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Characteristics of regenerated silk fibroin membrane in its application to the immobilization of glucose oxidase and preparation of a p-benzoquinone mediating sensor for glucose

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Abstract. The structures of the blend membrane of regenerated silk fibroin and glucose oxidase were investigated by means of FT-IR spectra, electronic absorption spectra and SEM. The structures of the membranes were partly transferred from silk I to silk II after the membrane had been treated with ethanol. It was found that the glucose oxidase in the membrane existed in molecular aggregates and the blend membrane had an islands sea structure. p-Benzoquinone in solution was employed to speed up the electron transfer between glucose oxidase in a regenerated silk fibroin membrane and a glassy carbon electrode. The effects of pH, temperature, concentration of p-benzoquinone and applied potential on the sensor were examined. The major advantage of water dispersed regenerated silk fibroin is its ability to immobilize the enzyme without any significant loss of enzymatic activity.

Introduction

Immobilization of enzymes onto electrode surfaces as applied to amperometric sensors has received active attention in recent years. The methods of immobilizing enzymes on electrodes include cross-linking polymers [1], mixing into carbon paste [2–4], gel matrix [5], entrapment by ion-exchange polymers [6, 7], conducting polymers such as polypyrrole and its derivatives [8, 9], polyaniline [10, 11] and polyindole [12] and non-conducting polymers such as poly(o-phenylenediamine) [13]. The natural membrane materials used so far are K-carrageenan, chitin, chitosan, collagen and gelatin. Recently, silk fibroin obtained directly from Bombyx mori larvae has been found to be a good immobilization matrix for enyzmes because it offers many advantages over the natural or synthetic materials in biosensor systems, including its stability to most organic solvents and water, good tensile strength and elasticity. As an enzymatic immobilization matrix, its major merit is its capability of entrapping the enzymes by using physical, chemical or mechanical treatment (e.g. change in temperature, pH, solvent, mechanical shear or stretch), without using the usual crossing-linking chemicals, which can partially deactivate the activity of the enzymes. It has been utilized to immobilize glucose oxidase [14–17], peroxidase [18], alkaline phosphatase [19] and invertase [20]. However, the silk fibroin obtained directly from Bombyx mori larvae is only available several times a year, and how the enzymes exist in the silk fibroin membrane is not clear. Biosensors coupling enzymes immobilized in Bombyx mori silk fibroin with an oxygen probe or a platinum electrode have been made to monitor either the consumption of oxygen or the formation of the hydrogen peroxide. However, they are originally affected by the ambient concentration of oxygen in the sample or required a high overpotential, causing interference. Moreover, hydrogen peroxide enzymatically generated can inhibit and irreversibly inactivate glucose oxidase. With this in mind, we employed the regenerated silk fibroin prepared from waste silk as an enzyme immobilization matrix, investigated the structure and morphology of the regenerated silk fibroin membrane containing glucose oxidase and examined the feasibility of p-benzoquinone transferring electrons between the immobilized enzyme and the glassy carbon electrode.

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Experimental

Materials. Glucose oxidase (EC 1.1.3.4, 150,000Ug-1, from aspergillus niger) was obtained from Sigma. D-glucose was purchased from Shanghai Chemical Reagent Company and glucose solution was stored overnight to reach mutarotational equilibrium before use. All other chemicals used were of analytical reagent grade.

Regenerated silk fibroin solution: The waste silk of a silk mill was treated with 0.5% $NaHCO_3$ aqueous solution at 100° C for 30 min, then washed with de-ionized water. The silk was dissolved in 9.3 mol/l LiBr aqueous solution. After dialysis against de-ionized water for 3 days, the solution was filtered and the aqueous solution of regenerated silk fibroin was collected.

Membranes were prepared by casting the regenerated silk fibroin solution or the mixed solution of the regenerated silk fibroin and glucose oxidase on glass plates at room temperature in air.

Construction of the glucose sensor. A glassy carbon electrode (4 mm in diameter) was polished with 0.5, 0.3, and 0.1 μm Al_2O_3, rinsed thoroughly with de-ionized water between each polishing step, sonicated in 1:1 nitric acid/acetone and de-ionized water successively, and dried in air before use. 20 mg glucose oxidase was completely dissolved in 0.25 ml of the regenerated silk fibroin solution. Aliquots (15 μl) of the solution were pipetted onto the treated glassy carbon electrode. When dried in air, the sensor was kept in air at 4° C.

