POROUS CARBON COMPOSITE/ENZYME GLUCOSE MICROSENSOR

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TABLE OF CONTENTS

1. ABSTRACT

An enzyme glucose microsensor using a glucose oxidase-immobilized porous carbon/Teflon composite microelectrode was developed. The microsensor was fabricated by etching a platinum microelectrode (platinum, radius of 25 and 50 micrometers) in hot aqua regia to create a cavity at the tip and then packing a porous carbon/Teflon composite, which was made from acetylene black and Teflon emulsion, into the cavity. Nafion was impregnated into the inner surface of porous carbon/Teflon composite electrode following immobilization of $Os(bpy)_3^{+2/+3}$ as electron transfer mediators. The loading amount of $Os(bpy)_3^{+2/+3}$ in the Nafion/porous carbon/Teflon composite electrode was found to be 7.0×10^{-8} mole cm^{-2}, which is much higher than that in polymer modified electrodes reported in literatures. The microsensor was further dipped overnight in buffer solution containing glucose oxidase for enzyme modification. With both glucose oxidase and mediators in the porous carbon/Teflon composite surface, the sensor performance was evaluated in buffer solutions containing different glucose concentrations and serum samples for glucose determination. The microsensor showed directly electrochemical glucose oxidation on the $Os(bpy)_3^{+2/+3}$ impregnated enzyme/porous carbon/Teflon composite surface with linear response over concentration range of $0 - 15$ mM and Machaelis behavior. Reliability and reproducibility were conducted in serum samples and glucose buffer solution, and the results demonstrated there was no significant decrease of amperometric response in air-saturated solution for one month. The sensor demonstrated potential in clinical diagnostic applications.

2. INTRODUCTION

Enzyme-based biosensors have been extensively studied for wide applications in clinical diagnostics, environmental protection, food quality control and agriculture. Particularly, determination of glucose in blood plays a crucial role in the clinical diagnosis and treatment therapy of diabetes. The amperometric glucose sensor is one of the most important enzyme-based biosensors (1-14) due to their simple, sensitive and inexpensive merits. In amperometric glucose sensors, solid electrode surface is chemically modified by a sensing layer containing redox centers of glucose oxidase (GOD), or both GOD and electron mediators for glucose determination (15-17). Effective immobilization of GOD and electron mediator onto electrode surface is the most important task in design and fabrication of the amperometric glucose sensor for sensitivity, specificity, precision, reproducibility and lifetime in successful applications. Different methods such as physical adsorption (18), covalent linking (15, 19-22), self-assembly (23, 24), and impregnation in polymers (25 -29) have been reported for the immobilization. However, there are needs to further increase the amount of reaction centers for higher sensitivity and to develop advanced material for better stability.

Novel porous materials with high specific surface area such as platinum nanoparticles (30), colloidal gold/carbon past (31), carbon nanotubes (30), carbon/silica composite (32), carbon past (31, 33), graphite past (34, 35), silica so-gel film (36), titania sol-gel membrane (37), redox-active clay film (38), and wood ceramic film (39), have been investigated to incorporate GOD for improvement of immobilization efficiency and loading amount of redox reaction centers. Some of these methods are relatively complicated, environmentally unattractive and costive. In order to make low cost and high performance glucose sensors, new immobilization schemes and advanced materials are highly desired to have simple fabrication, environmentally friendly process, high sensitivity and great reproducibility.
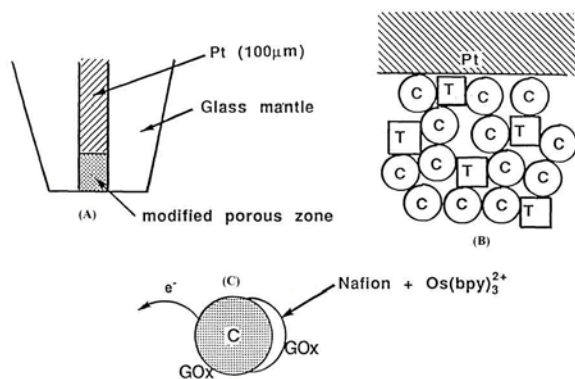
Carbon Composite Glucose Enzyme Microsensor

Figure 1. Schematic of construction of the porous carbon/Teflon composite/enzyme glucose microsensor

