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Immobilization of Glucose Oxidase with the Blend of Regenerated Silk Fibroin and Poly(vinyl alcohol) and Its Application to a 1,1'-Dimethylferrocene-Mediating Glucose Sensor

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ABSTRACT

The structure and properties of the blend of regenerated silk fibroin (RSF) and poly(vinyl alcohol) (PVA) were investigated. The two polymers in the blend are in the state of phase segregation. Infrared (IR) spectra indicate that the RSF in the blend maintains its intrinsic properties, thus, ethanol treatment can transfer silk I structure of RSF to silk II structure. The water absorption property and mechanical property of the blend are improved in comparison with those of RSF. The blend maintains the major merit of RSF, that is, it can immobilize glucose oxidase on the basis of the conformational transition from silk I structure to silk II structure. The properties of the immobilized enzyme are examined. Moreover, the second generation of glucose sensor based on the immobilized enzyme is fabricated and it has a variety of advantages including easy maintenance of enzyme, simplicity of construction, fast response time and high stability.

Index Entries: Regenerated silk fibroin; PVA; blend membrane; sensor; Immobilization of enzyme.

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INTRODUCTION

Enzymes possess extremely high catalytic activity and unique substrate specificity. It is these merits that make enzymes the application in many fields. However, their wide application is hindered by the fact that enzymes require extremely caustic conditions for reactions, such as narrow ranges of pH, temperature and ion strength, and so on. Therefore, since the early 1960s, immobilization of enzymes has been a topic of ongoing research. A large number of methods have been developed to immobilize enzymes onto an electrode surface, for example application to amperometric sensors has received active attention in recent years. The methods include cross-linking polymers *(1)*, entrapment with ion-exchange polymers *(2,3)*, conducting polymers such as polypyrrole and its derivatives *(4,5)*, polyaniline *(6,7)* and polyindole *(8)*, and nonconducting polymers such as poly(ophenylenediamine) *(9)*. Recently we have successfully immobilized glucose oxidase with regenerated silk fibroin (RSF) from waste silk by entrapment *(10)*. As an immobilization matrix of enzymes, major merit of regenerated silk fibroin is that the immobilization of enzyme is based on its conformational transition from water-soluble silk I structure to water-insoluble silk II structure. However, it becomes brittle in dried state or in ambient air exposure for a long time. In order to amend its inferior property, polymer blending is one of the convenient methods. Poly(vinyl alcohol) (PVA) is a nontoxic synthetic polymer, and possesses good filmforming property, impact strength and weather durability. Our interest is that the blend of RSF and PVA is prepared to improve mechanical property and to maintain their merits. In this paper, the structure and properties of the blend are investigated and the blend is used to immobilize glucose oxidase. Moreover, the properties of the immobilized enzyme are checked.

MATERIALS AND METHODS

Materials

Glucose oxidase (EC1.1.3.4. 150,000 U/g, from *Aspergillus niger*) was obtained from Sigma (St. Louis, MO), 1,1'-dimethylferrocene was purchased from Aldrich (Milwaukee, WI). A solution of Eastman-AQ-55D polymer (28% dispersion) was obtained from Eastman Kodak (Rochester, NY). D-glucose and PVA were purchased from Shanghai Chemical Reagent Company, Shanghai, China. Glucose solutions were stored overnight to allow to reach mutarotational equilibrium before use. PVA was heated and stirred at 85°C for 24 hours, so as to be completely dissolved in de-ionized water. It was then filtered and a 5% PVA solution was obtained. All other chemicals used were of analytical reagent grade. The preparation of the aqueous solution of RSF was described elsewhere *(10)*.

The membranes of RSF, PVA, or their blend were cast in the given weight ratio on polytetrafluoroethylene plates at room temperature in air.

Fabrication of 1,1'-Dimethylferrocene-Modified Electrode

The glassy carbon electrode (3.5 mm in diameter) was polished with 0.3, 0.1, and 0.05 μm Al_2O_3, rinsed thoroughly with de-ionized water between each polishing step, sonicated sequentially in nitric acid, acetone 1:1 and doubly distilled water, and dried in air before use. 1,1'-dimethylferrocene was coated on the electrode by pipeting 5 μL of 0.1 *M* 1,1'-dimethylferrocene acetone solution onto the electrode surface and letting it dry in air.