Measurements. IR spectra were recorded on an FT-IR 5DX spectrometer at room temperature. Spectra of the regenerated silk fibroin membranes in dried state were obtained by the reflection method and those of the glucose oxidase disc by the transmission method.

Electronic absorption spectra were measured on a Beckman DU-7 spectrophotometer at room temperature. Air was used as the reference for all the samples.

Scanning electron microscopy was carried out on a Hitachi S-520 at 20.0 kV.

Stationary potential measurements were accomplished on a FDH 3204 cyclic voltamperograph (Scientific Equipment Co. of Fudan University, P. R. China) in conjunction with a type 3086 x-y recorder (Tokoy, Japan). A three-electrode assembly was used in all experiments incorporating the glucose sensor working electrode, a saturated calomel (SCE) reference electrode and a laboratory-constructed platinum wire counter electrode. All experiments were performed in a thermostatted, stirred cell containing 5 ml 0.1 mol/l phosphate buffer. In the constant potential experiments, successive additions of stock glucose solution in the buffer were made after a constant residual current had been established. Changes in the measured oxidation current were recorded as function of time, following the addition of glucose. The sensor's response was measured as the difference between total and residual current.

Calculation of Michaelis-Menten constant. The apparent Michaelis-Menten constant K_M^{app} can be determined from the electrochemical Eadie-Hofsttee form of the Michaelis-Menten equation [21].

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

where j_{ss} represents the steady-state catalytic current, j_{max} refers to the maximum current measured under saturating substrate conditions, C is the glucose concentration and K_M^{app} represents the apparent Michaelis-Menten constant of the system as a whole, not that of an intrinsic property of glucose oxidase.

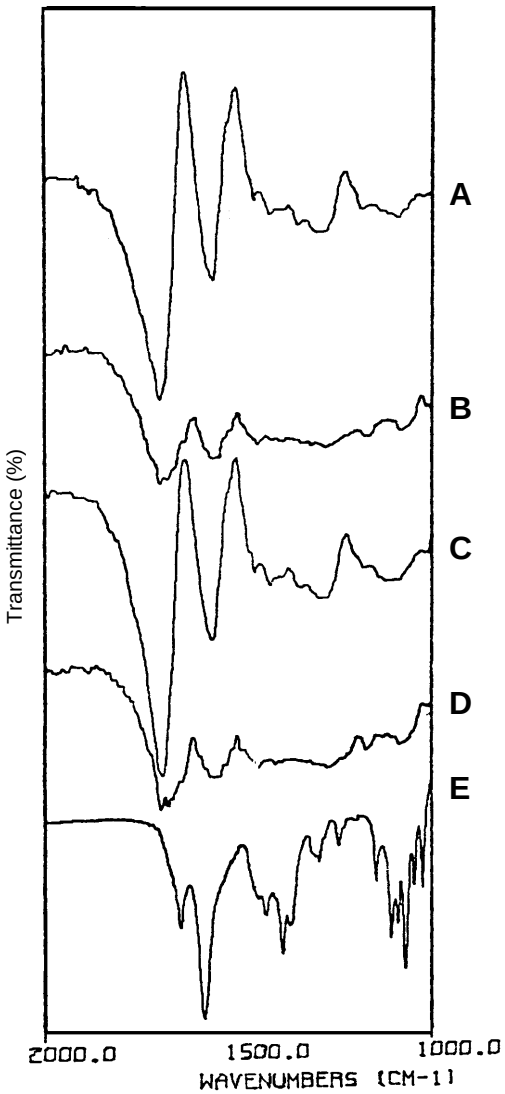

Fig. 1 A–E. IR spectra of the menbranes and KBr disc. **A** Regenerated silk fibroin membrane. **B** Regenerated silk fibroin membrane treated with ethanol. **C** Regenerated silk fibroin membrane containing 7.5% glucose oxidase. **D** Regenerated silk fibroin membrane containing 7.5% glucose oxidase after treatment with ethanol. **E** Glucose oxidase KBr disc

Results and discussion

IR spectra of the samples

Figure 1 shows that the regenerated silk fibroin membrane before ethanol treatment exhibits absorption bands at 1706 cm^{-1} (amide I), 1571 cm^{-1} (amide II), 1293 cm^{-1} (amide III), which is characteristic of the silk I structure. When treated with ethanol, the membrane shows mixed structures of two groups of absorption bands, one group is at 1706 cm^{-1} (amide I), 1575 cm^{-1} (amide II), 1312 cm^{-1} (amide III), characteristic of silk I structure, and the other is at 1687 cm^{-1} (amide I), 1559 cm^{-1} (amide II), 1275 cm^{-1} (amide III), characteristic of silk II structure. This indicates that a partial conformational transition from silk I to silk II of the regenerated silk fibroin membrane results from the treatment with ethanol. Compared with the corresponding amide bands of Asakura's membranes [22], our amide bands are located at higher wavenumbers.