Size is another feature to affect performance of the glucose sensor. Conventional amperometric glucose sensors use membrane to produce steady-state diffusion current for reproducible measurements. Microelectrode based sensors have a number of advantages, which require much less amount of sample for analysis than conventional sensors, could be implantable and can produce steady-state diffusion limiting current without forced convection for highly reproducible analysis as a microelectrode has the high mass transport rate per unit area (40). Membrane used in conventional amperometric sensors for the diffusion limiting current increases cost and decreases the sensitivity. Apparently, amperometric microsensors are superior to the conventional sensors. In addition, a porous electrode material could increase the sensitivity, due to its high specific surface area, resulting highly apparent loading amount of both enzyme and electron mediators. In this work, we report a novel glucose microsensor, which was made from a porous carbon/Teflon composite modified by Nafion/glucose oxidase and electron transfer mediator for glucose measurements.

3. METERIAL AND METHODS

3.1. Materials and solutions

Glucoseoxidase [EC1.1.3.4] (SIGMA, catalno. G8135), D-glucose anhydrous(Mallinckrodt), Nafion solution (5% Nafion 117 solution with methanol, Aldrich), osmium(II) chloride (Aldrich) and serum from SeraChem. (Fisher, Catalog no.2905-63) were used as received. Zonyl FSN fluorosurfactant was generously donated by Dupont. The deionized water used in all experiments was produced by a Millipore milli-Q water purification system. All other chemicals were obtained from common commercial supplies.

$Os(bpy)_3(ClO4)_2$ was synthesized essentially as the method described in [41, 42]. In our experiments, $Os(bpy)_3(ClO4)^2$ was obtained through recrystallization by adding an aqueous solution of sodium perchlorate to a hot concentrated solution of the osmium salt in 1:1 ethanol + water, and cooling the new solution in an ice bath (42). The filtered crystals were washed with cold ethanol and then dried under vacuum.

The serum was reconstituted before the measurements. The reconstituting procedure was conducted in terms of the instruction from SeraChem (43). After removing seal and stopper of a vial containing lyophilized serum, a volumetric pipette was used to add 5.0 ml of deionized water into the vial. Replacing the stopper, the vial was swirled gently to mix the serum into water. 10-15 minutes without excessive shake of the vial were taken for complete reconstitution of serum before use.

3.2 .Electrodes and Modifications

The starting material was a sheet (thickness = 0.3mm) of conductive porous carbon/Teflon composite made from acetylene black and Teflon emulsion. The method of fabrication and properties of this material has been reported in (44). A kind of porous carbon paste containing 10% Teflon and 90% acetylene black was made first. The sheet was then fabricated by extruding the carbon paste through an extrusion press like a noodle machine. Then the sheet was recycle-extracted in acetonitrile for 48 hours. The sheet had a pore volume of approximately 85%. The diameters of the pore were in the range 1-100 micrometers. The sheet was first treated by soaking in a solution containing 0.1% Zonyl$^@$FSN fluoro-carbon surfactant (45) for 4 hours to make the carbon composite surface hydrophilic for applications in this glucose microsensor.

The fabrication of porous microelectrodes was described previously by two of us (46 - 48). For making a porous microelectrode, a Pt microelectrode was fabricated first. Different fabrication methods of an ultramicroelectrode are described in literature (40). In our experiments, we sealed ultra fine platinum wires (with radius of 25 and 50 micrometers) into glass capillary tubing by torch-heating the glass to make a solid microelectrode (46). The glass capillary tip was polished to expose a flat platinum disc surrounded by the capillary wall. The flat disc microelectrode was etched in hot aqua regia to create a cavity with 2-5 micrometers at the tip of the electrode, then the cavity was packed with porous carbon/Teflon composite material described above by grinding the tip against small lumps of the material on a clean flat surface such as a glass plate. The tip section view of the porous carbon/Teflon composite microelectrode is shown in Figure 1a. The structure of the conductive porous composite material in the cavity is shown in Figure 1B, indicating the Teflon molecules distribute uniformly in the composite material as a binder for the carbon particles and the porous carbon particles contact the platinum wire for sensing current flow. For immobilization of GOD and electron mediator, the porous carbon/Teflon composite microelectrodes were soaked in 2.5% Nafion solution for 15 minutes and dried in 70^0C oven before loading $Os(bpy)_3^{+2/+3}$ and modifying with glucose oxidase. The loadings of $Os(bpy)_3^{+2/+3}$ into the Nafion/carbon composite layer were carried out by cyclovoltommetry over potential range of 200 mV – 900 mV for 100 cycles at a scan rate of 10 mV. The glucose oxidase solution was prepared to contain 400 mg glucose oxidase/ml in 0.1M sodium phosphate + 0.15 M sodium chloride buffer solution (pH 7.4). After washing in deionized water the porous
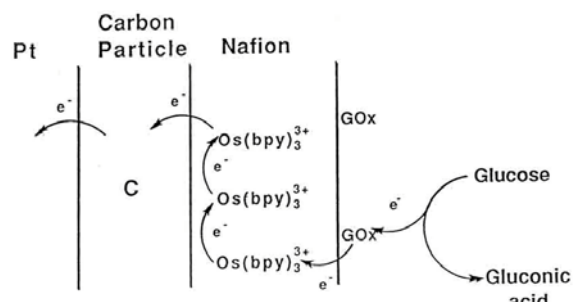
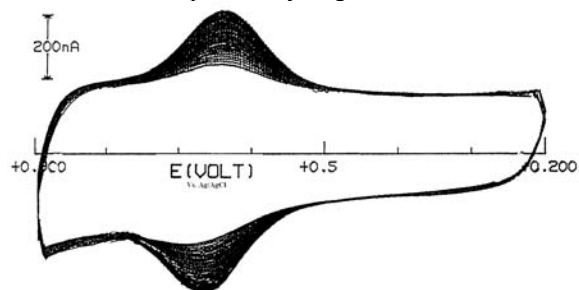**Figure 2.** Schematic of reaction scheme at the porous carbon/Teflon composite/enzyme glucose microsensor

