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STRUCTURE AND PROPERTIES OF THE COMPOSITE MEMBRANE OF REGENERATED SILK FIBROIN AND PVA AND ITS APPLICATION TO AMPEROMETRIC TETRATHIAFULVALENE-MEDIATING GLUCOSE SENSOR

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ABSTRACT

The structure of a composite of regenerated silk fibroin (RSF) and poly(vinyl alcohol) (PVA) is characterized with FT-IR spectra. PVA has little influence upon the structure of RSF in the composite since it exhibits the same structure as those of pure RSF. The water absorption property and maximum strength of the composite are dependent upon its component. An amperometric glucose sensor using tetrathiafulvalene as an electron transfer mediator was constructed to test the feasibility and workability of the composite of RSF and PVA as an immobilization matrix of glucose oxidase. The effects of scan speed, pH, and temperature on the electrocatalytic oxidation of glucose at the sensor are dis-

cussed. The enhanced hydrophilicity of the membrane results in fast response of the sensor to glucose (less than 40 seconds). Moreover, the sensor is useable for more than 2 months.

INTRODUCTION

Recently, silk fibroin from *Bombyx mori* silkworms has been used to immobilize enzymes [1–13]. The process is accomplished with physical, chemical, or mechanical treatment without using the usual crosslinking chemicals. Hence, their activities are readily maintained. However, it becomes brittle in the dry state or in ambient air exposure. In order to improve this property, it was blended with other natural or synthetic polymers, such as syndiotactic rich (PVA) [14], PVA (MW = 2,000) [15], chitosan [16, 17], poly(sodium glutamate) [18], and sodium alginate [19].

PVA is a nontoxic water-soluble synthetic polymer. It possesses good film-forming property, impact strength, and weather durability. Since silk fibroin obtained directly from *Bombyx mori* larvae is available only several times a year, RSF is now prepared from waste silk. In this paper we report the structure and properties of composite membranes of RSF and PVA, and explore the feasibility of using the composite membrane as the immobilization matrix of glucose oxidase. An amperometric glucose sensor was constructed by coupling immobilized glucose oxidase with an Eastman-AQ-TTF modified electrode to test the immobilization efficiency and the catalytic effectiveness. The RSF and PVA composite is more hydrophilic, and consequently its strong swelling power offers a favorable environmental condition for the immobilized enzyme and reduces mass-transfer resistance to the substrate and reaction product.

EXPERIMENTAL

Materials

Glucose oxidase (EC 1.1.3.4, 150,000 μg^{-1}, from *Aspergillus niger*) and tetrathiafulvalene were obtained from Sigma. A solution of Eastman-AQ-55D polymer (28% dispersion) was obtained from Eastman Kodak Co. D-Glucose and PVA were purchased from Shanghai Chemical Reagent Company. Glucose solutions were stored overnight to allow them to reach mutarotational equilibrium before use. All other chemicals used were of analytical reagent grade.

Regenerated silk fibroin (RSF) solution: The silk waste of a silk mill was treated with 0.5% $NaHCO_3$ aqueous solution at 100°C for 0.5 hour and then washed with distilled water. The silk was dissolved in 9.3 M LiBr aqueous solution. After dialysis against distilled water for 3 days, the solution was filtered and the aqueous solution of RSF was collected.

Membranes were obtained by casting the RSF solution or a solution of RSF, PVA, and glucose oxidase on glass plates at room temperature in air.

Fabrication of Eastman-AQ-TTF Modified Electrode

The glassy carbon electrodes were polished with 0.3, 0.1, and 0.05 μm Al_2O_3, rinsed thoroughly in deionized water between each polishing step, sonicated in 1:1 nitric acid, acetone, and de-ionized water sequentially, and dried in air before use. Eastman-AQ-TTF was coated on the electrode by pipetting 8 μL of diluted Eastman-AQ [1:25 (v/v) Eastman-AQ:acetone] polymer solution containing 0.08 M TTF onto the electrode surface and letting it dry in air.

Construction of the Glucose Sensor

Glucose oxidase (20 mg) was completely mixed in 0.40 mL of the blend solution of RSF and PVA. Aliquots (30 μL) of the solution were pipetted onto the Eastman-AQ-TTF modified electrode. After drying in air, the sensor was kept in air at 4°C in a refrigerator.

