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An optical biosensor for the rapid determination of glucose in human serum

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Abstract

An optical glucose biosensor based on the immobilization of glucose oxidase in a sensing film was developed. The sensing film consisted of an organically modified silicate film embedded with tri(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) perchlorate and a polyvinyl alcohol sol–gel matrix with immobilized glucose oxidase. A kinetic curve simulation method was employed for glucose measurements, as a result of which the measurement time was less than 1 min for each sample analysis. The detection limit (S/N = 3) of the glucose optical sensor was 3.6×10^{-6} mol L^{-1} with linear ranges from 0.00 to 0.50 mmol L^{-1} ($Y = 358.25X - 2.93$, $R^2 = 0.9954$) and 0.50 to 3.00 mmol L^{-1} ($Y = 430.12X - 46.43$, $R^2 = 0.9972$). In addition, the appearance of the sensing film, and effects of the amounts of immobilized enzyme, pH, temperature, ionic strength, and co-existing substances (including heavy metal ions and organic compounds) were investigated. The glucose values estimated by this optical biosensor correlated well with those determined using the conventional method for human serum samples.

Keywords: Glucose optical biosensor; Glucose oxidase; Kinetic measurement; ORMOSIL–PVA; Serum

1. Introduction

Diabetes is a disease whereby the body cannot produce or properly use insulin. Insulin is a hormone that is necessary to convert sugar, starches and other food into the daily energy requirement. Many people have diabetes, but unfortunately, nearly one-third of them are unaware of this. Although the fasting plasma glucose test (FPG) and oral glucose tolerance test (OGTT) have been suggested for the diagnosis of pre-diabetes or diabetes, the development of easier, faster and cheaper equipment for glucose determination is still necessary.

Enzyme catalyst reactions have been widely applied in both FPG and OGTT detections due to their high efficiency and specificity, but enzyme is consumed in their application, which implies high cost and inconvenience. This drawback limits rapid glucose determination. Construction of a glucose biosensor using immobilizing enzyme is a promising approach to overcome this problem, and many immobilization methods, such as covalent attachment [1–4], cross-linking [5,6], entrapment [7–9] and physisorption methods [10,11], have been proposed and have gained acceptance as practicable techniques. However, in the physisorption method, leakage of enzyme still occurs; the covalent attachment method changes the activation of the enzymes; and the entrapment method causes a long response time. Recently, great attention has been paid to the method involving organically modified silicate (ORMOSIL)–polyvinyl acetate (PVA) to create a matrix for the immobilization of microorganisms [12–14] due to its ease of preparation and modification. In the ORMOSIL–PVA material, PVA associates well with silica via a hydrogen-bonding interaction [15], which gives the ORMOSIL–PVA material an excellent biocompatibility for the immobilization of microorganisms or enzymes [16].

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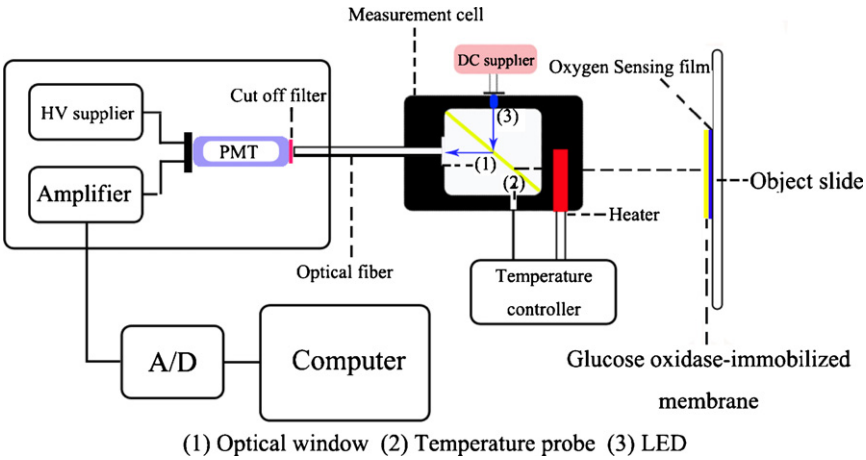

Fig. 1. Setup for the optical sensing measurement of glucose.