Compared with the membrane of pure regenerated silk fibroin, the IR spectra of the blend membranes show no additional absorption bands and no band shift before and after treatment with ethanol. The characteristic absorption bands of glucose oxidase are overlapped. It is suggested that the intermolecular interactions between the two kinds of macromolecules are fairly weak in the blend membranes.

Electronic absorption spectra of the samples

Glucose oxidase has a characteristic electronic absorption band at 450 nm. However, the blend membranes of the two kinds of macromolecules have some absorption bands

Table 1. Electronic absorption bands of the membranes containing glucose oxidase and the solution of glucose oxidase

State	Concentration (%)	Absorption bands
Solution	0.08	450
Solution	0.26	450
Solution	0.29	449, 551, 576
Solution	0.41	449, 551, 576
Solution	1.43	449, 549, 579, 582
Membrane	0.8	476, 484
Membrane	2.1	478, 491
Membrane	7.5	475, 490, 508, 522

at longer than 450 nm, such as 476, 484, 508, 522 nm etc., but no absorption band at 450 nm (see Table 1). These are assigned to molecular aggregates of glucose oxidase. During the process of casting membranes for immobilizing the enzyme, accompanied by water evaporation, a clear one-phase solution is converted into a heterogeneous solution. A gel is formed and the microphase is segregated between the two kinds of macromolecules. Therefore, the electronic absorption bands of the blend membrane are those of molecular aggregates of glucose oxidase. In order to test this explanation, we studied the electronic absorption spectra of aqueous glucose oxidase solutions. Table 1 shows that these solutions always have a band at 450 nm, characteristic of the enzyme molecules of all the chain segments unfolded and fully solvated. When the concentration of glucose oxidase is raised to 0.29%, extra absorption bands appear at 551 nm and 576 nm, up to 1.43% solution in addition a band at 582 nm. This phenomenon results from partial interpenetration and entanglement of glucose oxidase molecules and from their molecular coil and segments partly held together by both intramolecular and intermolecular forces. This means that the bands in concentrated solutions of glucose oxidase are partly similar to those in the blend membranes and the molecules of glucose oxidase partly exist in aggregates.

Scanning electron microscopy (SEM) of the blend membrane surface

SEM (Fig. 2) also supports the fact that glucose oxidase molecules are aggregated in the blend membranes. It is obvious that the bright regions are the glucose oxidase domains, which are incompatible with the silk fibroin. The glucose oxidase aggregates are in random and distributed like islands in sea. Some are larger, some are smaller. The glucose oxidase molecules in the silk fibroin sea are relatively free because of the very weak molecular interaction between glucose oxidase and silk fibroin. Furthermore, there are empty spaces in the domain, suggesting that the glucose oxidase molecules seem to be incompact and their conformations may be in the most favourable state in which the activity is retained.