Figure 3. Voltammograms at Nafion-modified porous carbon/Teflon composite microelectrode in 0.1mM $Os(bpy)_3(ClO4)^2$ + 0.01M $LiClO_4$. Scan rate: 10 mV/second, microelectrode r = 50 micrometers
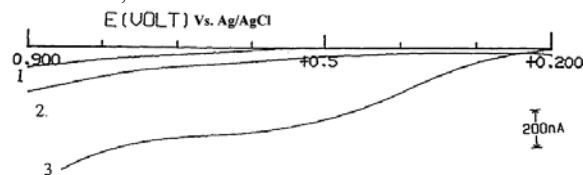

Figure 4. Voltammograms at porous carbon/Teflon composite microelectrode (Curve 1), glucose oxidase/Nafion-modified porous carbon/Teflon composite microelectrode (Curve 2), and $Os(bpy)^{+2/+3}$/glucose oxidase/Nafion-modified porous carbon/Teflon composite microelectrode (curve 3), respectively in 30 mM glucose + 0.1M KH_2PO_4 + 0.1M K_2HPO_4. Scan rate: 10 mV/second, Radius of electrode r = 50 micrometers

carbon/Teflon composite microelectrodes were dipped overnight in the glucose oxidase solution at 4^0C. Figure 1C schematically shows the porous carbon/Teflon composite with immobilization of GOD and $Os(bpy)^{+2/+3}$.
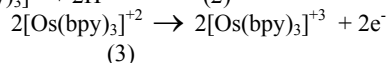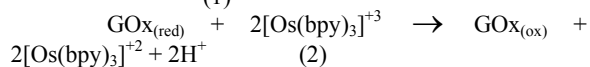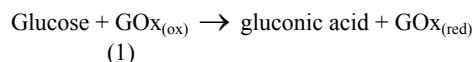
3.3. Electrochemical Experiments

Cyclic voltammetry and amperometric measurements at constant potential were used to evaluate the characteristics of the micro glucose sensor. The electrochemical experiments were performed using a commercially available BAS-100B Electrochemical Analyzer with a preamplifier unit. All experiments were carried out using a three electrode system. A Ag/AgCl, saturated KCl reference electrode was used. A salt bridge containing a saturated solution of potassium chloride was used to separate the reference electrode from the electrochemical cell. A coiled platinum wire was used as the counter electrode. All experiments were conducted under room temperature (ca. 24^0C).

4. RESULT AND DISCUSSION

4.1. Electrocatalytic reaction scheme

The reaction mechanism at the porous carbon/Teflon composite/enzyme glucose microsensor could be schematically shown in Figure 2 with the following reactions:

$$Glucose + GOx_{(ox)} \rightarrow gluconic\ acid + GOx_{(red)}$$
$$(1)$$
$$GOx_{(red)} + 2[Os(bpy)_3]^{+3} \rightarrow GOx_{(ox)} + 2[Os(bpy)_3]^{+2} + 2H^+ \qquad (2)$$
$$2[Os(bpy)_3]^{+2} \rightarrow 2[Os(bpy)_3]^{+3} + 2e^-$$
$$(3)$$

In this scheme, glucose is oxidized by glucose oxidase following electron transfer between reduced glucose oxidase and diffusional electron mediator, $[Os(bpy)_3]^{+3}$, and then sinks the electrons to porous carbon particle by $[Os(bpy)_3]^{+3/+2}$.