To date, the development of biological-based rapid glucose assays has centered on the development of glucose biosensors. This approach involves immobilizing enzyme on a membrane (bio-film) placed in close, intimate contact with an amperometric oxygen electrode or an optical oxygen sensor, which relies on measuring the enzymatic oxidization rate in the bio-film [17]. Although the conventional glucose sensor test has certain benefits and no expensive equipment is needed, it seems that the performance of these sensors is still not satisfactory. Limitations include the depletion of oxygen occurring during glucose measurement with electrochemical oxygen sensors such as the Clark electrode, and lack of more effective immobilization of the enzyme for long-term usage. It is therefore of considerable interest to develop an alternative method that could circumvent the weakness of the conventional glucose test. In recent years, a glucose sensor based on fluorescence intensity quenching has generated a great interest since it provides high sensitivity, and little or no damage to the host system. Moreover, the advantages of optical oxygen sensing-based glucose sensors (over those with an electrochemical oxygen sensor like the Clark electrode) are small size, no consumption of oxygen, no requirement for reference cells and inertness against sample flow rates and stirring. In the fluorescent biosensing approach, glucose is oxidized into gluconic acid by the enzyme, and oxygen is simultaneously consumed, and the change of dissolved oxygen (DO) in the glucose sensor causes a quenching fluorescent intensity [18–20]. The signal change is proportional to the content of glucose. Measurement approaches including the fluorescence lifetime, and some special fluorescence techniques, which provide information about the structure and micro-environment of molecules [21], can also be applied for optical glucose sensors.

Up to now, there are more than 40 kinds of commercial blood glucose meters based on enzyme and electrochemical reaction [22]. These glucose meters make daily glucose measurement a reality, but several difficulties including their impairing responses, unpredictable drift of signal in vivo, skin incursion and inaccuracies, which have restricted their widespread use in diabetes diagnosis [23], still remain. Thus, new approaches to glucose sensing have been attracted to fluorescence-based sensors because of their high sensitivity, stability and lower drift. An optical needle-type enzyme sensor for rapid and simple determination of glucose level in fish blood has been developed [24]. The sensor was constructed as a needle-type, since this meant the enzyme could be easily exchanged for long-term use. In application, the reproducibility of measurements was reduced when the sensor was applied in different ways. In addition, the response became unstable when the fish moved. Yang and colleagues reported another fluorescent glucose biosensor, which was constructed by immobilizing glucose oxidase on a bamboo inner shell membrane [27]. They successfully applied this to the measurement of glucose in commercial wines and medical glucose injections, but obvious errors occurred at low concentrations of glucose due to the tiny fluorescence intensity change in the glucose sensor. Thus, establishing an accurate, logical measurement mechanism and constructing an optical glucose sensor with good selectivity, low detection limit, easy usage, good reproducibility and stability become important.

In this study, ORMOSIL–PVA was employed as a matrix to immobilize glucose oxidase for construction of an optical glucose sensor. A series of experiments characterizing the sensor were carried out, including measurement mode, reproducibility, linear range and effects of temperature, pH and co-existing substances. The proposed sensor was successfully applied to the determination of glucose in human serum samples.

2. Experimental

2.1. Chemicals and instruments

Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*) with a specific activity of 210 U/mg of lyophilized solid was purchased from Sigma (St. Louis, MO, USA); tetramethoxysilane (TMOS) and PVA were purchased from Aldrich (Milwaukee, WI, USA). Dimethyldimethoxysilane (DiMeDMOS) was obtained from Fluka AG (Buchs, Switzerland). $Ru(Ph_2phen)_3(ClO_4)_2$ [Ph_2phen = 4,7-diphenyl-1,10-phenanthroline, $Ru(dpp)_3^{2+}$] used as the oxygen-sensing indicator was synthesized and purified in the laboratory of the Department of Applied Biology and Chemical Technology,