Pretreatment of the Glucose Sensor

After fabrication of the sensor and prior to experiments, the sensor response was stabilized by scanning between +0.45 and −0.2 V (vs SCE) in phosphate buffer (pH 6.5) over a 10-minute time period. TTF was oxidized to TTF^+ by this process when the potential was positive enough. Meanwhile, the Na^+ ions in the ester sulfonate group of Eastman-AQ were exchanged with TTF^+ and entered the phosphate buffer. The electrochemical reaction of TTF in the Eastman-AQ film can be described as

$$TTF + SO_3/Na^+ \longrightarrow SO_3/TTF^+ + Na^+ + e$$

Measurements

IR spectra were recorded on a FT-IR 5DX spectrometer at room temperature. The spectra of the membranes in the dry state were obtained by the reflection method.

The water absorbability was obtained by the following procedure. The membranes in their dried state were soaked in deionized water for 24 hours to achieve swelling equilibrium. The absorption water content was calculated by the equation

$$w = (w_1 - w_0)/w_0 \times 100\%$$

where w is the absorption water content, and w_0 and w_1 are the weights of the membranes in the dried and soaked states, respectively.

The maximum strength was measured by an electrical strain gauge apparatus (Instron 1121) and recorded at a constant drawing rate of 50 mm/min. The membranes dried in vacuum were cut into 10 × 50 mm rectangles. The measurement was carried out for the five membranes obtained under the same conditions.

All experiments were carried out with a three-electrode configuration comprising a glucose sensor as the working electrode, a saturated calomel reference electrode, and a platinum wire auxiliary electrode. The electrodes were connected to FDH 3204 and FDH 3206 cyclic voltammetry apparatus (Scientific Equipment Co.

of Fudan University, China), and the signal was recorded on a type 3086 x-y recorder (Tokyo, Japan) for cyclic voltammetric and amperometric measurements, separately. All experiments were carried out in a thermostatted, stirred electrochemical cell containing 5 mL of 0.1 M phosphate buffer (pH 7.0) at 25.0 ± 0.5°C. All experimental solutions were thoroughly deoxygenated by bubbling nitrogen through the solution for 10 minutes. In the constant potential experiments, aliquots of glucose stock solution were successively injected into the cell while the current was monitored after a constant residual current had been established. A calibration curve was obtained by applying a standard addition method, with measurements at 95% of the steady-state current.

RESULTS AND DISCUSSION

IR Spectra of the Membranes

Figure 1 is the IR spectra of the membranes. Before ethanol treatment, the membrane of pure RSF possesses absorption bands at 1706 cm^{-1} (amide I), 1571 cm^{-1} (amide II), and 1293 cm^{-1} (amide III), attributable to the characteristic silk I

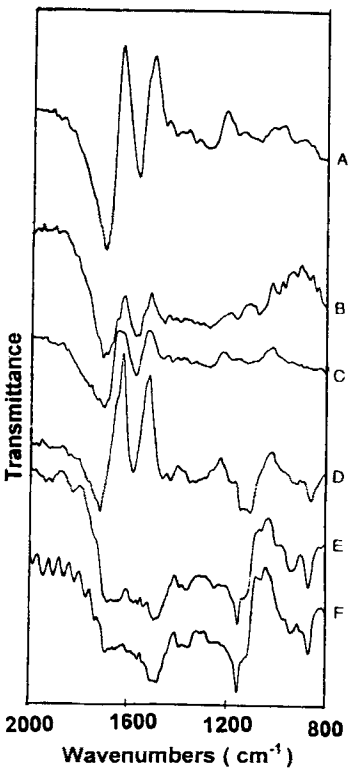

FIG. 1. IR spectra of the membranes. (A) Pure RSF before ethanol treatment, (B) pure RSF after ethanol treatment, (C) RSF/PVA = 2:1, (D) RSF/PVA = 1:5, (E) RSF/PVA = 1:9, (F) pure PVA.

structure. After ethanol treatment for 24 hours, the absorption bands of the membrane are split into two groups of absorption bands. One group is the same as those of the silk I structure, the other is 1687 cm^{-1} (amide I), 1559 cm^{-1} (amide II), and 1275 cm^{-1} (amide III), attributable to the silk II structure. The membrane of pure PVA shows a strong absorption band at 1150 cm^{-1} (C—O stretching). In the composite membranes of RSF and PVA, the absorption band at 1150 cm^{-1} increases with the proportion of the PVA in the composite. All composite membranes show the absorption bands of mixed structures, the silk I structure, and the silk II structure since they have two groups of absorption bands and the composite membranes consist mostly of the silk I structure. This suggests that the intermolecular interactions are not strong and that PVA has little influence on the structure of RSF. In addition, the membrane RSF/PVA = 1:5 possesses most elements of the silk I structure. It appears that RSF and PVA are immiscible, forming phase segregation [15].