Fabrication of the Glucose Sensor

Twenty mg of glucose oxidase was completely disolved with 0.40 mL of the blend solution of RSF and PVA. Aliquots (25 μL) of the mixture were deposited on the 1,1'-dimethylferrocene modified electrode and allowed to dry in ambient conditions for 20 h. Then 2 μL of 2% Eastman-AQ-55D ethanol-diluted solution was pipeted onto the surface of the sensor, letting it dry. The sensor was kept dry in air at 4°C in refrigerator between the measurements.

Measurements and Methods

IR spectra of the dried membranes were recorded by transimission method on a MAGNA-IR550 (Nicolet) spectrometer at room temperature. Scanning electron microscopy of the surface of the membranes was performed on a Hitachi S-520 operating at 20.0 kV. The fracture cross surfaces of the membranes were achieved by cooling them in liquid nitrogen.

The water absorption property of the membranes was examined according to the following. The different membranes obtained under the same conditions were dried in vacuum in order to remove free water in the membranes. They were soaked in deionized water at room temperature for 24 h to achieve swelling equilibrium. The absorption water content was calculated by using the equation:

$$W = (W_1 - W_0)/W_0 \times 100\% \quad (1)$$

Where W is absorption water content, W_0 and W_1 are weights of membranes in dried state and in soaked state, respectively.

The maximum strength was measured on an electrical strain gauge apparatus (Instron 1121) at room temperature and recorded under a constant drawing rate at 50 mm/min. The membranes obtained under the same conditions were dried in vacuum, then they were cut into the 10 × 50 mm five strips.

Cyclic voltammetry and amperometric measurements were carried out with FDH 3204 and FDH 3206 cyclic voltammetry apparatus (Scientific

Equipment Fudan, P. R. China) in line with a type 3086 x-y recorder (Tokyo, Japan). All experiments were performed in a thermostatted, stirred electrochemical 5 mL-cell at 25.0 ± 0.5°C, which was equipped with a glucose sensor as a working electrode, plus a saturated calomel reference electrode and a platinum wire auxiliary electrode. In the constant potential experiments, successive additions of stock glucose solution in the buffer were made and the current-time data were recorded after a constant residual current had been obtained. The sensor response was measured as the differences between total and residual current.

Pretreatment of the Glucose Sensor

After fabrication and prior to experiments, the electrode response was stabilized by scanning between +0.50 and –0.2 V (vs. SCE) in phosphate buffer (pH 7.0) over 15 min.

RESULTS AND DISCUSSION

IR Spectra of the Membranes

Figure 1 shows IR spectra of the samples before ethanol treatment. The membrane of RSF displays the absorption bands at 1653/cm (amide I), 1543/cm (amide II), 1243/cm (amide III) and 669/cm (amide V) as shown in Fig. 1A attributed to the characteristic of silk I structure. The membrane of PVA illustrates the absorption bands at 1446/cm and 1332/cm (O-H bending), 1143/cm and 1094/cm (C-O stretching), 655/cm (O-H twisting) as shown in Fig. 1E. The blend membrane of RSF and PVA has the absorption bands at 1651/cm (amide I), 1539/cm (amide II), 1238/cm (amide III) and 667/cm (amide V), which are the absorption bands of RSF but shifts to lower wavenumber, and at 1452/cm and 1334/cm (O-H bending), 1143/cm and 1093/cm (C-O stretching), which is the absorption bands of PVA but shifts to higher wavenumber as shown in Fig. 1B, C, D. These facts indicate that the RSF and PVA maintain their own structures respectively although a little intermolecular interaction between them exist in the blend membranes.

