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Improved Biosensor for Glucose Based on Glucose Oxidase-Immobilized Silk Fibroin Membrane

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ABSTRACT

Based on glucose oxidase-immobilized silk fibroin membrane and oxygen electrode, the authors have developed an amperometric glucose sensor in flow-injection analysis. After the sensor was improved by the configuration of oxygen electrode and a temperature control system was added to the electrode body, its sensitivity, analytical precision, and stability were enhanced greatly. The authors first introduced a tailing inhibitor-ion pair reagent into a buffer system in the biosensor so as to eliminate all interference from hemacyte, macromolecules, and small mol wt charged species besides electroactive specie ascorbate in complex matrices. A considerably serious tailing of the biosamples, such as whole blood, plasma, serum, or urine on the sensor, based on enzyme electrode, entirely disappeared, their response times were shortened, and base lines became more smooth and stable. The glucose sensor has a broad range of linear response for glucose (up to 25.0 mmol/L) and a good correlation (γ = 0.999) under conditions of control temperature 32.0°C and 1.6 mL/min 0.02 mol/L phosphate buffer containing 0.5% tailing inhibitor (v/v). Recoveries of glucose in these biosamples are within the range of 93.71–105.88%, and its repeatabilities for determining glucose, repeated 100 times, human blood dilution 125 times, and serum 128 times, are 1.81, 2.48, and 2.91% (RSD), respectively. The correlation analysis for 200 serum samples showed that the correlation (γ) is 0.9934 between the glucose sensor and Worthington method for determining serum glucose used conventionally in a hospital laboratory. Moreover, the enzyme membrane used in the biosensor can be stored for a long time (over 2 yr)

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and measured repeatedly over 1000 times for biosamples. The glucose sensor is capable of detecting over 60 biosamples/hr.

Index Entries: Glucose sensor; glucose oxidase-immobilized fibroin membrane; oxygen electrode; immobilized enzyme; flow-injection analysis; ion pair reagent.

INTRODUCTION

Silk fibroin derived from silkworm *Bombyx mori* is a natural macromolecular protein to which much attention has been paid as biomaterial for enzyme immobilization. Since Miyairi et al. reported immobilization of β-glucosidase in fibroin membrane in 1978 *(1)*, several enzymes, such as glucose oxidase (GOD), peroxidase (POD), and lipase, have been trapped and immobilized in silk fibroin membrane *(2–4)*. Asakura and Demura et al. investigated in detail its mechanism for enzyme immobilization and membrane potential of fibroin membrane induced by an immobilized enzyme reaction by means of infrared spectra, nuclear magnetic resonance, and spin-labeling electron spin resonance *(5–7)*. It is confirmed that the silk fibroin membrane is a good biomembrane material for enzyme immobilization. However, there are few reports on the enzyme-immobilized silk fibroin membrane for practical use in biosensors. In recent years, the authors have prepared three POD-, GOD-, and uricase-immobilized silk fibroin membranes and described their characteristics in spectrophotometry and electrochemistry *(8–10)*. Moreover, the authors have prepared another GOD- or POD-immobilized silk fibroin membrane whose cost is cheaper than the one just mentioned and demonstrated that this enzyme membrane also has good performance and very high recovery for enzyme activity *(9,11)*. In these experiments the authors have found a key property of the two kinds of enzyme-immobilized fibroin membranes: they can be stored for a long time (over 2 yr) and keep their original activities. Recently, the authors have developed an amperometric biosensor in flow-injection analysis for determination of glucose, based on these GOD-immobilized membrane and oxygen electrode *(12)*. This biosensor has a broad linear-response range for glucose and good repeatability, and is capable of detecting about 30 human serum samples per hour (50 μL serum per injection). However, the biosensor suffered the influence of hemacytes, some macromolecules, and smaller molecular charged species, besides interference from endogenous electroactive substances in biosamples, as with other biosensors. It induced a considerably serious lag of the difference current response peak of biosamples on sensor and unstable baseline, which resulted in the analytical rate for samples decreasing and loss of its practical value. The present article describes in detail the elec-

trochemical investigation of a variety of characteristics after the glucose sensor based on enzyme-immobilized fibroin membrane was undergone to being improved in oxygen electrode's configuration and control temperature system, and introduced a tailing inhibitor into buffer system for enzymatic reaction.

MATERIALS AND METHODS

Preparation of GOD-Immobilized Silk Fibroin Membrane

The *B. mori* cocoons were degummed twice with 0.5% Na_2CO_3 solution at 100°C for 30 min, and then washed repeatedly with distilled water. After lyophilization, the degummed silk fiber was dissolved in 9.0 mol/L LiBr solution, and the aqueous fibroin solution was obtained. GOD-immobilized silk fibroin membrane was prepared with the aqueous fibroin solution and GOD derived from *Aspergillus niger* (EC 1.1.3.4, 23900 U/g, Type II, Sigma), as reported previously *(9)*. It is pointed out here that the GOD-immobilized fibroin membrane, prepared on August 9, 1994, was stored at 4°C until use for all tests in this article in August 1996.