4.2. Loading of Osmium complex electron mediators

Loading $[Os(bpy)_3]^{+2/+3}$ into Nafion-modified porous carbon/Teflon composite was conducted in 0.1mM $Os(bpy)_3(ClO_4)_2$ + 0.01M $LiClO_4$ by cyclic voltammetry. The voltamograms are shown in Figure 3. The reduction/oxidation peaks in the voltammograms are almost symmetric, indicating negligible iR drop in the porous carbon/Teflon composite electrode. The loading value of the electron mediators could be calculated from the area of peaks and was 7.0×10^{-8}mol/cm^2 for the apparent geometric electrode surface(outer surface area). This value is 35 times higher than the value obtained at a Nafion-coated pyrolytic graphite electrode (1.9×10^{-9} mol/cm^2) (49). Therefore, this technique is a very efficient way of introducing a significantly high concentration of catalytically active charge-transferring redox sites per unit of apparent area of electrode surface by using porous electrode materials. This is a great advantage of the enzyme microsensor since the electrochemical response could be significantly increased.

4.3. Effect of electrode modification on glucose detection

Porous carbon/Teflon composite microelectrode without modification, glucose oxidase/Nafion modified porous carbon/Teflon composite microelectrode and $[Os(bpy)_3]^{+2/+3}$/glucose oxidase/Nafion modified porous carbon/Teflon composite electrode were made by the method described in 3.2, respectively and were investigated with voltammetry measurements in 30 mM glucose + 0.1M KH_2PO_4 + 0.1M K_2HPO_4. The results are shown in Figure 4. The electrochemical responses on the porous carbon/Teflon composite electrode (Figure 4, curve 1) and glucose oxidase/Nafion modified porous carbon/Teflon composite microelectrode (Figure 4, curve 2) did not show well-defined diffusion-control current, and the current was much smaller than that obtained at the $[Os(bpy)_3]^{+2/+3}$/glucose oxidase/Nafion modified porous carbon/Teflon composite microelectrode (Figure 4, curve 3), indicating glucose could not have significant electrochemical reactions on these porous carbon/Teflon
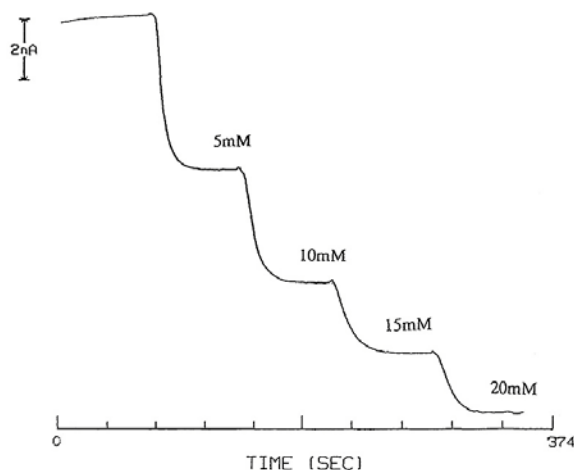
Figure 5. Voltammograms measured by single potential time base at Os(bpy)$_3^{+2/+3}$/glucose oxidase/Nafion-modified porous carbon/Teflon composite microelectrode in 0.1M KH$_2$PO$_4$ + 0.1M K$_2$HPO$_4$ by adding concentrated glucose solution for different glucose concentration. Applied potential = 0.6 V vs. Ag/AgCl, Radius of electrode r = 25 micrometers
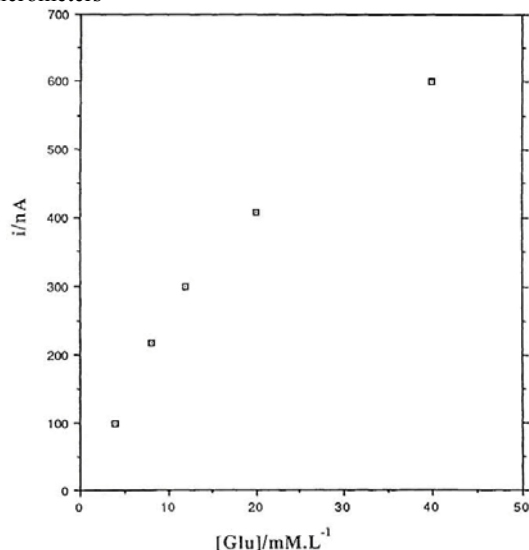

