I. The Michaelis constant, K_m, of the immobilized GOD without a spacer (PAAc–GOD) is higher than that of the immobilized GOD with a spacer (PAAc–PEO–GOD) and the native one. This may be due to the limitation of diffusion resistance. Glucose is apt to decrease for immobilized GOD without a spacer owing to the steric hindrance as compared to the case of immobilized GOD with a spacer or the native GOD. The V_{max}, namely, GOD > PAAc–PEO–GOD > PAAc–GOD, is also due to factors associated with diffusion.

Stability of Immobilized Enzymes

Thermal Stability

The thermal stability of immobilized enzymes is one of the most important criteria of their application. The enzyme activity in most immobilized enzyme preparations, especially in covalently bonded systems, is more resistant against heat and other denaturing agents than that of the soluble form.[13] The effect of temperature upon the stability of the three forms of GOD in acetate buffer is shown in Figure 8. The temperature maximum of the immobilized GOD is the same as that of the native GOD (30°C), but broader in the range of a higher temperature. The immobilized GOD at 65°C for 10 min is observed here to exhibit activity twice that of the soluble enzyme. However, the immobilized GOD without a PEO spacer (PAAc–GOD) is more stable than that with a spacer (PAAc–PEO–GOD). The higher stability of the immobilized GOD without a spacer than that with a spacer is shown in Figure 8 to be ascribed probably to the stabilization of the GOD molecule owing to the multipoint attachment of the GOD molecule to the surface of the PAAc-grafted PE membrane. This multipoint attachment, consequently, leads to a reduction in molecular mobility that is a common principle of enzyme stabilization.[14]

pH Stability

The pH effect on the activity of the immobilized and soluble forms of the enzyme was studied in adequate buffers (pH 2.8–9.6) and is presented in Figure 9. Two forms of immobilized GOD have the optimum pH of the native form (pH 5.6), but the pH

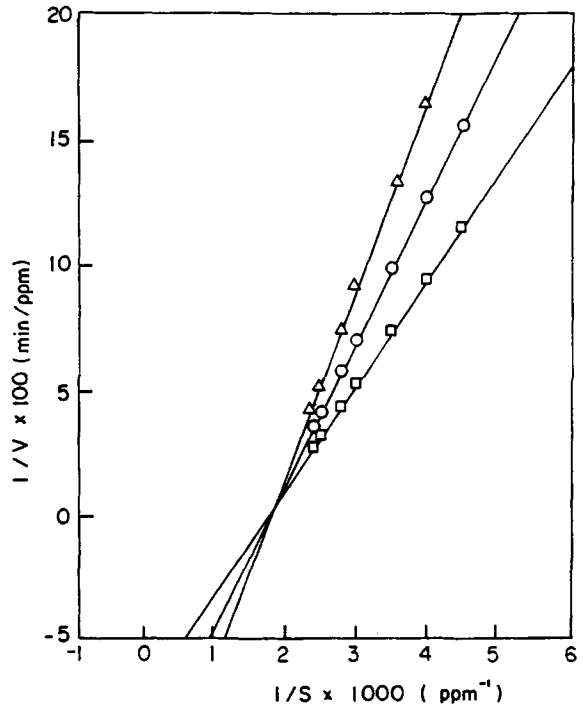


Figure 7 Lineweaver–Burk plots of (□) native GOD, (○) PAAc–PEO–GOD, and (△) PAAc–GOD.