Figure 2 shows IR spectra of the samples after ethanol treatment. Figure 2A indicates that the structure of RSF has been transferred from silk I structure to silk II structure since it has the absorption bands at 1627/cm (amide I), 1531/cm (amide II), 1236/cm (amide III), and 695/cm (amide V), which is the characteristic of silk II structure. Figure 2E manifests that ethanol treatment has no influence upon the structure of PVA since it has the same absorption bands as those before ethanol treatment. Blend membranes of RSF and PVA have absorption bands at 1626/cm (amide I), 1528/cm (amide II) and 1234/cm (amide III), which are the absorption bands of silk II structure of RSF but shifts to lower wavenumbers, 1448/cm and 1334/cm (O-H bending), 1144/cm and 1093/cm (C-O stretching),

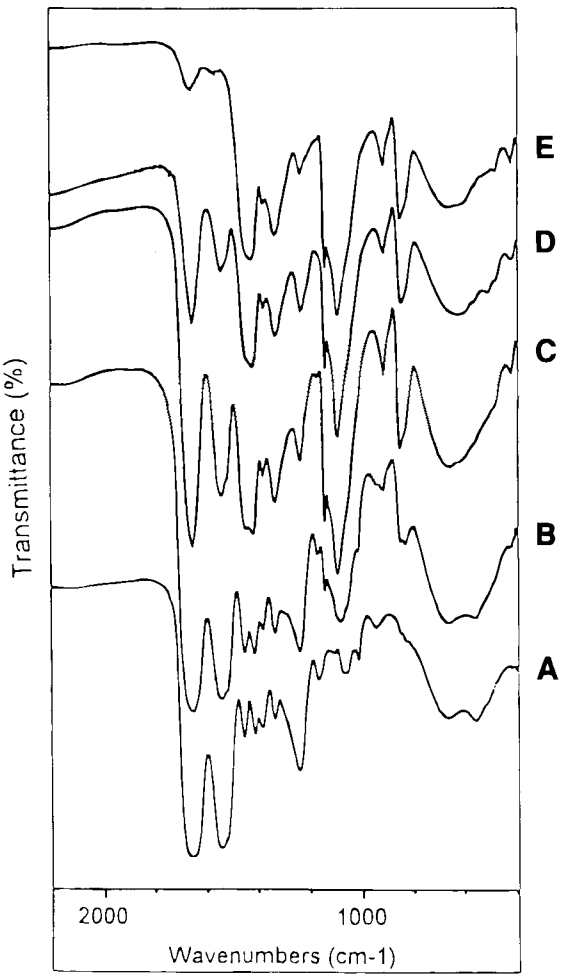

Fig. 1. IR spectra of the membranes before ethanol treatment **(A)** pure RSF **(B)** RSF/PVA = 2:1 **(C)** RSF/PVA = 1:5 **(D)** RSF/PVA = 1:9 **(E)** pure PVA.

which are the absorption bands of PVA (shown in Fig. 2B–D). These facts suggest that PVA has no influence upon the conformational transition of RSF and imply that the RSF in the blend maintains its intrinsic properties and that the blend can be used to immobilize glucose oxidase.

SEM Observations of the Fracture Cross Surface of the Membranes

When the components of the blend membranes are in the ratio of 2:1 or 1:9, RSF to PVA their fracture cross surfaces show that two kinds of structures exist in the blend membrane (shown in Fig. 3A,C). Major fracture surfaces are smooth with a series of concentric crack grown bands, which is a result of the slow crack development. Minor fracture surfaces are that the crack jumps from side to side, which results from the rapid crack development. However, the membrane with the ratio of RSF to PVA as 1:5 presents