Figure 6. Relation of amperometric response vs. glucose concentration at Os(bpy)$_3^{+2/+3}$/glucose oxidase/Nafion-modified porous carbon/Teflon composite microelectrode in 0.1M KH$_2$PO$_4$ + 0.1M K$_2$HPO$_4$ solutions containing different glucose concentrations. Radius of electrode r = 50 micrometers

lectrode surfaces without loading of electron mediators. However, the current of curve 2 is larger than curve 1, which might indicate that there was sluggish glucose enzymatic reaction at the glucose oxidase/Nafion modified porous carbon/Teflon composite microelectrode. In addition, a well-defined diffusion control current was observed at the [Os(bpy)$_3$]$^{+2/+3}$/glucose oxidase/Nafion modified porous carbon/Teflon composite microelectrode over potential range of 0.6-0.8 V vs. Ag/AgCl. Li reported that the steady-state diffusion – controlled current could be also obtained from a porous carbon/Teflon composite

microelectrode (39). The steady-state diffusion-controlled current obtained from high mass transport rate is insensitive to fluctuations from natural convection in bulk solution and to electrode potential drift in certain range. The experimental results showed that the micro porous carbon/Teflon composite electrode modified by Nafion, glucose oxidase and Os(bpy)$_3^{+2/+3}$ could be a novel sensor for glucose detection.

4.4. Amperometric responses

Voltammetric experiments were conducted with the enzyme porous carbon/Teflon microsensor in 30mM glucose + 0.1M KH$_2$PO$_4$ + 0.1M K$_2$HPO$_4$ solutions saturated with nitrogen, air and oxygen, respectively. There was no significantly different amperometric response of the microsensor to oxygen concentrations, indicating such a glucose microsensor is relatively independent of wide changes of concentration of oxygen. In early glucose sensors (50), the enzyme sensing electrode does not have electron-carrying mediators and determination of glucose relies on the liberation of hydrogen peroxide in the enzyme reaction. Obviously, oxygen concentration has significant effect on glucose detection. Therefore, the result of oxygen independence of glucose detection demonstrated that the directly electrochemical oxidation of glucose through glucose oxidase at the porous carbon/Teflon composite microsensor occurs as the reaction scheme shown in Figure 2 and Equations of 1, 2 and 3. Independence of oxygen for glucose detection could improve the signal/noise ratio and extend the sensor lifetime since H$_2$O$_2$ formed in reaction 4 could degrade the enzyme.

Voltammograms were measured by single potential time base at the porous carbon/Teflon/enzyme microsensor in 0.1M KH$_2$PO$_4$ + 0.1M K$_2$HPO$_4$ solutions containing different concentrations of glucose. The results are shown in Figure 5. In the measurements, aliquots of 1M glucose solution were sequentially injected into the measurement cell (solution volume 20ml) while the current was monitored. The volume of injecting aliquot was determined in such a way that actual glucose concentration in the electrochemical cell was increased by step of 5 mM after every addition of the glucose solution. Figure 5 shows well-defined diffusion-controlled current plateau. The plateau current stabilized in ca. 18s, which was the time required to reach a uniform distribution of glucose concentration throughout the cell and to achieve the diffusion control zone after the injection of concentrated glucose solution. The response time could be further reduced with a smaller measuring chamber due to shorter mass transportation time. However, even response time of 18 s. is fairly good for the clinical applications in glucose determination.

Figure 6 shows the relation of diffusion current vs. glucose concentration. The curve deviates from linearity above 15 mM glucose. The result may represent the typical Michaelis-Menten Kinetics behavior of an enzyme reaction (51). The Michaelis-Menten enzyme kinetics Equation can be expressed as

$$V = -K_m (V/[S]) + V_{max} \quad (4)$$

Carbon Composite Glucose Enzyme Microsensor

Table 1. Comparison of measurement results obtained at porous carbon/Teflon composite microsensor in sera with manufacture data