Configuration and Operation Fundamentals of Sensor

This glucose sensor in flow-injection analysis consists of a sample mixer, peristaltic pump, GOD-immobilized fibroin membrane-covered oxygen electrode, heat exchanger, microcell for enzyme reaction, circulating thermostat (±0.1°C), amplifier for oxygen electrode, and a Beckman 427 integrator. A given volume of sample solution (standard glucose solution, human blood dilution, plasma, serum, or urine) was injected into the flowing phosphate buffer (1.6 mL/min), and then the mixture was incubated at a given temperature in the heat exchanger. When running into a microcell, glucose in the flowing buffer solution was in contact with enzyme membrane at the base of the electrode and reacted continuously with O_2 by catalysis of immobilized GOD, so consumption of oxygen in the reaction solution could be monitored continuously by the oxygen electrode. The resulting difference current response peak was recorded in mV by an integrator. Shortly after glucose in flowing buffer left the microcell for enzyme reaction, the response current of the electrode returned once to original state, and the next injection began.

Improvement of Sensor

The configuration improvement for the glucose sensor, in comparison with the original sensor reported earlier *(12)*, focuses on the following four aspects: (1) To abate the measuring error induced by the fluctuation of envi-

ronmental temperature, the electrode body and buffer were all controlled at the same temperature as that of enzymatic reaction in heat exchanger by a circulating thermostat; (2) in order to increase the sensor's sensitivity to substrate glucose, the surface area of Pt cathode in oxygen electrode was enlarged fivefold than that of original electrode; (3) because the original sensor system at a higher work temperature suffers easily the influence of changing environmental temperature on enzyme electrode, the temperature for enzymatic reaction was decreased from the original 37 to 32°C; (4) a tailing inhibitor in the proportion of 0.5% (v/v) was first introduced into a phosphate buffer system at the biosensor to eliminate the lag of response current peak of biosamples at biosensor in flow-injection analysis.

Sensor's Operation Condition

All tests for the present article were carried out under the same experimental conditions, except a given description: polarizing voltage 0.640 V (Ag anode and Pt cathode), buffer 0.02 mol/L phosphate buffer containing 0.5% tetrabutylammonium hydroxide (v/v), pH 7.8, adjusted with 0.5 *N* HCl or NaOH solution, control temperatures for enzyme reaction, buffer, and electrode body 32°C.

Worthington Determination Method for Glucose

In the experiment of correlation analysis for glucose determination on 200 human serum samples, the authors adopted the GOD–POD Enzymatic-Colorimetric Method (Worthington method) recommended by the Clinical Examination Center of Chinese National Public Health Ministry *(13)*. Serum sample was measured repeatedly two times at a Spectrophotometer (Hitachi 200-20 Model, Japan) using a glucose reagent kit (Experimental Center for Clinical Diagnostic Reagents, Shanghai Institute of Biological Products, Ministry of Public Health Shanghai, China). An average level of glucose in serum sample was calculated with the linear regression equation ($y = 6.5076 + 0.0395\chi$, $\gamma = 0.99973$) derived from calibration curve for glucose.

RESULTS

Response Characteristics of Glucose Sensor

When injecting a series of various concentrations of 50 μL glucose standard solution into the biosensor, the original record of the response peaks of difference current is shown as Fig. 1. Table 1 illustrates in detail the response behaviors such as response current and response time. When glucose concentration is in the range of 0.25–12.5 mmol/L, the linear response of difference current of glucose on the sensor is fairly good (γ =

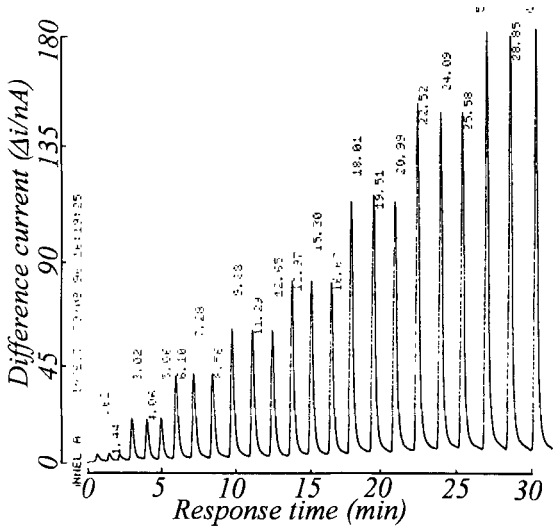

Fig. 1. Difference response current peaks of a series of standard glucose solution (0.25–12.5mmol/L) on the sensor for glucose. Electrode voltage (EV): 0.640 V, buffer: 1.6 mL/min 0.02 mol/L NaH_2PO_4, pH 7.80, heat exchanger and electrode body temperature 32.0°C. Sampling volume: 50 μL.