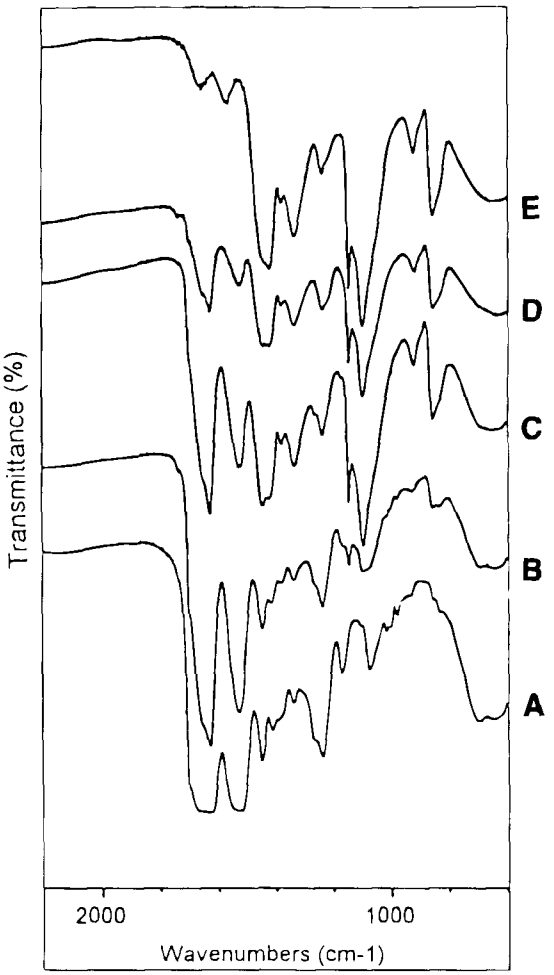

Fig. 2. IR spectra of the membranes after ethanol treatment **(A)** pure RSF **(B)** RSF/PVA = 2:1 **(C)** RSF/PVA = 1:5 **(D)** RSF/PVA = 1:9 **(E)** pure PVA.

only one kind of fracture surface, attributable to the result of the slow crack development (shown in Fig. 3B). In addition, its structure is island-like. The dark spaces are domains of RSF retained on the other cross fracture surface. The distribution and sizes of RSF domains are irregular. These display that phase segregation takes place in the process of forming the blend.

Properties of the Blend Membranes of RSF and PVA

Figure 4 shows the relationship between RSF and water content of the membranes. When RSF in the blend membranes is less than 16.7%, the water absorption content of the membranes is increased with increment of RSF. When RSF in the blend membranes is more than 16.7%, the water absorption content of the membranes is decreased with increase of RSF. It is obvious that the membrane with 16.7% RSF (RSF/PVA = 1:5) possesses highest water absorbability.