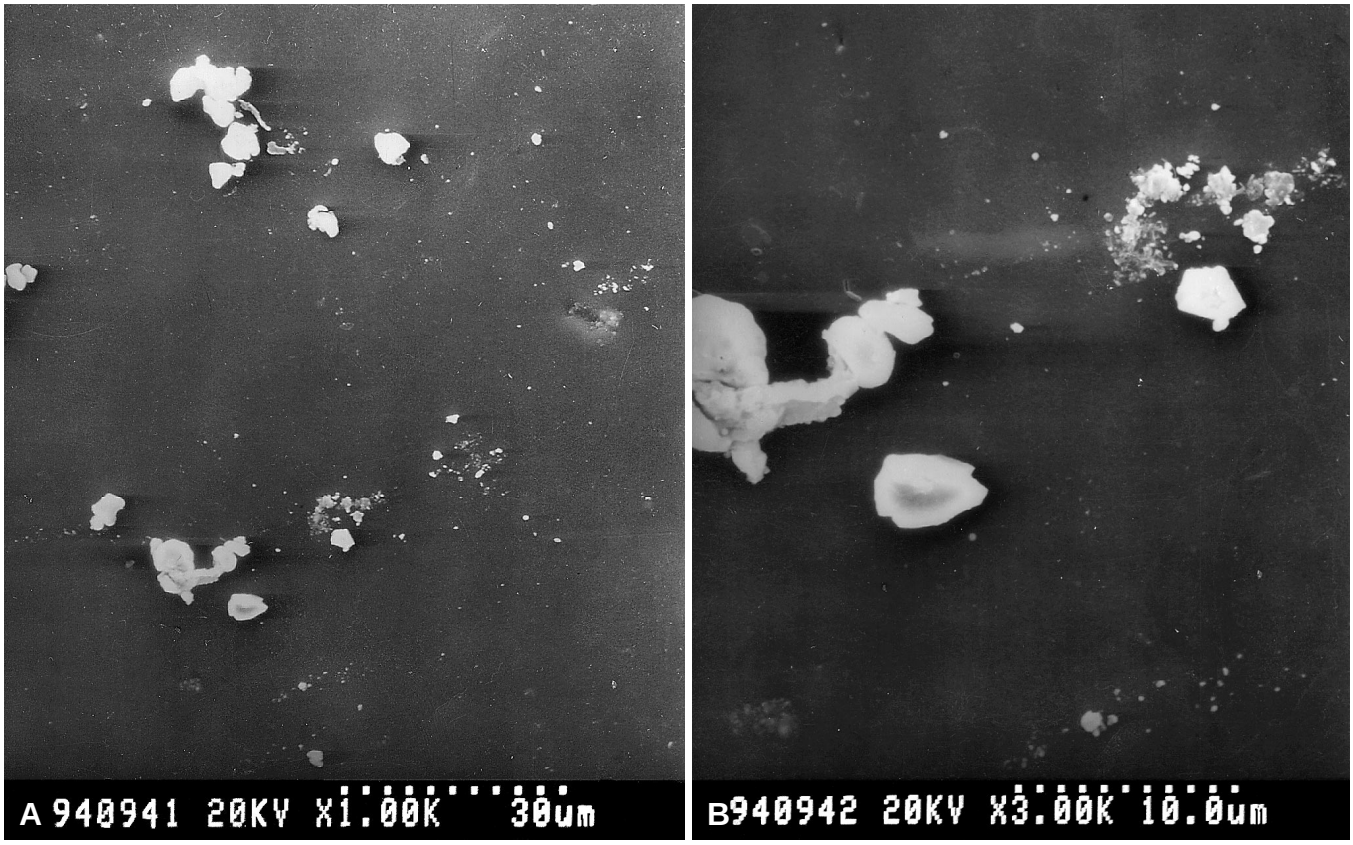

Fig. 2 A, B. Scanning electron microscopy of the regenerated silk fibroin membranes containing 0.8% glucose oxidase. **A** 1,000 times. **B** 3,000 times

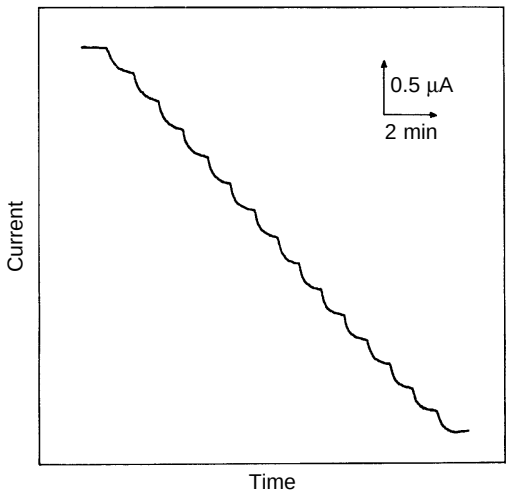

Fig. 3. Typical response of glucose oxidase on successive increase of 0.2 mmol/l glucose