0.99986). Figure 2 displays the calibration curves of different current responses for glucose on the biosensor. In the range of 0.5–25.0 mmol/L, the resulting linear regression equation is $y = 0.887254 + 14.787499\chi$, and its correlation coefficient (γ) can still arrive at 0.99956. Within the smaller scale of 0.5–10.0 mmol/L, a good linearity for glucose can be obtained ($\gamma = 0.999989$). Lower limit of detectability on this glucose sensor is 0.1 mmol/L glucose, which is entirely dependent on the amount and activity of immobilized enzyme in the silk fibroin membrane. The higher activity of immobilized enzyme, the lower the limit of detectability. In addition, can also find that there is a difference in the response time from the response peaks in Fig. 1 and Table 1 because of the different concentrations of glucose injected into the sensor. The higher the glucose concentration, the longer its response time. If running within a range of 0.25–5.0 mmol/L glucose solution, the sensor can detect no less than 60 samples per hour. Therefore, this glucose sensor, based on GOD-immobilized silk fibroin membrane and oxygen electrode, could rapidly determine a variety of analytical glucose samples.

Tailing Inhibitor

The glucose biosensor based on GOD-immobilized silk fibroin membrane and oxygen electrode suffered from many charged substances in the biosamples besides anionic electroactive species, as with other biosensors.

Table 1 Response Behavior of Glucose on Biosensor

50μl glucose (mM)	0.25	1.25	2.50	3.75	5.00	7.50	10.00	12.50	linearity equation and γ
Response (nA)	1.695	10.335	21.720	33.075	45.450	66.345	88.170	109.830	$y = -0.18378 + 8.84422x$
±SD	0.180	0.225	0.105	0.270	0.435	1.005	0.135	0.945	$\gamma = 0.99986$
Response time (S)	30	36	50	55	60	70	80	90	

The values in the table were an average of repeatedly measuring for three times. Electrode voltage: 0.640 V, Buffer: 1.6 mL/min 0.02 mol/L NaH_2PO_4 containing 0.5% additive, pH 7.80; heat exchanger, buffer, and Electrode body temperature: 32.0°C.

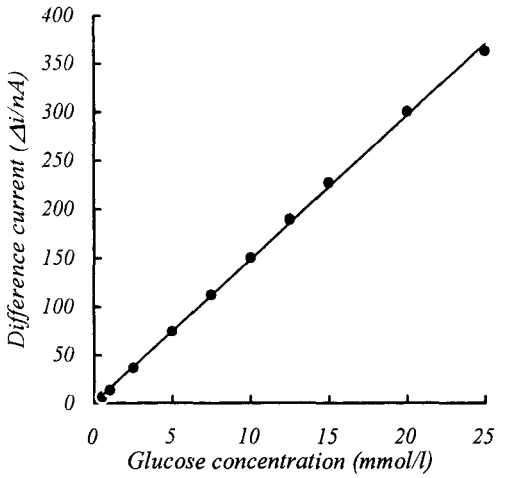

Fig. 2. Typical calibration curve of standard glucose on the biosensor based on GOD-immobilized fibroin membrane. All experimental conditions are the same as Fig. 1. The values in the table were an average of repeatedly measuring for three times.

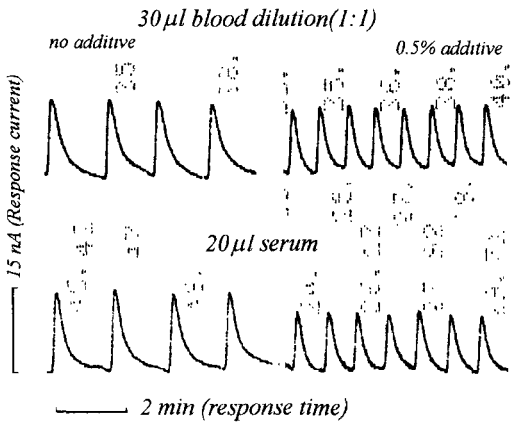

Fig. 3. Tailing inhibition of additive in buffer to the response peaks of human blood and serum on glucose sensor. All experimental conditions are the same as Fig. 1.