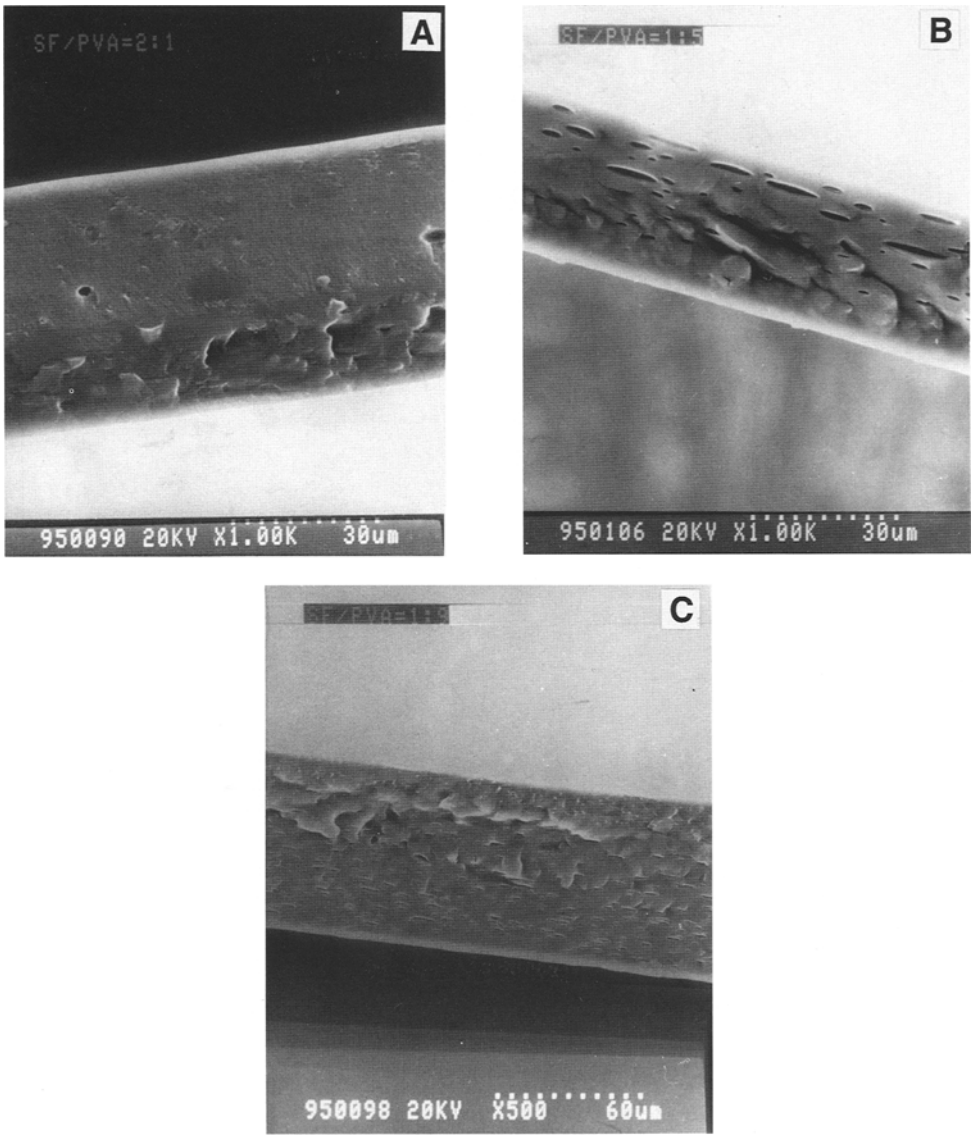

Fig. 3. Scanning electron microphotographs of the fracture cross surface of the blend membranes with different ratio of RSF to PVA. **(A)** RSF/PVA = 2:1 **(B)** RSF/PVA = 1:5 **(C)** RSF/PVA = 1:9.

The relationship between the maximum strength and RSF content of the membranes is shown in Fig. 5. When RSF in the blend is less than 16.7%, the maximum strength is increased with increment of RSF, which suggests that RSF in the blend plays the role of strengthening agent. When RSF in the blend is over 16.7%, the maximum strength is decreased with further increase of RSF. It is evident that the blend with the ratio of RSF to PVA as 1:5 is feasible as the material of immobilizing glucose oxidase.

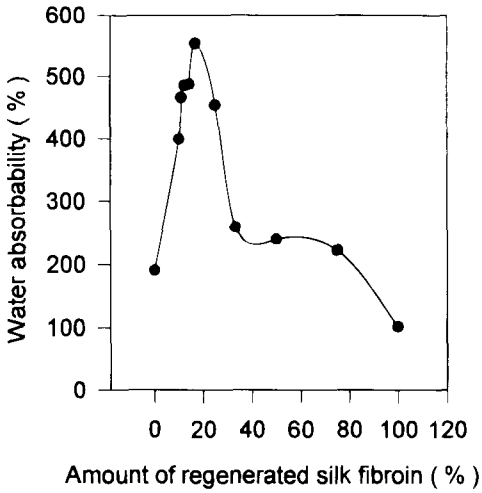

Fig. 4. Relation between the water content and RSF content of membranes.

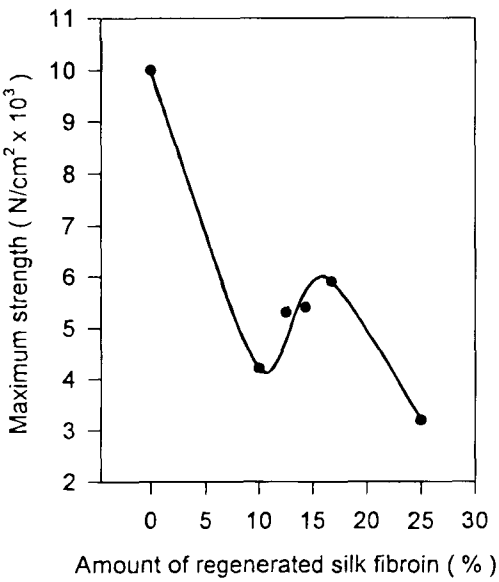

Fig. 5. Relation between the maximum strength (units, w/w) and RSF content of membranes.