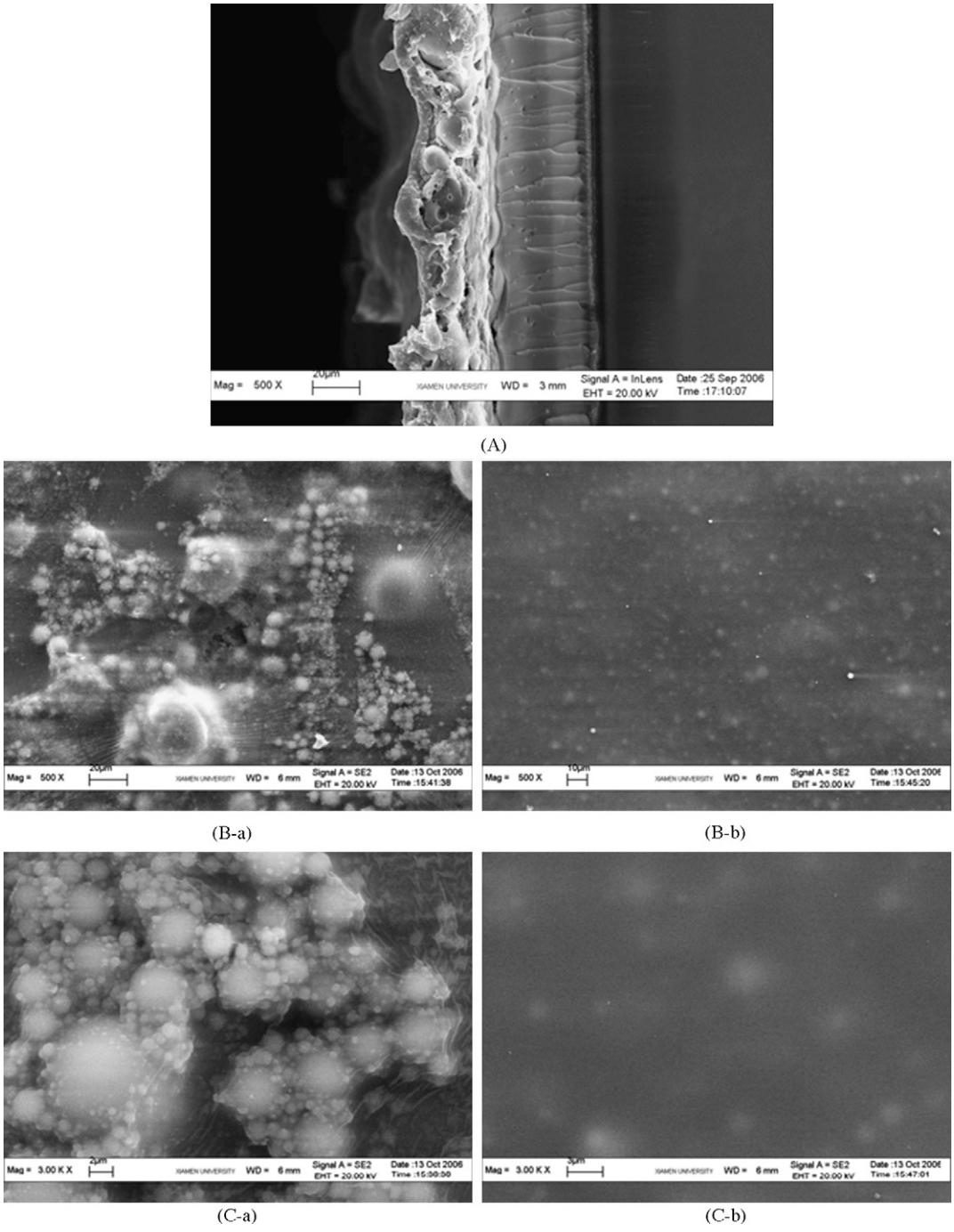

Fig. 2. Scanning electron micrograph of glucose sensor: (A) section and comparison of (a) with- and (b) without-immobilized glucose oxidase at (B) low and (C) high magnification.