The influence resulted in tailing phenomena of response current peak of biosamples on the sensor and an unstable baseline. This tailing of response peak can become more and more serious, with the analytical time delayed and the amount of sample detected increasing (the left of Fig. 3). Finally, the response time lengthened gradually so that biosamples could not be determined continuously. It is possible that there were some interactions between hemacytes, some macromolecules, or lower mol-wt charged species in blood, serum, or urine, and the fibroin membrane-covered elec-

trode, so that the enzymatic reaction between glucose and immobilized GOD was delayed slightly, which result in a tailing peak of response current for biosamples on biosensor. This tailing phenomenon influences directly the sensor's capability for detecting glucose in biosamples and limits its practical application to clinical diagnoses. In general, an ultrafiltration film was added and covered just outside enzyme membrane-covered electrode to isolate the membrane from hemacytes and some macromolecules. In this case, even though the substrate glucose can pass through the film to react with O_2 at the catalysis of immobilized GOD, the enzymatic reaction subjects to a considerable effect. Moreover, it can not isolate some lower mol-wt charged species, such as amine, amino acid, and nucleotide from enzyme membrane. Here, the authors have first introduced a tailing inhibitor, tetrabutylammonium hydroxide, into the buffer system at enzyme electrode-based biosensor in flow-injection analysis, to resolve the tailing problem on the response current peaks of biosamples on sensor. When adding 0.5% tailing inhibitor to buffer, it eliminated entirely the tailing of response current difference peak of biosamples, such as blood dilution, plasma, serum, urine, on the sensor, baseline stabilized, and response time shortened, so that the response behaviors of these biosamples on the sensor were almost the same as that of standard glucose solution, whose response time was about 50 s (see the right of Fig. 3). Thus, this glucose sensor is capable of detecting over 60 biosamples per hour, and can satisfy fully a variety of analytical needs to determine glucose in biosamples. It is very clear that this ion pair reagent are capable of combining with interferents in the complex matrices as those known applications to reverse-phase liquid chromatography. When 0.5% $(CH_3[CH_2]_3)_4$ NOH was added to phosphate buffer, $(CH_3[CH_2]_3)_4N^+$ in the flowing buffer could combine with all interferents with negative charge, including hemacytes, macromolecules, and lower molecular species in the complex matrices, to form a neutral complex, some interactions or absorption stated above disappeared, the substrate glucose, along with these neutral complexes, left simultaneously the surface of enzyme membrane with the flowing buffer. Thus, the response behaviors were the same as that of substrate standard solution.

In addition, the authors found that the response peaks of blood dilution and serum on the sensor, using the buffer system containing 0.5% tailing inhibitor, decreased in size in comparison with using control buffer, as in Fig. 3. To clarify the problem, the dissolved oxygen concentrations in the buffer containing 0.5% additive were investigated by the electrochemical method reported previously *(2)*. The result showed that dissolved oxygen concentration in the buffer containing the additive was lower, by about 5%, than that of control. Figure 4 explores the effect of oxygen concentration in buffer on the response current values of biosamples, including stan-

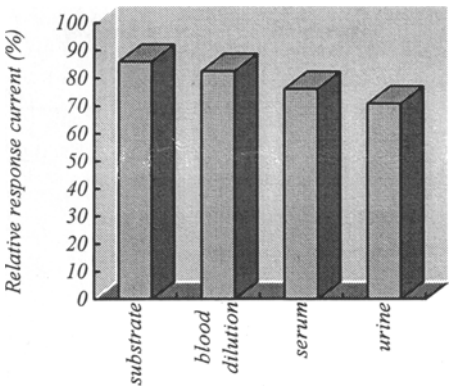

Fig. 4. Effect of additive on the responsive current values of biosamples on glucose and urate biosensors. The buffer contained 0.5% tailing inhibitor. These response current values obtained with control buffer were set 100% respectively.

dard glucose solution on the biosensor. Relative response current of these samples declined from 15 to 25%, in which that of standard glucose solution on the biosensor decreased about 15% and urine about 27%. From the results above, it is certain that tailing inhibitor present in buffer reduces the size of the signal of biosamples on the sensor is involved in decrease of dissolved oxygen concentration. In other words, the decrease of the signal size of biosamples on the biosensor is mainly contributed to change of dissolved O_2 in the buffer when the tailing inhibitor is added. On the other hand, the parts of current signal of biosamples decreased more than the standard glucose solution result from those interferents described above to be inhibited by the tailing inhibitor.

Effect of Variety of Buffer on Current Response of Biosensor

In order to determine whether buffer would adapt to the enzyme membrane-based glucose sensor, five buffers used daily were investigated in the experiment, and the result are shown in Table 2. Judging from the response peak values, their biosamples at all buffers responded well at the sensor, except Tris-HCl buffer. Phosphate buffer was used in the glucose sensor because of its buffering capability and is the strongest in these buffers.