Electrochemical Characterization of the Glucose Sensor

Figure 6 displays cyclic voltammograms of the sensor in the absence of glucose. The enzyme gives no response and the voltammogram retains reversible characteristics: the cathodic and anodic peak separation (ΔE_p) of the cyclic voltammogram is 60 mV and the peak current is proportional to the square root of the scan rate, consistent with cases *(11)*. The symmetrical surface waves and fast kinetics indicate that the presence of the blend

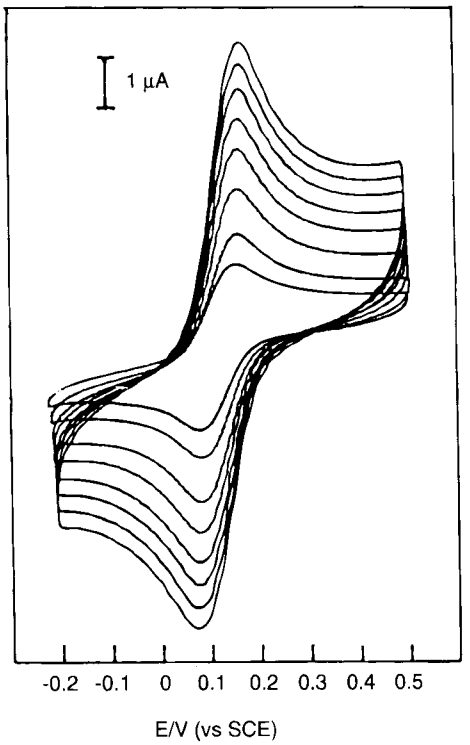

Fig. 6. Cyclic voltammograms of the glucose sensor arriving at steady state at various scan rates (from inner curve to outer curve): 15, 25, 45, 65, 85, 105, 125, 145 mV/s in 0.1*M* phosphate buffer (pH 7.0).

membrane of RSF and PVA does not appreciably affect the electrochemical behavior of 1,1'-dimethylferrocene.

Electrocatalytic Oxidation of Glucose at the Glucose Sensor

There is no electrocatalytic oxidation current at the 1,1'-dimethylferrocene modified electrode in the presence of glucose. Figure 7 displays typical cyclic voltammetric results for the glucose sensor. Without glucose in the buffer, the glucose oxidase gives no response and only typical oxidation and reduction peak for 1,1'-dimethylferrocene is observed in Fig. 7 (A). Addition of glucose gives a rise to an increase in the anodic current with a concomitant decrease in the cathodic wave Fig. 7(B). Comparison of the voltammograms with and without glucose present illustrates that 1,1'-dimethylferrocene can expedite an electron exchanger between the FAD/$FADH_2$ centers of glucose oxidase in the blend membrane and a glassy carbon electrode. The flavin adenine dinucleotide (FAD) of glucose oxidase (GOD) is reduced by the glucose penetrating the membrane:

$$\beta\text{-D-glucose} + \text{GOD(FAD)} \longrightarrow \delta\text{-gluconolactone} + \text{GOD}(FADH_2)$$

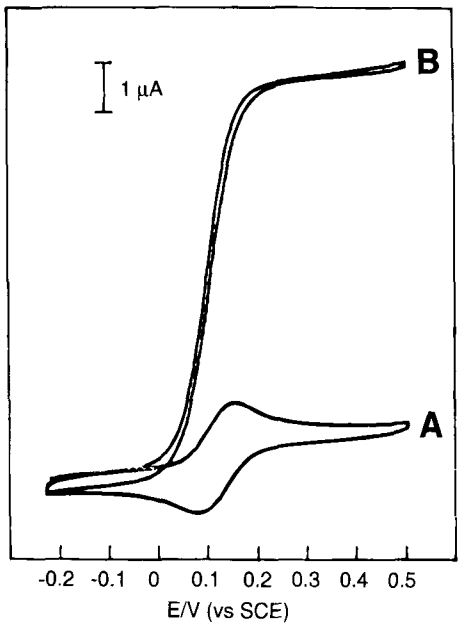

Fig. 7. Cyclic voltammograms of the glucose sensor at a scan rate of 3.0 mV/s in 0.1*M* phosphate buffer (pH 7.0) in absence of glucose **(A)** and in presence of 15 m*M* glucose **(B).**