Water Absorbability and Mechanical Property of the Blend Membranes

The relationship between the RSF and water contents of the membranes was investigated. At first, the water absorbability of the membranes increased with an increase of RSF. When RSF is 16.7% in the composite membrane (RSF/PVA = 1:5), water absorbability reaches its highest value. The water absorbability decreases with any further increase of RSF.

The relationship between RSF and the maximum strength of the membranes was tested. At first, the strength of the composite membranes increased with an increase of RSF. When RSF is increased up to 16.7% (RSF/PVA = 1:5), the strength reaches its highest value. The strength decreases with any further increase of RSF. These properties are evidently dependent upon the components of the composite membranes. Therefore, we chose the composite with the RSF to PVA ratio of 1:5 as the immobilization matrix of glucose oxidase, and we investigated the properties of the enzyme entrapped in the composite membrane.

Electrochemical Characterization of the Glucose Sensor

Figure 2 displays cyclic voltammograms of the glucose sensor in 0.1 M phosphate buffer (pH 7.0). With glucose absent from the solution, the enzyme contributes no response and only TTF at the sensor generates voltammograms indicating a reversible electron redox agent since separation of the oxidation and the reduction peaks (ΔE_p) below a scan rate of 165 mV/s remained constant (60 mV at 25°C) as did the peak current vs square root of the scan rate. The presence of the immobilized enzyme film does not change the electrochemical propriety of the mediator couple.

Electrocatalytic Oxidation of Glucose at the Sensor

There is no electrocatalytic oxidation current at the Eastman-AQ-TTF modified electrode when glucose is added to the phosphate buffer. Curve a in Figure 3 is a cyclic voltammogram of the Eastman-AQ-TTF modified glucose sensor in 0.1 M phosphate buffer without glucose. The cyclic voltammogram of the glucose sensor displays two oxidation waves with E_p values of about 150 and 460 mV, respectively.

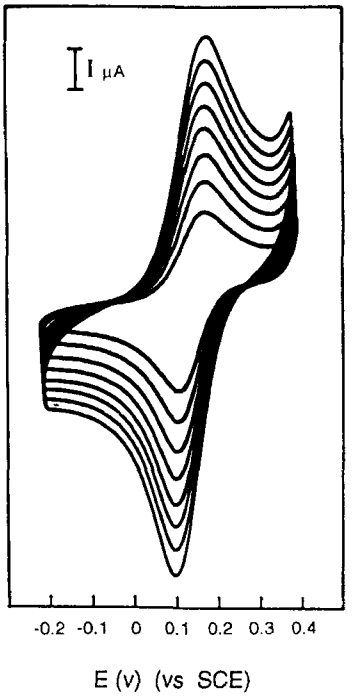

FIG. 2. Cyclic voltammograms of the glucose sensor reaching steady state at various scan rates (from inner curve to outer one): 25, 45, 65, 85, 105, 125, 145 mV/s in 0.1 M phosphate buffer (pH 7.0).

The first cyclic wave (TTF to TTF^+) exhibits reversible electrochemical behavior with a peak separation of 60 mV; the second does not. With successive additions of glucose to the solution, a gradual increase of two electrocatalytic oxidation waves is observed, accompanied by a gradual decrease in the cathodic current (Fig. 3, curves b to l), which indicates that both TTF^+ and TTF^{2+} can oxidize the $FADH_2$ of glucose oxidase. However, it is better if the working potential does not surpass +0.40 V because TTF^{2+} is less stable than TTF^+. Figure 4 presents typical cyclic voltammetric results for the sensor with the scan window between −0.20 and +0.40 V. When no glucose is present, the voltammetry displays the usual oxidation and reduction peaks for TTF. With successive additions of glucose to the solution, a gradual increase in oxidation current along with a gradual decrease of reduction current is observed. Comparison of the voltammograms with and without glucose present indicates that TTF can facilitate electron communication between the FAD/$FADH_2$ centers of glucose oxidase immobilized in the blend membrane and a glassy carbon electrode.

