IMMOBILIZATION OF GLUCOSE OXIDASE IN SILICA SOL-GEL FILM FOR APPLICATION TO BIOSENSOR AND AMPEROMETRIC DETERMINATION OF GLUCOSE

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ABSTRACT

In this study, an electrochemical biosensor was developed by using a sol-gel coating solution. The modified platinum electrode used in the study was constructed by immobilization of glucose oxidase under a layer of sol-gel film. The sol-gel coating solution was prepared by using GLYMO, TEOS, and MTEOS. Electrochemical measurements were carried out amperometrically by determining hydrogen peroxide produced by the enzymatic reaction between glucose and glucose oxidase. The amperometric responses of the resulting enzymatic electrode to glucose were rapid. It was observed that the amperometric response of the enzymatic electrode was linear for glucose concentrations in the range from 2 to18 mM with 50 s response time. LOD and LOQ for the enzymatic electrode were calculated to be 0.055 mM and 0.184 mM, respectively. It was determined that the developed biosensor had an acceptable reproducibility. The selectivity of the biosensor was determined in the presence of some interfering substances, such as lactose, sucrose, urea, uric acid, oxalic acid, and ascorbic acid. The stability of the biosensor was investigated, and it was found that the sensor response decreased by 59% of its initial response over a period of 30 days of storage in dry conditions at 4 ºC.

Keywords: Amperometry, biosensor, glucose, glucose oxidase, sol-gel

INTRODUCTION

Electrochemical biosensors are the most commonly used class of biosensors. They may be divided into conductometric, potentiometric, and amperometric biosensors depending upon the electrochemical property to be measured by detector system. The amperometric biosensors are more attractive than the others due to their high sensitivity and wide linear range. An amperometric biosensor measures the resulting current changes on the working electrode due to direct oxidation of the products of the biochemical reactions[1, 2].

Enzyme-based amperometric biosensors have attracted a great attention in recent years. The most important factor in the development of an enzyme-based biosensor is the immobilization of enzyme on the transducer surface. A number of immobilization techniques, such as physical entrapment, chemical immobilization in an inert matrix, and covalent attachment to electrode surfaces have been used to immobilize the relevant enzyme in the construction of the amperometric biosensors[3-5]. Among the various modification procedures, the sol-gel process has attracted much attention for immobilization of biomolecules in the design of the biosensor because of its distinct advantages, such as low temperature requirement, chemical inertness, negligible swelling, optical transparency, low-temperature encapsulation, tunable porosity, thermal stability, and biocompatibility[6-9].

Hybrid coatings prepared by means of the sol-gel process using organic-inorganic silanes are a type of composite materials in which the inorganic and organic components are combined at the molecular level. The organic part of the hybrid material improves the adhesion between the coating and substrate while the inorganic part maintains the hardness and chemical durability of the coating. These coatings have been successfully used as enzyme immobilization matrix due to their biocompatibility. They can be readily prepared by hydrolysis and condensation of alkoxysilanes and organoalkoxysilanes[10, 11].

The routine analysis of glucose in various physiological fluids is one of the most frequent operations in clinical chemical laboratories. The convenient, rapid, safe and precise determination of blood sugar in diabetes patients is important for the treatment and control of diabetes. Therefore, the studies intended for the development of the amperometric glucose sensor have been a subject of interest in recent years[12-17].