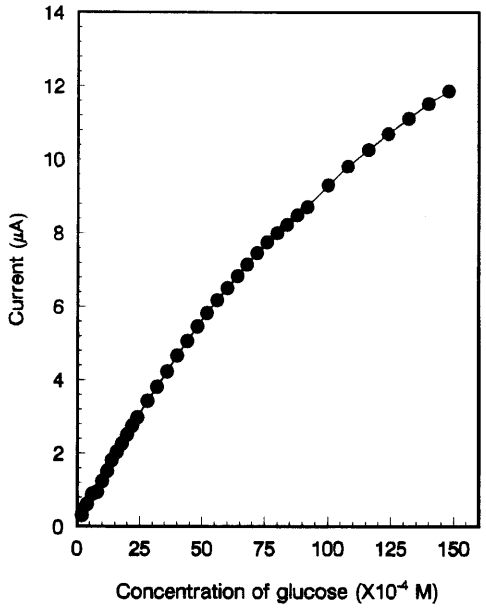

Fig. 4. Calibration curve of the sensor in 0.1 mol/l phosphate buffer (pH 7.0) containing 1.0 mmol/l p-benzoquinone at 0.4 V and at 20° C

Analytical characteristics of the sensor

Figure 3 shows the typical response of the glucose sensor. It plainly displays the quick response and good sensitivity to glucose. The time required to reach 95% of the steady state is within 12 s after injection of the glucose sample. Successive additions of equivalent glucose give the same responses. Figure 4 shows the plot of current response as a function of glucose concentration.

Effects of pH and temperature on the sensor

The experiment shows that the pH of the buffer has no influence upon the linear relationship of the sensor, but exerts an influence on its sensitivity. The sensor has an optimum response in the pH range between 6.5 and 8.5 and arrives at a maximum response at 7.5 (Fig. 5). This demonstrates that the sensor can be used in a wide range of pH.

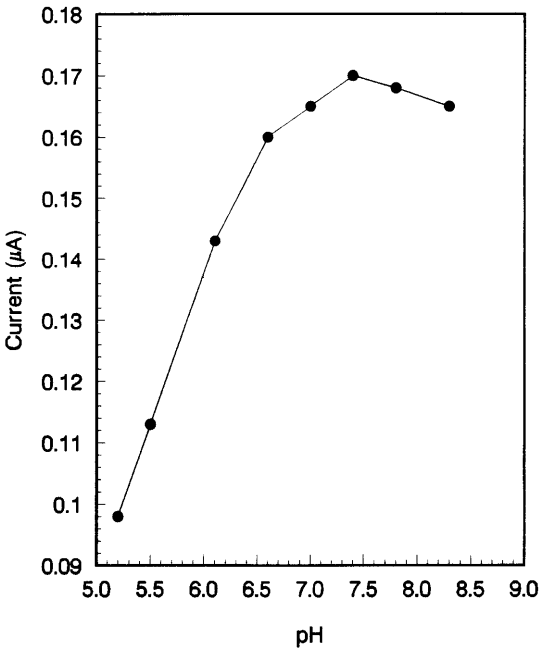

Fig. 5. Effect of pH on the current response of the sensor in 0.1 mol/l phosphate buffer containing 1.0 mmol/l p-benzoquinone and 0.2 mmol/l glucose at 20° C and at + 0.4V

Table 2. K_m^{app} at various temperatures

Temperature	20° C	25° C	30° C	40° C	45° C
K_m^{app} (mmol/l)	22.1	30.9	45.6	51.4° C	57.8° C

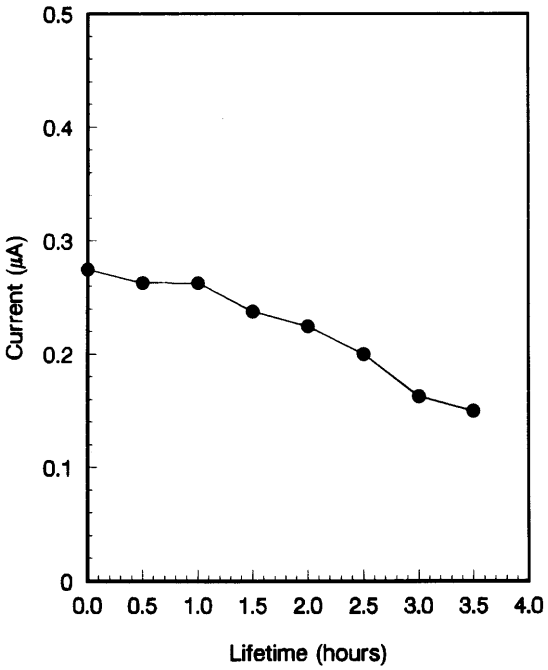

Fig. 6. Stability of the sensor at 50° C in 0.1 mol/l phosphate buffer (pH 7.0) containing 1.0 mmol/l p-benzoquinone at + 0.4V