Sensor#	1	2	3	4	5	Average	Max.Variation (%)
Measured (mM)	5.97	6.03	5.59	5.87	5.76	5.84	4.6
Manufacture Range (mM)	4.22-6.38	4.22-6.38	4.22-6.38	4.22-6.38	4.22-6.38	5.30	17
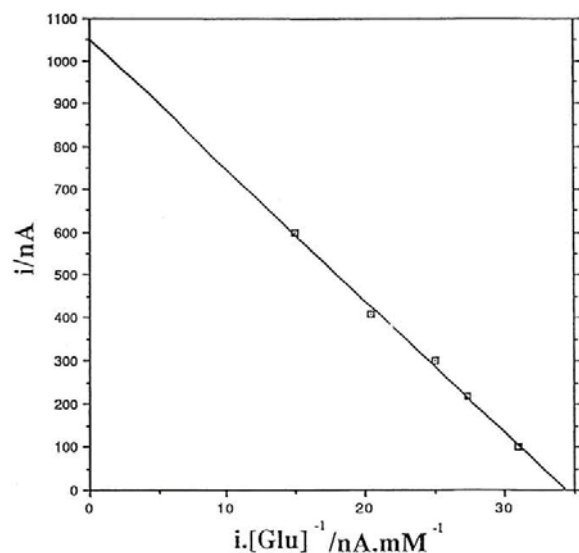
Figure 7. Relation of enzyme reaction rate (i) vs. rate(i)/glucose concentration(C) at $Os(bpy)_3^{+2/+3}$/glucose oxidase/Nafion-modified porous carbon/Teflon composite microelectrode in 0.1M KH_2PO_4 + 0.1M K_2HPO_4 solutions containing different glucose concentrations. Radius of electrode r = 50 micrometers

Where V is the enzyme-catalyzed reaction rate, [S] is the substrate concentration, V_{max} is the maximum reaction rate and K_m is the Michaelis constant. The results obtained at the composite microsensor were plotted in a form of i vs. i/[S], shown in Figure 7, giving a straight line as the Equation 4 predicts. According to Equation 4, the intercept of 1052 nA in Figure 7 equals to i_{max} (i.e. V_{max}). Thus, the maximum current density based on the apparent area of outer surface of the microelectrode (7.85×10^{-5} cm^2) is 13.4 mA. The maximum reaction rate of this micro glucose sensor is 16 times at least higher than that of 0.1 to 0.8 mA reported in (11). It might be due to the higher mass transportation rate and larger true surface area of the porous carbon/Teflon microelectrode for greater apparent diffusion-control current density. The linear range of this micro enzyme sensor is over 0-15 mM, which is shown in Figure 6.

4.5 Diagnosis application and sensor reproducibility

The porous carbon/Teflon enzyme glucose microsensors were used to determine the glucose concentration in serum samples. The known constituent values of serum from the manufactory were 4.22-6.38 mM (43). Measurements were conducted in serum solutions with five micro porous carbon/composite based glucose sensors, showing the average glucose concentration in the serum was 5.84 mM, and maximum deviation from the average value was 0.27 mM. The results are shown in Table 1, which compares the measured results with the concentration range reported by the manufactory (43) and shows that the results are in good agreement with the reported data. The results also demonstrated the standard deviation of the sensors was 0.17 mM, indicating that the novel glucose microsensors had fairly good reproducibility.

The lifetime of the porous carbon/Teflon composite enzyme microsensors were investigated. The microsensors were used to determine the glucose concentration in 7.5 mM glucose + 0.1M KH_2PO_4 + 0.1M K_2HPO_4 twice a week for three months. The microsensors were stored in 0.1M KH_2PO_4 + 0.1M K_2HPO_4 buffer solutions after every measurement following thoroughly rinse with the same storage buffer solution. Analytical results demonstrated that the sensitivity of this glucose microsensors changed less than 5% in a month. After one month, the sensitivity gradually decreased to 30% of the initial value at the end of three months. However, the linear relationship of the diffusion-control current vs. glucose concentrations was always obtained by the measurements in all three months.

5. CONCLUSION

A simple and effective method was developed to immobilize both enzyme and electron-carrying mediator onto a porous carbon/Teflon composite electrode for a glucose microsensor. This technique increased the amount of loading of enzyme and electron mediator for sensitivity improvement. The experimental results also demonstrated that the microsensor was independent of existence of oxygen and had reproducible results in one month. The microsensor was used to detect glucose concentration in sera and showed good agreement with the manufacturer's data. This demonstrated its potential applications in clinical diagnosis applications for glucose determination. This technique for immobilization of enzyme and mediator onto porous carbon/Teflon composite electrodes appears to be very simple and promising for other enzyme electrode applications.

6. ACKNOWLEDGEMENT

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