Hong Kong Polytechnic University. A buffer solution of pH 7.0 was prepared using 0.5 mol L^{-1} Na_2HPO_4 and KH_2PO_4. All other chemicals used in this study were analytical reagent grade and all solutions were prepared with deionized distilled water.

The excitation and emission wavelengths of oxygen-sensing film were 468 and 589 nm, which were measured using an F-4500 spectrofluorimeter (Hitachi, Japan). A scanning electron microscope (SEM; LEO1530, Leo, Germany) was used to obtain scanning electron micrographs of the glucose sensor film.

2.2. Oxygen-sensing film preparation

The preparation of oxygen-sensing film for the glucose sensor was performed according to the previous description [16]. Typically, 1.0 mL of TMOS and 1.8 mL of DiMe-DMOS were placed in an open vial. After the mixture was magnetically stirred for approximately 1 min, 1.5 mL of 0.01 mol L^{-1} hydrochloric acid was added. The vial was then immersed in a 60 °C water bath and stirred for about 3 h. An emulsion formed during this step. Two hours later, 0.2 mL of 1.5 g L^{-1} $Ru(dpp)_3^{2+}$ in THF was added.

The mixture was then vigorously stirred for 20 min to ensure a homogenization. Films were prepared by pipetting 60 μL of the mixture onto a glass slide (25.5 mm × 26.5 mm), which had been soaked in concentrated nitric acid for 12 h and washed with distilled water and ethanol. The resulting film which was sensitive towards oxygen was left undisturbed under ambient conditions for 0.5 h [25], and finally, thermally cured in an oven for 24 h at 80 °C, and then cooled to room temperature ready for use.

2.3. Immobilization of glucose oxidase

ORMOSIL was prepared by mixing TMOS, DiMe-DMOS and 0.01 mol L^{-1} HCl (1:1.2:1, v/v). The mixture was stirred at 60 °C for about 4 h. Then 500 μL ORMOSIL was mixed with 500 μL 5% (w/w) PVA. After laying aside for 5 min, 200 μL ORMOSIL–PVA mixture and 200 μL buffer solution with 250 units of glucose oxidase at pH 7.0 were added into a 0.5 mL centrifuge tube. After vigorously stirring for 1 min, the mixture was daubed (well-proportioned) onto the optical oxygen-sensing film produced previously. The glucose sensing film was dried at room temperature for 24 h and stored in pH 7.0 phosphate buffer solution at 4 °C before use.

2.4. Assembly of a setup for glucose optical sensing measurement

A setup for the optical sensing application is shown schematically in Fig. 1. In order to effectively reduce the effect of the excitation light from a blue LED (wavelength 465 nm), the sensing film was placed in the detection cell at a 45° angle to the excitation light direction. The emission light passed through a cut-off filter with a half bandwidth of 10 nm at 580 nm, and was transferred by an optical fiber to a R928 PMT (Hamamatsu, Japan), then the fluorescent signal was converted into electrical signal and recorded by an Echrom 98 chromatogram workstation (Dalian Elite Scientific Instruments Co. Ltd., China). Temperature was kept constant using a temperature controller (Xiamen Yudian Automation Technology Co. Ltd., China) with a precision of ±0.2 °C.

2.5. Experimental procedures

The glucose sensing film was inserted into a black, airtight detection cell in which the sample solution was kept motionless. The temperature of the detection cell was maintained at 35 °C. When the fluorescence intensity reached a steady state in the air, 20 mL of the sample solution was rapidly added into the detection cell. After the cell was covered, the fluorescent signal was recorded and a typical kinetic curve was obtained.