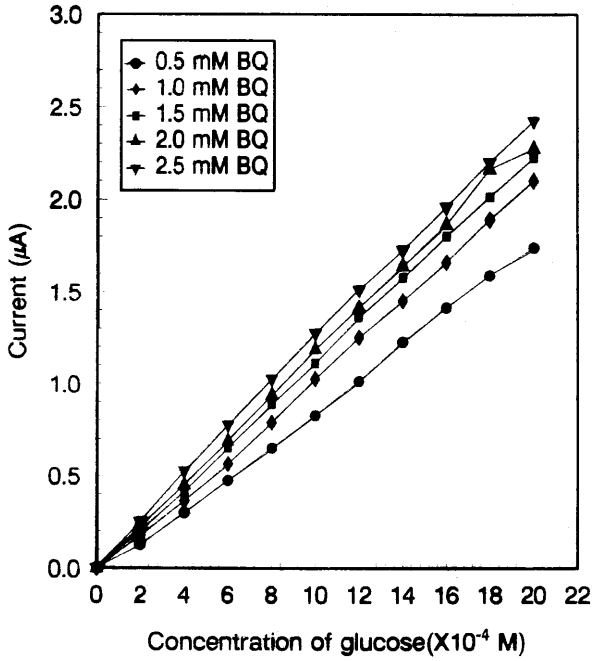

Fig. 7. Effect of the concentration of added p-benzoquinone on the sensor's response in 0.1 mol/l phosphate buffer (pH 7.0) at 20° C and at + 0.4V

Table 3. K_m^{app} at various potentials

Potential (V)	0.20	0.25	0.30	0.35	0.40	0.45
K_m^{app} (mmol/l)	10.87	13.61	17.54	22.68	28.71	31.73

The experiment confirms that the temperature does not affect the linear relationship of the sensor, but its sensitivity and the response time. The sensitivity increases and the response time is shortened with increasing temperature. The steady state current response increases with temperature, arriving at a maximum value between 45 and 55° C. Table 2 shows that the Michaelis-Menten constant is a function of the temperature. Its value increases with temperature.

Figure 6 shows the thermal stability of the sensor at 50° C. 95% of its original activity was maintained for 1 h continuous operation. The activity declined to 82%, 50% of its initial value after 2 and 3.5 h, respectively. These results indicate that the sensor can be used in an extensive range of temperature, even at higher temperature.

Effect of the concentration of added p-benzoquinone mediator on the sensor

The dependence of current response on the concentration of the mediator added is shown in Fig. 7. The concentration of p-benzoquinone does not influence the linear relationship of the sensor, but the steady-state current response increases with the concentration of the mediator. However, the higher the concentration of the mediator, the larger is the baseline current, and the optimum concentration of the mediators is 1.0 mmol/l.

Effect of the applied potential on the sensor

The sensitivity, linear range of detection and Michaelis-Menten constant are dependent on the working potential. The enhanced linear range and the increased sensitivity with the working potential can be attributed to the increased driving force for the fast reoxidation of the $FADH_2$ of glucose oxidase. Table 3 shows that the Michaelis-Menten constant increases with temperature.

Reproducibility and lifetime of the sensor

The sensor showed good reproducibility. The standard deviation of repeated experiments (10 times) was 2.7% for a solution of 0.2 mmol/l glucose. The response remained at 80% for 4 h continuous operation. The lifetime of the sensor was tested by keeping it in air at 4° C. The response to glucose was determined at 5-day intervals. The sensor had a long lifetime: the enzyme activity remained almost unchanged for 20 days and retained of 86% its original value for 60 days.

Conclusion

Regenerated silk fibroin prepared from waste silk has been successfully used to immobilize glucose oxidase by treatment with ethanol in spite of its weak intermolecular interaction and immiscibility. The glucose oxidase in the blend membrane exists in aggregates and the blend membrane has an islands-sea structure. We have demonstrated the feasibility of p-benzoquinone as electron transfer mediator between glucose oxidase in regenerated silk fibroin membrane and a glassy carbon electrode. The protective effect of regenerated silk fibroin for glucose oxidase against autoinactivation and thermodeactivation can be ascribed to a close moulding of the structure of the enzyme with regenerated silk fibroin.

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