the reduced GOD($FADH_2$) is oxidized by 1,1'-dimethylferrocene ion ($DMFc^+$):

$$GOD(FADH_2) + 2DMFc^+ \longrightarrow GOD(FAD) + 2DMFc + 2H^+$$

$DMFc^+$ is reoxidized at the electrode, giving a rise to oxidation current:

$$2DMFc \longrightarrow 2DMFc^+ + 2e$$

Figure 8 displays cyclic voltammograms for glucose sensor in the presence of glucose at various scan speeds. Increasing scan speed brings about an increase of the catalytic current. At slow scan speeds, the cathodic peak disappears completely and the anodic peak becomes a plateau, whereas, at scan speeds of greater than 120 mV/s, a hysteresis appears and a reduction wave is also observed. The appearance of the hysteresis and the reduction wave is dependent on film composition and thickness, scan speed, substrate concentration, temperature, and ionic strength.

Steady-State Amperometric Response of the Sensor to Glucose

A well-defined and fast amperometric response is observed at 0.20 V with successive injections of glucose and the time required to reach 95% of maximum response is less than 40 s (Fig. 9). The trace (current versus time) plainly displays the rapid response and good sensitivity of the sensor to glucose. Fast response of the sensor to glucose results from the hydrophilicity of

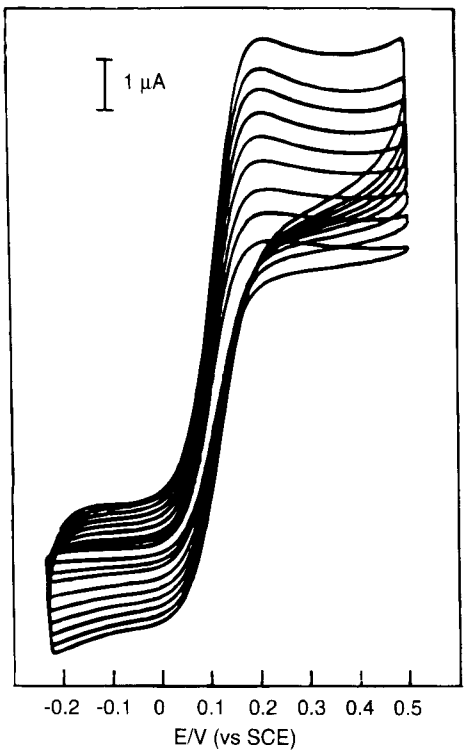

Fig. 8. Cyclic voltammograms of the glucose sensor at various scan rates in 0.1 *M* phosphate buffer containing 10 m*M* glucose. The scan rates (from inner curve to outer one) are 15, 25, 45, 65, 85, 105, 125, 145, 165 mV/s, respectively.

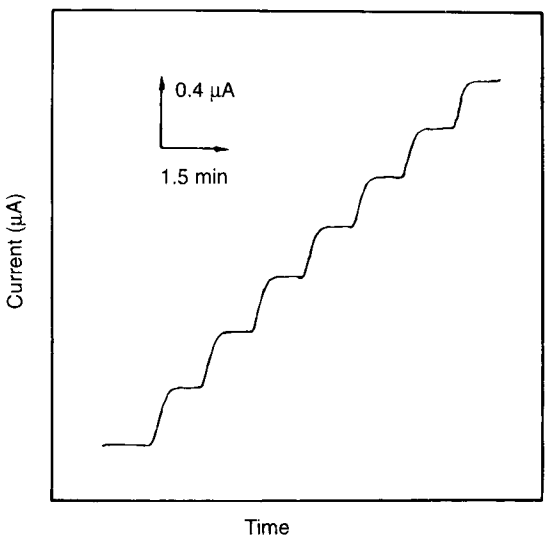

Fig. 9. Typical response of the glucose sensor to successive increase of 2.0 mM glucose at the applied potential of + 200 mV.