3. Results and discussion

3.1. SEM images of the glucose sensing film

Typically, in the construction of a glucose sensor, a glucose-oxidase-immobilized layer closely covered an oxygen-sensing layer prepared by TMOS, DiMe-DMOS and 0.01 mol L^{-1} HCl (1:1.2:1, v/v). According to the SEM observations shown in Fig. 2A, the oxygen-sensing layer with a thickness of 42 μm was generated by a dip-coating method for a higher fluorescent intensity change in the glucose measurement. It should be mentioned that prepared films in the same batch have good reproducibility with a R.S.D. less than 5%, but comparing different batches of prepared films, fluorescence intensity deviation exists due to the different viscosity of ORMOSIL. The second layer (made by ORMOSIL–PVA for glucose oxidase immobilization) presented a thickness of about 40 μm. From Fig. 2B and C, we found that the addition of glucose oxidase brought an obvious change of surface appearance. A rough surface with many different sizes of spheres was generated when glucose oxidase was immobilized in the layer. In the highly magnified SEM images, many small spheres adhering to the large globes on the surface could be found. It could be considered that the molecules of glucose oxidase were embedded into the small ORMOSIL–PVA spheres, and these spheres attached to the large globe formed by PVA [29]. The multi-protuberant and incompact microstructure implied that ORMOSIL–PVA material contributed a very large surface for glucose oxidase immobilization and good permeability for the sensing response.

3.2. Typical response curves and linear characters of glucose sensing film

Generally, there are two measuring techniques, including the dynamic balanceable and the kinetic method [26,27], available for glucose sensors. Compared with the dynamic balanceable mode, measurement in the kinetic mode presents better characteristics, such as much faster response, more convenience, higher sensitivity and without oxygen saturation. For most enzyme catalyst reactions, the Michaelis–Menten equation ($\nu = \bar{\nu}_m[S]/K_m + [S]$) applies (where ν is the reaction rate, [S] the substrate concentration, $\bar{\nu}_m$ the maximum rate and K_m is the Michaelis–Menten constant). When $K_m \gg [S]$, the equation can be simplified as $\nu = \bar{\nu}_m[S]/K_m$. The reaction can be taken as a pseudo-first order reaction. In the enzyme catalyst reaction of glucose oxidation, K_m for the enzyme is between 11 and 41.8 mmol L^{-1} [28]. The reaction can be simplified as a first order reaction under low concentration of glucose (lower than 0.50 mmol L^{-1}). A fit linear simulation for the initial velocity of the reaction could be applied when a typical kinetics curve is obtained.

As in the previous description, oxygen concentration gradually decreased in the catalytical reaction of glucose oxidase, which could be measured by the fluorescent change in oxygen-sensing film. A typical response curve with the fluorescent intensity and the measurement time for the glucose sensing film is shown in Fig. 3. In the insert in Fig. 3, fluorescent intensities in the initial 6 s present a good linearity with the measurement time. A velocity of the simulation line (beeline in insert in the figure) was considered as the initial velocity of the reaction. There was a good linear relationship between the initial velocity and the concentration of glucose. In order to simulate the initial velocity of the reaction, the short measurement time used in the simulation method was necessary. Simulation with different measurement

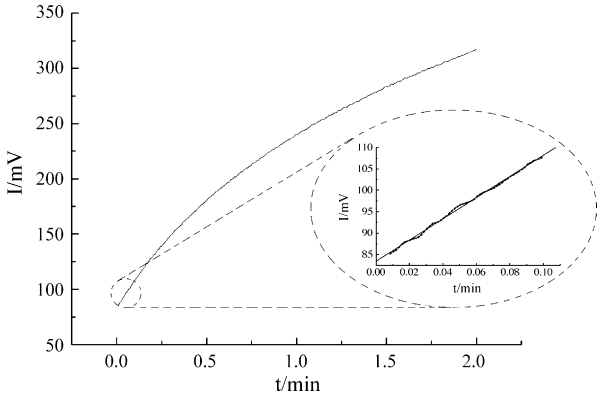

Fig. 3. Typical response curve in 0.50 mmol L^{-1} glucose solution and the corresponding linear simulation.