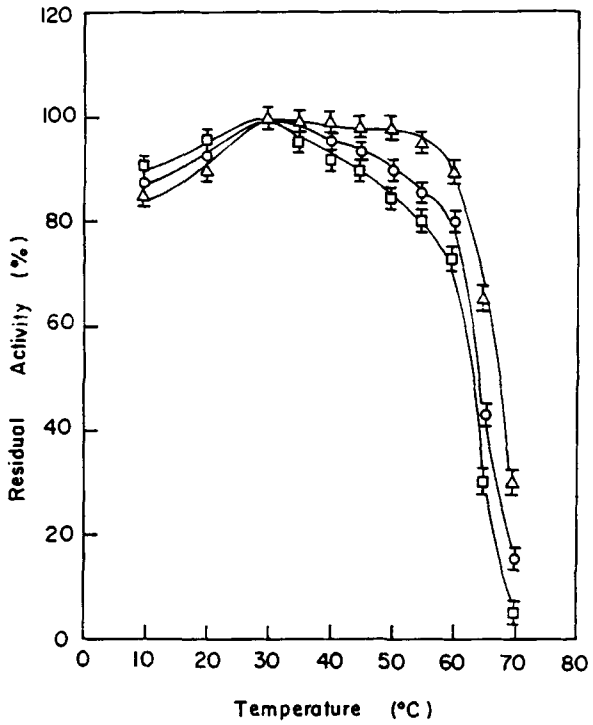


Figure 8 Effect of temperature upon the residual activity of glucose oxidation at pH 5.6 and 30°C: (□) native GOD, (○) PAAc–PEO–GOD, and (△) PAAc–GOD based on 10 min reaction at various temperatures in 0.05*M* acetate buffer (pH 5.6).

profile is considerably widened owing to diffusional limitations of the immobilized enzyme molecules.[15] PAAc–GOD displays the greatest stability at higher pH values than those of PAAc–PEO–GOD and native GOD.

CONCLUSION

The novel synthesized acrylic enzyme carriers PAAc-grafted PE and PEO-modified PAAc-grafted PE were observed in this study to be a promising GOD carrier. The bioactivity of PEO-modified PAAc-grafted PE membrane (PAAc–PEO–GOD) was higher than that of PAAc-grafted PE membrane (PAAc–GOD). The Michaelis constant, K_m, values were larger for immobilized GOD than for the free forms, whereas V_{max} values were smaller for the immobilized GOD. The optimum pH value of GOD was not affected by the immobilization reaction. However, the activity of immobilized GOD was observed to be considerably widened. The immobilized enzyme demonstrated a reduced sensitivity to the thermoinactivation as compared to that of the free form. The immobilized GOD without a spacer (PAAc–GOD) exhibited a higher thermal stability than that with a spacer (PAAc–PEO–GOD).

Table I Michaelis Parameters K_m and V_m at pH 5.6 and 30°C

Sample	K_m (mM)	V_m (mM/min)
GOD	23.58	5.62
PAAc-GOD	31.44	4.17
PAAc-PEO-GOD	27.33	4.75

NOMENCLATURE

PAAc	Poly(acrylic acid)
PEO	Poly(ethylene oxide)
GOD	Glucose oxidase
PAAc–PEO	PEO spacer modified PAAc grafted PE membrane
PAAc–GOD	GOD immobilized PAAc grafted PE membrane

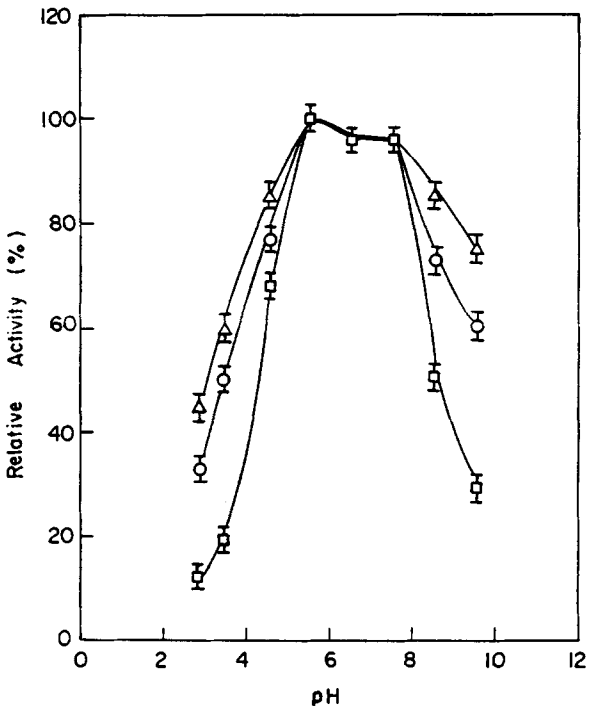


Figure 9 Effect of pH of the reaction medium upon the residual activity of glucose oxidation at 30°C: (□) native GOD, (○) PAAc–PEO–GOD; (△) PAAc–GOD.

PAAc–PEO–GOD	GOD immobilized PEO spacer modified PAAc grafted PE membrane
CMC	1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate
Mohr's salt	Ammoniumeisen(II)-sulfate-6-hydrate
K_m	Michaelis constant
V_{max}	maximum reaction velocity

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