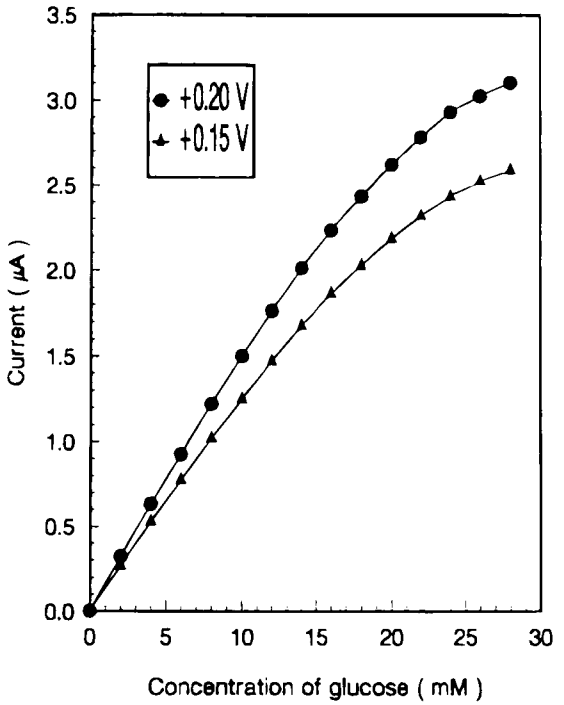

Fig. 10. Calibration plots for the glucose sensor. Steady-state current is measured in 0.1*M* phosphate buffer (pH 7.0) at two applied potentials.

the blend membrane which reduces the mass-transfer resistance to the substrate and reaction product. Figure 10 presents curves of current response as a function of glucose concentration at various applied potentials. Increase of applied potential results in the enhanced linear range and the increased sensitivity owing to an increased driving force for the fast reoxidation of the $FADH_2$ of glucose oxidase. The apparent Michaelis-Menten constant K_M^{app} can be obtained from the Michaelis-Menten equation

$$j_{ss} = j_{max} - K_M^{app}(j_{ss}/C) \quad (2)$$

Where j_{ss} represents the steady-state catalytic current, j_{max} is the maximum current measured under saturating substrate conditions, C refers to the glucose concentration and K_M^{app} stands for the apparent Michaelis-Menten constant of the system as whole, not that of glucose oxidase itself, provides a measure of glucose concentration range over which the response of the sensor is approximately linear. The result gives K_M^{app} = 22.5 m*M* at 0.2 V.

Effects of pH and Temperature on the Glucose Sensor

When the pH of the solution is either very low or very high, the level of response current of the sensor is low. An optimum and maximum response current is observed between pH 6.0 and 8.0. The response of the sensor to 5.0 m*M* glucose is measured at five intervals from 15 to 55°C. The experiment

displays that the steady-state current response increases with the temperature, reaching a maximum value at about 50°C. Albeit response is higher at higher temperatures, the analyses are performed at 30°C since extended exposure to high temperature causes more rapid denaturation of the enzyme.

The Stability of the Glucose Sensor

The reproducibility of the current response of the sensor is examined at different glucose concentrations and the relative standard deviations for eleven repetitive measurements are 2.7, 2.6, and 2.1% for the solutions of 2.0, 5.0, 7.5 m*M* glucose, respectively. The operational stability is examined by recording over 50 successive assays of 5.0 m*M* glucose, the relative standard deviation is 6.2%. The sensor displays good storage characteristics. The sensor has been stored under dry conditions at 5°C for two and half months, after which it displays decreases of 8–12% in current response.

CONCLUSION

The blend of RSF and PVA retains the intrinsic property of RSF, that is, the immobilization of glucose oxidase is carried out on basis of its conformational transition from silk I structure to silk II structure. The mechanical property of RSF is amended in the blend. The composite material allows for a simple and straightforward immobilization of glucose oxidase on the surface of the electrode and offers a favorable environmental condition to the enzyme. Moreover, the hydrophilicity of the blend membrane reduces the mass-transfer resistance to the substrate and reaction product, and consequently, it enhances the response of the sensor.

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