time (3, 6, 7.5, 15, 30 or 60 s) was contrasted using the differential method. The results indicated that the simulation method was available for the measurement of glucose concentrations from 0.01 to 0.50 mmol L^{-1} when measurement times of 6, 7.5 and 15 s were selected. The data obtained from simulation methods had an S.D. less than 5% after three reduplicate measurements, which indicated that errors from experimental operations could be neglected. Comparatively, a measurement time of 6 or 7.5 s was optional when the concentration of glucose was from 0.50 to 3.00 mmol L^{-1}. In order to extend the application range of the glucose sensor, the unification simulation method with a 6 s measurement time was selected. The simulation method was not available as soon as the concentration of glucose was above 5.00 mmol L^{-1} since the quick velocity change caused a large measurement error.

The response behavior of the biosensor towards different concentrations of glucose is displayed in Fig. 4. The linearity between the glucose concentration and initial velocity value included two parts. As shown in Fig. 4, the glucose optical sensor had linear ranges from 0.00 to 0.50 mmol L^{-1} ($Y = 358.25X - 2.93$, $R^2 = 0.9954$) and 0.50 to 3.00 mmol L^{-1} ($Y = 430.12X - 46.43$, $R^2 = 0.9972$), respectively. As mentioned above, there was a linear relationship between ν and [S] as shown in the concentration range 0.00–0.50 mmol L^{-1}. With increasing concentration of glucose in the range 0.50–3.00 mmol L^{-1},

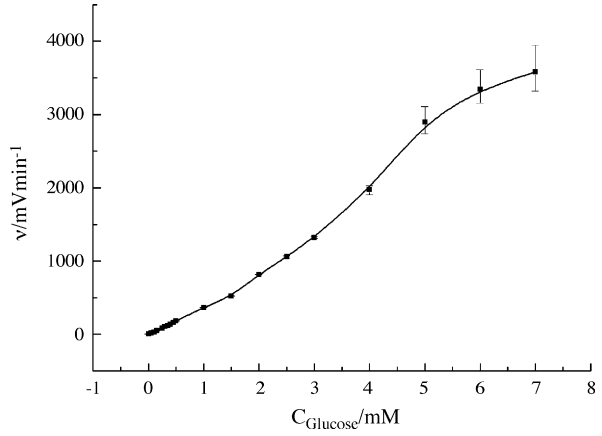

Fig. 4. Responses of the biosensor towards various concentrations of glucose.

fortunately, there existed another linearity between the concentration of glucose and the initial velocity of the reaction. When $K_m \ll [S]$, the equation could be simplified as $\nu = \bar{\nu}_m$. The reaction could be considered as a pseudo zero order reaction, and the initial velocity became a constant. When the glucose concentration exceeded 5.00 mmol L^{-1}, the quick enzyme catalyst reaction made a flexural curve in the first 6 s due to large deviation, resulting in the data obtained from the linear simulation method being not credible. In addition, the unneglectable errors from experimental operation made the linear simulation method unsuitable.

3.3. Effect of immobilization of the enzyme

The amount of immobilized enzyme in the sensing films played an important role in their response characters. Different units of enzyme (10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350 and 400) were selected and immobilized in the sensing films (25.5 mm × 26.5 mm), and their response characters were investigated in 0.05 and 3.00 mmol L^{-1} glucose solution. Results demonstrated that the response velocity and sensitivity of the glucose sensor increased gradually with the increasing amount of immobilized enzyme. The change in initial velocity became constant when the amount of immobilized enzyme in the sensing film was more than 250 units. Furthermore, when the amount of immobilized enzyme was less than 100 units, a large deviation occurred (S/N < 3) in the measurement of 0.05 mmol L^{-1} glucose solution. As a result, 250 units of enzyme was selected and immobilized in the sensing film.