Effect of pH on Biosensor

Figure 5, shows that those optimum scales of pH for two standard glucose solutions are very broad. Within the range of pH 6.0–8.5, percentage difference from their response values of different currents at each point was less than 10%. As to human serum or urine sample, pH value at the

Table 2
Effect of Various Buffers on Response Peak Values (mV) of Biosamples at the Sensor

Buffer[a]	Glucose	Serum	Urine
barbital	1.806 ± 0.009	2.019 ± 0.023	1.460 ± 0.037
acetate	1.919 ± 0.024	2.149 ± 0.045	1.358 ± 0.065
tetraborate	1.720 ± 0.033	1.957 ± 0.043	1.249 ± 0.006
Tris-HCl	1.601 ± 0.037	1.683 ± 0.008	0.864 ± 0.049
phosphate	1.728 ± 0.032	1.994 ± 0.090	1.050 ± 0.015

[a] Concentrations of a variety of buffer all were 0.02 mol/L (pH 7.80). The values in the table were an average of repeatedly measuring for three times. Sampling volume per time is 50 μL 5.0 mmol/L glucose solution, 20 μL human serum and 50 μL human urine, respectively. Other experimental conditions are the same as Table 1.

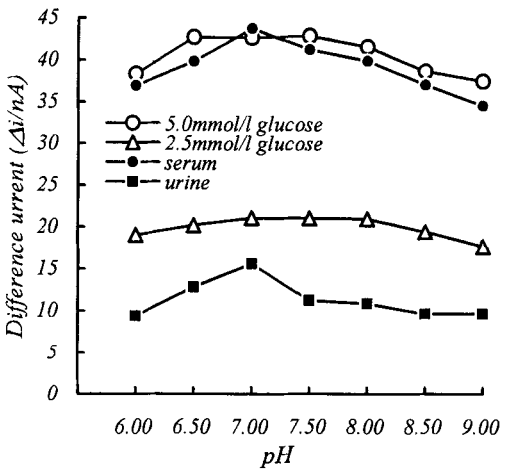

Fig. 5. Effect pH on the response difference current of biosamples on the biosensor. The values in the table were an average of repeatedly measuring for three times. Sample volume: 20 μL glucose solution, 20 μL human serum, 50 μL human urine, other experimental condition is the same as Fig. 1.

top response value of different currents was at pH 7.0. However, the response time of peak at this point was not the shortest of all because of much interference present in biosamples. Thus, in consideration of these two aspects, selected the buffer pH 7.8 but pH 7.0.

Temperature Effect

Biosensors suffer seriously the influence of temperature focus mainly on the two aspects of electrode itself and enzymatic reaction. As men-

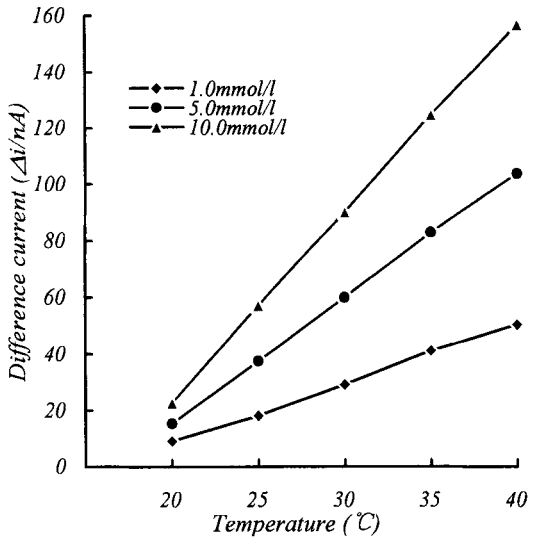

Fig. 6. Effect of temperature on the response current of glucose on the sensor. The three glucose concentrations are shown in the figure. The values in the table were an average of repeatedly measuring for three times. Other experimental conditions are the same as Fig. 1.

tioned at Materials and Methods, the region for temperature control was enlarged, the electrode body explored at air originally and buffer in the environmental temperature were controlled at the same temperature as that of enzymatic reaction in heat exchanger. So, it made biosensor's analysis error induced by changing environmental temperature decreasing largely and stability enhancing.

Even if the response values of glucose on biosensor rose with the temperature in a scale of 20–40°C (Fig. 6), as to stability of electrode work, a lower temperature fitted stable work of biosensor for a long time. Because of the construct improvement of oxygen electrode, it resulted in enzyme-electrode's sensitivity to substrate glucose to be enhanced fairly. Therefore, work temperature for the sensor from original 37.0°C went down to 32.0°C so that the sensor still remains a higher sensitive to substrate and runs more smoothly.

Effect of PO2 in Buffer on Response Current of Glucose Sensor

Due to presence of the partial pressure of oxygen (PO_2) difference in varieties of biosample solution such as human blood or serum, it is necessary to investigate whether the current response of the sensor suffers the dissolved O_2 concentration in a sample solution. The results obtained in the experiment explored that the current response of deaerated glucose solution (5.0mmol/L) increased about 5.0% than that of the gas-satured

sample solution. In essence, a large amount of dissolved O_2 in continuously flowing phosphate buffer acts as a buffer against changes in the dissolved O_2 concentration biosample solution. Therefore, the difference of dissolved O_2 concentration in varieties of biosample do not induce a significant influence on glucose measurement using this sensor.