Figure 5 presents the effect of scan rate on the cyclic voltammogram for the glucose sensor in 0.1 M phosphate buffer with glucose. Increasing the scan rate results in an increase in the catalytic current, which is still observable at a higher scan speed. The absence of reduction waves suggests that the production rate of TTF from TTF^+ by reaction with GOD($FADH_2$) is fast at a scan rate less than 145

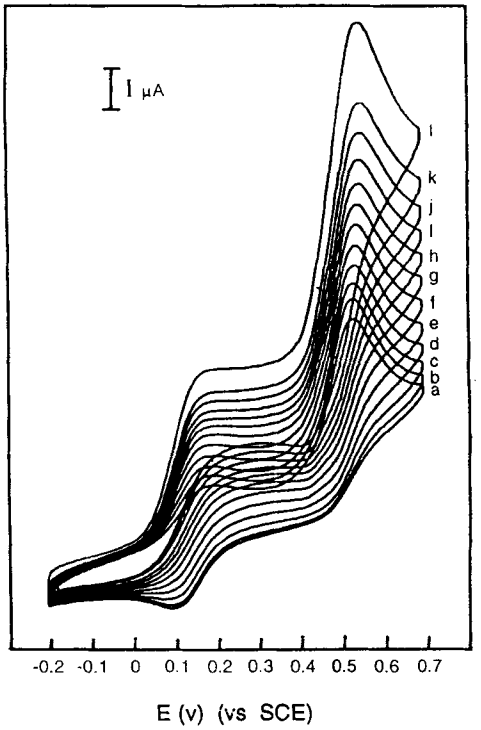

FIG. 3. Cyclic voltammograms of the glucose sensor with the scan potential window between −0.2 and +0.7 V at a scan rate of 35 mV/s in 0.1 M N_2-saturated phosphate buffer (pH 7.0) on successive increases of 0.5 mM glucose (from k to l for step increases of 1.5 mM glucose).

mV/s. However, at a scan rate greater than 200 mV/s, hysteresis appears and a reduction wave is also observed. The appearance of hysteresis and the reduction wave is a function of ionic strength, temperature, scan rate, film thickness, and substrate concentration.

Constant Potential Response to Glucose

Figure 6 gives a typical trace of the steady-state current-time response of the sensor at an applied potential of 0.20 V, with successive injections of glucose. The trace plainly illustrates the fast response and high sensitivity of the sensor to glucose, and the time required to reach 95% of maximum response of the sensor is short and calculated to be within 50 seconds. Figure 7 displays the calibration plot of the sensor response. The linear response is observed up to 15 mM. The detection limit of glucose, at a signal-to-noise ratio of 3, is found to be 0.04 mM.

These results demonstrate that the sensitivity and linear range of detection are dependent on the working potential. Increasing the applied potential results in an enhanced linear range and an increased sensitivity because of an increased driving force for the fast reoxidation of the $FADH_2$ of glucose oxidase.

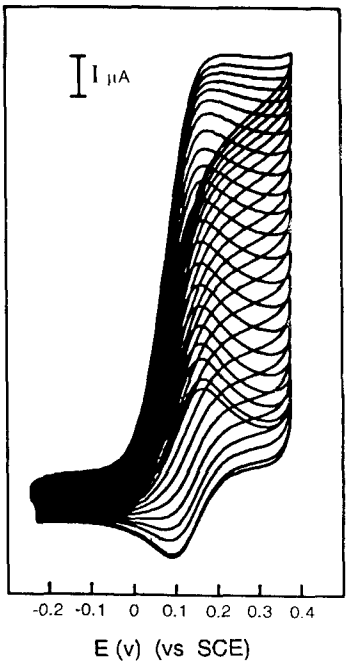

FIG. 4. Cyclic voltammograms of the glucose sensor with the scan potential window between −0.2 and +0.4 V at a scan rate of 35 mV/s in 0.1 M N_2-saturated phosphate buffer (pH 7.0) on successive increases of 0.5 mM glucose.

Effects of pH and Temperature on the Glucose Sensor

The effect of pH on the electrocatalytic current was investigated between pH 5.0 and 9.0. The substrate response displays an optimum at a broad pH, 6.5–7.5, and reaches a maximum at pH 7.0.