3.4. Characteristics of the glucose sensor

Generally, the glucose sensing film was stored in phosphate buffer solutions at 4 °C. Before use, the film was immersed in a 0.5 mmol L^{-1} glucose solution at 35 °C for 5 min in order that the enzyme recovered its activation and presented stable behavior in the following detection. After obtaining six signals with an S.D. less than 5%, the sensor was ready for use. The reproducibility of the glucose sensor in 0.50 and 2.00 mmol L^{-1} glucose buffer solutions at pH 7.0 was studied. The relative standard deviations (R.S.D.) were 1.50% and 1.89% in 20 continuous measurements, respectively. After being used continuously for a month, the sensor film maintained 80% of its initial response activity. Results demonstrated that glucose oxidase immobilized in the ORMOSIL–PVA matrix could not easily leak, and the sensor presented good reproducibility. The linear simulation method was suitable for data analysis.

3.5. Effect of pH

Since the activity of glucose oxidase greatly depended on the pH of the sample solutions, the effects of pH in glucose sensing detection were investigated from pH 4.49 to 9.18 in 0.50 mmol L^{-1} glucose solution (shown in supplementary Fig. S.1). The response velocity reached its maximum value at pH 7.0 and decreased when pH was beyond 7.70. The sensor response remained constant in the pH range 5.50–7.70. It was reported

Table 1
Effect of co-existing substances and heavy metal ions on the sensing film response

Organic compound	ΔF (%)	Organic compound	ΔF (%)	M^{n+}	ΔF (%)	M^{n+}	ΔF (%)
Citric acid	−0.45	Oxalic acid	0.62	Ag^{+}	−31.89	Mg^{2+}	2.63
D-Fructose	1.07	Sucrose	1.26	Al^{3+}	−2.87	Mn^{2+}	−6.64
Ethanol	−7.22	Tartaric acid	0.50	Ba^{2+}	−2.11	Ni^{2+}	−1.58
Ethylene glycol	3.13	Urine	−6.37	Ca^{2+}	−4.27	Pb^{2+}	−25.87
Glycerol	−4.44	α-Lactose	1.00	Co^{2+}	−4.99	Sn^{2+}	−0.07
L-Glutamic acid	6.58	1,2,4-Benzene tricarboxylic acid		Cu^{2+}	−6.21	Sr^{2+}	2.07
Maltose	90.20		−0.84	Fe^{3+}	5.75	Zn^{2+}	2.88
Methanol	−6.05			Fe^{2+}	4.68		

Metal ions were all at a concentration of 1.0 μmol L^{-1} and organic compounds at 5.0 mmol L^{-1} in a 0.5 mmol L^{-1} glucose buffer solution at pH 7.0. ΔF (%) = $(v - v_0/v_0) \times 100$, where v is the velocity of response curve in the first 6 s after co-existing substance added, and v_0 is the velocity of response curve in the first 6 s without co-existing substance.

that glucose oxidase immobilized in membranes presented a correspondingly broad working pH range [27,30]. The activity of glucose oxidase was kept in a definite working pH range when it was immobilized in ORMOSIL–PVA membrane, which allowed the sensing film to present a good response character in this range. When pH overstepped the working range, the enzyme lost its activity, resulting in an obvious decrease of sensing film response.

3.6. Effect of temperature

Temperature is an important factor which has a significant effect on enzyme activity. An appropriate temperature will promote the activation of glucose oxidase and reduce deviations in its measurement. At low temperature, lower enzyme activity causes a weaker response and results in a large deviation in its measurement. Conversely, an inappropriate high temperature will change the enzyme conformation; even causing protein aggregation and solidification, resulting in the loss of enzyme activity. The activity of glucose oxidase was studied in the range 4–60 °C in order to find the optimum temperature (shown in supplementary Fig. S.2). Results indicate that the rate of activity increased rapidly in the range 4–35 °C, but decreased quickly when the temperature was higher than 35 °C. As a result, an optimum temperature of 35 °C was chosen for all future studies. Furthermore, temperature also affect the luminescent property of $Ru(dpp)_3^{2+}$ due to the effect of thermal quenching. It is reported that the oxygen indicator $Ru(dpp)_3^{2+}$ immobilized in polyacrylonitrile (PAN) microbeads which are gas-impermeable can be used as a temperature-sensitive probe for the severely precise temperature sensoring [31,32], temperature effect can be neglected when $Ru(dpp)_3^{2+}$ was embedded in sol–gel matrix which has a good gas-permeable character, was mainly used as an oxygen indicator and has good stability towards temperature during detection.