Recovery Test and Repeatability Analysis

The results for recovery test of glucose in the biosamples human blood, serum and urine were shown in Table 3. Glucose standard solution mixed with human blood dilution, serum or urine in different proportions of volume. The results showed that recoveries of glucose at these biosamples were within a range of 93.71–105.88%. In order to determine the stability of the device, carried out the repeatability tests with glucose standard solution, blood dilution, plasma, serum, and urine samples. As shown in Table 4, relative standard deviations (RSD) for measuring repeatedly glucose solution, blood dilution, plasma, and serum were 1.81, 2.48, 2.98, and 2.91%, respectively. The partial original chart records of the response peaks of standard glucose solution, human blood, plasma, and serum on the sensor for repeatability tests of hundred times were shown in details as Fig. 7. The response behaviors of blood dilution, plasma, and serum samples all displayed their base line stable and response time about 50 s as same as that of standard glucose. Therefore, this sensor, based on GOD-immobilized fibroin membrane and electrode, is able to satisfy needs to monitoring rapidly glycemia in human blood, plasma, serum, or urine for clinical diagnoses of diabetes and glucose concentration in fermentation industry.

Chemical Specificity and Interference from Other Molecules

As there exists interference of some endogenous electroactive species in biosamples, it is investigated those effects of ascorbate, glutamate, and urate on the sensor. As shown in Fig. 8, we can find urate and glutamate had almost no current response at the device except ascorbate. There was an evident influence of ascorbate on enzyme electrode because the membrane covered at the base of oxygen electrode is a polyethylene film but an acetate cellulose. Galactose and urca have no response current. But there were some responses of sucrose, lactose, and maltose on this glucose sensor. In the previous paper *(12)*, the authors had discovered these sugars had no current response on the original sensor system. Here is two possible factors. First, because of the improvement for electrode configuration, the sensor's sensitivity increased greatly so as to be able to monitor a trace glucose present at these reductive sugar reagents in analytic or chemical grade used in this experiment. Second, when this GOD-immobilized

Table 3
Recovery Test for Human Blood, Serum and Urine on Sensor

Sample	Mixture (V/V)[a]	Measured value[b] (mmol/L)	Expected value[b] (mmol/L)	Recovery (%)
Blood	5:2	12.310	12.208	100.84
dilution[c]	5:1	8.305	7.944	105.88
	10:1	5.775	5.812	99.36
	1:1	8.673	8.707	99.61
	2:1	7.613	7.443	102.28
Serum	3:1	6.785	6.811	98.85
	4:1	6.630	6.431	103.09
	5:1	6.262	6.179	101.34
	1:1	7.035	6.880	102.25
	2:1	5.170	5.006	103.28
Urine	3:1	3.885	4.069	95.48
	4:1	3.341	3.507	95.27
	5:1	2.935	3.132	93.71

[a]Whole blood dilution (3.680 mmol/L) was mixed 25.0 mmol/L glucose solution in the proportion above. Serum (4.9142 mmol/L) or urine (1.2589 mmol/L) was mixed with 12.5 mmol/L standard glucose solution.

[b]The values in the table were an average of repeatedly measuring for three times.

[c]Blood dilution means whole blood was mixed with 4% trisodium citrate solution in same volume (1:1). Other experimental conditions are the same as Table 1.

Table 4 Repeatability of Biosamples at the Biosensor

Sample[a]	Repeated times	Average value (mV)	±SD	RSD (%)
Glucose	100	1.385	0.025	1.81
Blood dilution	125	0.899	0.022	2.48
Plasma	50	0.777	0.023	2.98
Serum	128	1.307	0.038	2.91
Urine	70	0.639	0.033	5.19

[a]Sampling volume per time is 50 μL 2.0 mmol/L glucose solution and blood dilution (1:1), 15 μL plasma, 25 μL serum and 50 μL human urine, respectively. Other experimental conditions are the same as Table 1.

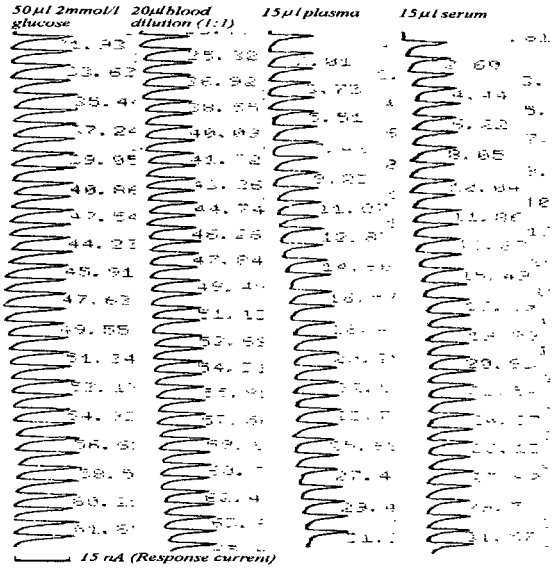

Fig. 7. The partial original records of biosamples on the biosensor for repeatability test. The number in the figure is the retention time (min) per sampling. Other experiment condition is the same as Fig. 1.