The effect of temperature on the sensor was examined between 15 and 60°C. At 35°C the immobilized enzyme loses 11% of its initial activity in 5 hours. The experiment shows that the steady-state current response increases with temperature, reaching a maximum value at 45°C. Any further increase in temperature causes a decrease of the response.

Reproducibility and Storage Stability of the Sensor

The sensor displays good reproducibility. After 100 measurements the standard deviation of response is within 4.1%. The lifetime of the sensor was investigated by keeping it dry in air at 4°C and determining its response at 5-day intervals. The sensor exhibits good storage characteristics; the current response was maintained almost unchanged for 2 months. The enhanced stability of the sensor is attributed to the favorable environmental conditions provided for the immobilized enzyme by the RSF and PVA composite.

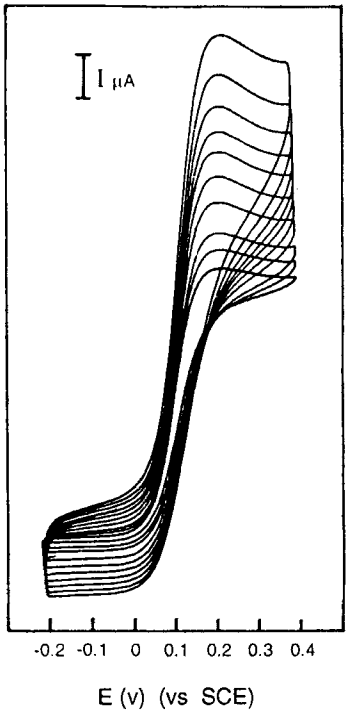

FIG. 5. Cyclic voltammograms of the glucose sensor at different speeds in 0.1 M N_2-saturated phosphate buffer with 12.5 mM glucose at scan rates of 15, 25, 45, 65, 85, 105, 125, 145, 165 mV/s (from inner to outer), respectively.

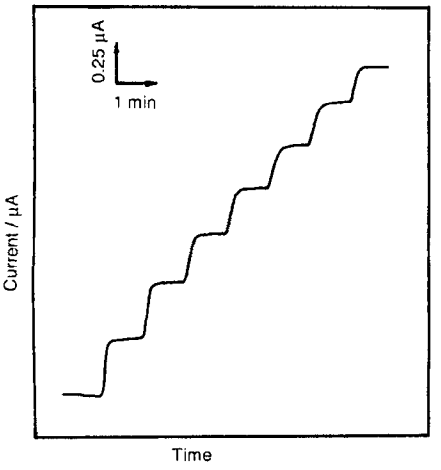

FIG. 6. Typical response of the modified glucose sensor to successive increases of 1 mM glucose at an operating potential of +200 mV in the N_2-saturated solution.

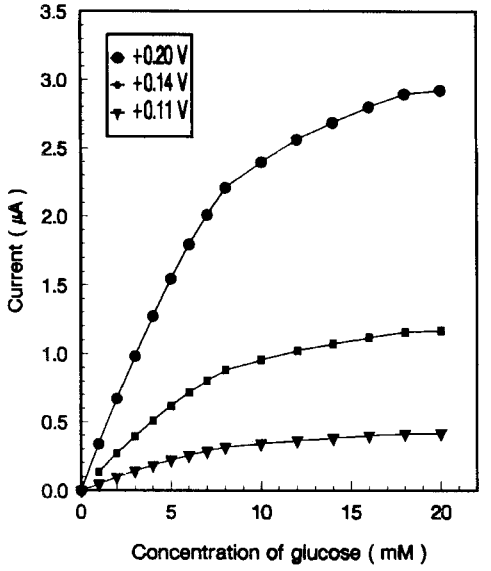

FIG. 7. Calibration plots for the glucose sensor. Steady-state current is measured in 0.1 M N_2-saturated phosphate buffer (pH 7.0) at several applied potentials.

CONCLUSIONS

Entrapment of glucose oxidase in a RSF and PVA composite provides a favorable microenvironmental conditions for the immobilized enzyme. The water absorption property and maximum strength of the composite (RSF/PVA = 1:5) are better than those of pure RSF. Moreover, its enzyme immobilization is superior to that of pure RSF. Consequently, the amperometric tetrathiafulvalene-mediating sensor of glucose has advantages of high stability and fast response to glucose. The simple method of sensor construction should be applicable to other enzyme-substrate systems for a variety of practical situations.

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