3.7. Effect of ionic strength of the medium and co-existing substances in the medium

Most biosensors with immobilized enzyme are influenced negatively in a higher ionic strength medium. Generally, human serum maintains a certain ionic strength in order to ensure the balance of vital activity. In 0.5 mol L^{-1} Na_2HPO_4 and KH_2PO_4 buffer solution at pH 7.0, the effect of ionic strength on the glucose sensing response for 0.50 mmol L^{-1} glucose solution was investigated by adding various amounts of sodium chloride. With increasing NaCl concentration (higher ionic strength), the results indicated that the response rate was slightly decreased. In the test solution containing 1.50 mol L^{-1} NaCl, the initial response rate decreased by 10% compared with the original value without NaCl. However, interestingly, the results revealed that the sensing film recovered its original response ability to glucose after the second measurement. The same phenomenon was obtained when the tests were performed in NaCl concentrations lower than 1.50 mol L^{-1}. This implied that the ionic strength change of the sample did not permanently affect the response characteristics of the sensing film, and glucose oxidase immobilized in the sensing film was able to accommodate the ionic strength change. As a result, the selected 0.5 mol L^{-1} Na_2HPO_4 and KH_2PO_4 buffer solution provided a suitable ionic strength for glucose detection.

In general, various organic substances and trace amounts of metal ions exist in human serum. Their effects on the sensor were investigated using co-existing organic substances at a concentration of 5.00 mmol L^{-1} and metal ions at a concentration of 1.0 μmol L^{-1}. The results, summarized in Table 1, show that there were no obvious effects for most co-existing organic substances, except maltose. Except for silver and lead cations, our results indicated that the prepared glucose sensing film was almost independent of metal ions. Although silver and lead cations greatly inhibited the activity of glucose oxidase because of their toxicity, fortunately, the lower contents in human serum limit these effects.

3.8. Sample analysis

The proposed glucose sensor was employed to detect glucose concentrations of human blood serum samples. Before determination, all samples obtained from the Fuzhou University Hospital (Fujian, China) were diluted 100 times using phosphate buffer at pH 7.0. Data for contrast were obtained from ASCA Chemistry System (Mindray Bio-Medical Electronics Co. Ltd., USA). As shown in Table 2, the results obtained from the glucose sensor agreed well with those obtained by the

Table 2
Comparison of results from the ASCA Chemistry System and the proposed optical fiber glucose sensor

No.	ASCA Chemistry System (mmol L^{-1})	Proposed glucose sensor (mmol L^{-1})	Error rate (%)
1	4.2	4.12	−1.9
2	4.5	4.38	−2.7
3	5.8	5.94	2.4
4	6.4	6.42	0.3
5	11.0	11.18	0.7
6	11.1	11.07	−0.3
7	13.7	12.63	−8.47

ASCA Chemistry System. The errors (between −8.47% and 2.4%) indicated that the sensor performed well in the detection of glucose in serum and most of the results were accurate and credible. Recovery tests for glucose in serum samples were carried out by adding known amounts of glucose to mixed serum sample solutions. The results demonstrate that the recovery rate ranged from 97.1% to 103.5%, indicating that the glucose sensor had an excellent performance for glucose detection in human serum.

4. Conclusions

A new optical glucose biosensor based on glucose oxidase immobilized in ORMOSIL–PVA has been developed. The biosensor has the advantages of short response time, lower detection limit, high sensitivity, and stability. It is easy to fabricate and use. The cost is very low and there is no need to saturate with oxygen. The glucose measurements were carried out in the kinetic mode under optimized conditions. A kinetic curve simulation method was employed for data analysis, and this method was proved to be convenient and precise, and it only took about 1 min to analyze a sample. This optical biosensor has potential application in the fast detection of glucose in human serum.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2007.09.079.

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