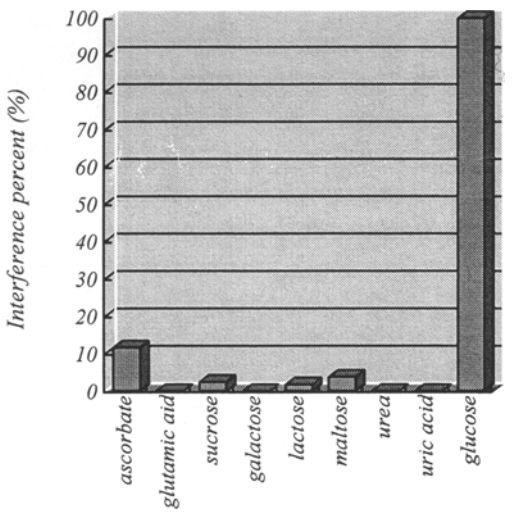

Fig. 8. Interference from other molecules on the sensor for glucose. All interference percentages in the figure were calculated by response current value of 5.0 mmol/L glucose at the biosensor as 100%. These compounds used in the experiment were analytical or chemical grade. Sample volume: 20 μL. The values in the table were an average of repeatedly measuring for three times. Other experimental conditions are the same as Fig. 1.

fibroin membrane prepared, GOD reagent used is the product derived from *Aspergillus niger* (EC 1.1.3.4, 23900 U/g, Type II, Sigma) in which it contained a certain amount of some oxidases for these reductive sugars according to its product description. Fortunately, no matter what factor, this reductive sugar's current response is not large enough to influence the analytic result of glucose on the sensor. As to the tailing phenomena of other macromolecules present in biosamples on the biosensor, as described ahead, the problem was settled satisfactorily by tailing inhibitor added to buffer system used at the sensor.

Biostability

In the practical application to biosensor based on enzyme membrane and electrode, the main elements that limit its application are the cost for preparing enzyme membrane and its stability. We have reported previously the simple method and low cost for making enzyme-immobilized fibroin membrane, described their characteristics in spectrophotometry and electrochemistry. With tested for the late years, this GOD membrane had a higher activity recovery and used repeatedly for measurement of glucose in a phosphate buffer for several mouths. This enzyme membrane can be stored over 2 yr, and enzyme membrane used once could be still stored for subsequent use for detecting glucose. A piece of enzyme membrane, less than 10 mm in diameter, can be used repeatedly for detecting glucose at biosamples for over 1000 times. For example, One piece of GOD-immobilized membrane was used for the most of tests at this paper. This piece of enzyme membrane, subjected to immerse in pH 5.0–10.0 buffer solution and at 20–50°C and to contact with a variety of buffers and additives in buffer, was used repeatedly for measurement 1445 times, in which it detected glucose solution for 713 times and human blood, plasma, serum or urine for 742 times between one-half and 2 mo. Moreover, when calibrated with a standard glucose solution, this piece of GOD membrane can still be used for the determination of biosamples.

Correlation Analysis

Two hundred human serum samples, provided by Suzhou 7th Hospital, Jiangsu Province, China, were quantified for monitoring glucose level using two disparate techniques. The first utilized the glucose sensor based on GOD-immobilized fibroin membrane described here and the seconds, Worthington determination method *(13)* used conventionally at hospital laboratory. Every sample was measured repeatedly two times for glucose level. The correlation distribution plot for monitoring glycemia to 200 serum samples with both methods above was explored in Fig. 9. The

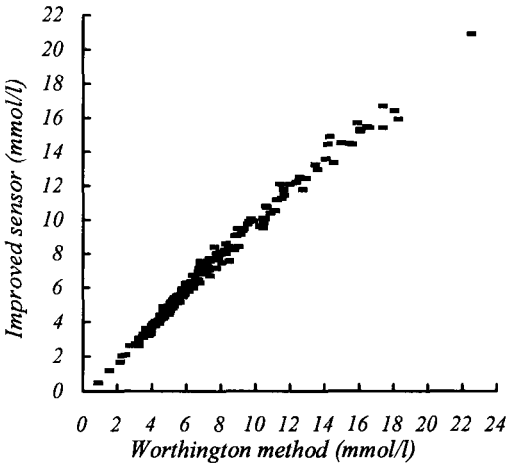

Fig. 9. Correlation analysis for glucose values obtained with Worthington method and the sensor determination for 200 serum samples. All values in the figure were an average of repeated measurements for two times with both methods above, respectively. The resulting correlation coefficient (γ) is 0.993445. Other experimental conditions are the same as Fig. 1.

resulting correlation coefficient (γ) was 0.993445. These results indicate that this biosensor described here is able to apply to clinical diagnoses for diabetes.

DISCUSSION

In the development of enzyme-immobilized membranes and electrode-based biosensors, enzyme immobilization is accomplished by various means including electrochemical immobilized, covalent binding crosslinking and entrapment in a gel, polymer, and nature protein. These works often require implicating elaboration and time-consuming preparation procedures (e.g., chemical and physical pretreatments) which may results in decrease of the enzyme activity *(14,15)*. Furthermore, these enzyme membranes are often lack of good stability and cost highly for the preparation of immobilized enzyme. In the present glucose sensor in flow injection analysis, glucose oxidase was entrapped in natural silk fibroin membrane whose preparation method is very simple and cost is low *(9)*. After only simple physical treatment such as soaking in methanol solution for a moment without chemical treatment, this fibroin membrane could tightly fixed the enzyme. The resulting enzyme-immobilized fibroin membrane is of a higher recovery of enzyme activity and good biostability. It is more important that this enzyme membrane can be stored for long time (over 2 yr) and not contaminated by microbiol. In operation of the glucose

sensor using this enzyme membrane and electrode, this enzyme membrane, about 20 μm thick and less than 10 mm in diameter, can be used for repeatedly measuring samples over 1000 times. Moreover, the membrane is easily assembled and replaced on the present sensor. Therefore, it is possible that biosensor based on enzyme-immobilized fibroin membrane come into use in practical clinical diagonese for glucose determination.

Biosensors based on immobilized enzyme and electrode often suffer interferences from hemacytes, some macromolecules and lower mol wt charged substances besides endogeneous electroactive species in complex matrices such as whole blood, plasma, serum, and urine. A conventional method is to add a membrane with restricted permeability such as porous ultrafilter film or commercial dialysis membrane at outer of enzyme membrane of electrode for isolating enzyme membrane from some biomolecules at biosamples *(16–20)*, which could avoid restricted diffusion from the macromolecules into the enzyme membrane, but not isolate restricted diffusion from smaller molecules into it. Moreover, enzymatic reaction between immobilized enzyme and its substrate analyst was affected considerably by the isolating membrane, which resulted in serious decrease of response signal of the analyst in biosamples on sensor. As described previously by Manowitz et al. *(21)*, their galactose biosensors using composite polymers was suffered by an interference from plasma some lower mol wt charged species. In amperometric sensor for glucose in flow injection analysis based on enzyme-immobilized silk fibroin membrane and oxygen electrode, had also faced these questions which resulted in the considerably serious tailing phenomena of response current peaks of human blood, plasma, serum, and urine glucose, thereby delayed response time for serum and urine glucose and limited analytical speed *(12)*. Up to now, there was no study on eliminating or abating this interference from hemacyte, some macromolecules and lower mol wt charged substances besides use of a restricted permeable membrane mentioned above. In the present paper, we have first introduced a tailing inhibitor into a phosphate buffers at the amperometric sensor for glucose. The result has shown this tailing inhibitor can entirely eliminate the tailing phenomena induced by hemacyte, some macromolecules, and lower mol wt charged substances from human blood, plasma, serum, or urine. More recently, we have again met with success in eliminating the same tailing phenomena as described above at an urate sensor based on uricase-immobilized fibroin membranes *(22)*. Therefore, it is possible to apply this tailing inhibitor to other enzyme electrode-based biosensors in flow injection analysis.

In general, in enzyme electrode-based biosensors in flow injection analysis, temperature control for biosensors only limits to heat exchanger for enzymatic reaction. In fact, temperatures in carrier solution (e.g., buffer) and electrode body explored at air all influence more or less ana-

lytical result of biosensors determination. The present article has used a circulating thermostat controlling all temperatures for buffer, electrode body, and heat exchanger for enzymatic reaction. It makes the glucose sensor's accuracy and repeatability enhance largely.

Thus, the glucose biosensor based on GOD-immobilized fibroin membrane is capable of detecting over 60 blood, plasma, or serum samples per hour. Moreover, it had a better correlation with the method for determining glycemia used conventionally at hospital laboratory. Therefore, the biosensor can fully satisfy the analytic need of monitoring rapidly glycemia in clinical diagnoses for diabetes.

Nevertheless, there is still a question that remained to be solved in the further studies. Therefore, future work will include studies on using a cellulose membrane in place of PE film or improving the component of enzyme-immobilized fibroin membrane to prevent interference from ascorbic acid on the device.

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