There are several reports on the immobilization of glucose oxidase (GOx) within the sol-gel matrix for the development of glucose biosensor. A glucose biosensor based on the sol-gel encapsulation of glucose oxidase by using different oxysilanes compounds has been developed by Pauliukaite and Brett[18]. Tatsu et al.[19] developed an amperometric biosensor for glucose determination by encapsulating glucose oxidase in a silica sol-gel matrix. They monitored the level of glucose by using an oxygen electrode, which was covered by GOx-doped sol-gel film. Li et al.[13] reported the construction of a glucose sensor by using sandwich configuration in which GOx was immobilized between two sol-gel films. Pandey et al.[20] constructed a glucose sensor based on the sandwich configuration of organically modified sol-gel glasses. The sol-gel glass was developed by using 3-aminopropyltrimethoxy silane and 2-(3,4-epoxycyclohexyl)-ethyltrimethoxy silane. Kim et al.[21] reported the usage of the nanoporous composite sol-gel film derived by using zirconia and Nafion for the encapsulation of glucose oxidase on the surface of the platinized glassy carbon electrode. Pauliukaite et al.[10] developed an electrochemical biosensor based on the sol-gel enzyme encapsulation by applying protective polymer membranes over the sol-gel-enzyme layer to prevent enzyme leaching. Chitosan/silica organic–inorganic hybrid composite sol–gel films were used by Kang et al.[22] for the fabrication of a glucose biosensor. Florescu et al.[23] prepared an electrochemical enzyme biosensor using two types of sol-gel precursors to entrapment the glucose oxidase. They used combination of 3-glycidoxypropyltrimethoxy silane with methyl trimethoxysilane or tetra ethoxysilane to prepare the sol-gel mixture.

This paper reports a glucose biosensor prepared by immobilization of GOx on the platinum electrode surface by using a silica sol-gel film. The sol-gel layer was prepared by mixing of (3-glycidoxylpropyl)trimethoxysilane, tetraethoxysilane and methyltriethoxysilane precursors. Electrochemical measurements were carried out amperometrically. The optimal values of the working potential and pH of buffer solution were determined. Analytical parameters of the biosensor were investigated.

EXPERIMENTAL

Chemicals and solutions

(3-Glycidoxylpropyl)trimethoxysilane (GLYMO, 98%), tetraethoxysilane (TEOS, 98%), methyltriethoxysilane (MTEOS, 99%), and 2-butoxyethanol were supplied from Aldrich. α-D-(+) glucose and glucose oxidase (GOx) from *Aspergillus Niger* were purchased from Sigma. HCl (37%) was supplied from Riedel-de-Haën. Double distilled water was used throughout the preparation and dilution of all solutions.

Phosphate buffer solution (PBS) was prepared by using disodium hydrogen phosphate and potassium dihydrogen phosphate. The glucose stock solution (0.2 M) was prepared in distilled water and left at room temperature for 24 h prior to use to ensure the presence of β-D-glucose form.

Preparation of silica sol-gel solution

The sol-gel coating solutions were prepared by mixing 1 mL of GLYMO, 0.4 mL of TEOS, 0.4 mL of MTEOS, and 0.505 mL H_2O in a glass vial. A 0.044 mL aliquot of concentrated HCl solution (with 37%) was added to the mixture to accelerate hydrolysis of the silanes. The mixture in glass vial was stirred until a clear and homogeneous solution was obtained and stored at room

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temperature for 24 h. This solution was used as the stock sol solution. Then, a coating solution was prepared by mixing 1 mL of the stock sol solution and 3 mL of 2-butoxyethanol in a separate glass vial. This final solution was stirred for 2-3 h and stored at room temperature for 24 h. The solution diluted with alcohol was used for the immobilization of glucose oxidase.

Preparation of enzymatic electrode

The platinum electrode was chosen as working electrode for the preparation of the sol-gel modified glucose biosensor. Before modification of the platinum electrode, the surface of the bare Pt electrode was polished with 1.0, 0.3, and 0.05 μm alumina slurries, respectively, and then it was washed with distilled water. Afterwards, the polished platinum electrode was cleaned ultrasonically with acetone and distilled water in an ultrasonic bath for 3 min. Finally, the cleaned electrode was rinsed with double distilled water and dried at room temperature in air. Subsequently, the construction of the enzymatic electrode was accomplished as follows:

The enzyme solution was prepared by dissolving 5.1 mg of glucose oxidase in 50 μL of 0.1 M PBS solution (pH=7) at room temperature. A volume of 2 μL of this enzyme solution was dropped on the platinum electrode surface (2 mm diameter) and allowed to dry at room temperature for 30 min. After that, aliquots of 8 μL of the solution diluted with alcohol was carefully dropped on the enzyme adsorbed onto the surface of the platinum electrode and allowed to dry at room temperature for 48 h. The resulting enzyme biosensor was stored at 4 °C in a refrigerator when not in use.

Methods and instruments

Electroanalytical measurements were carried out with a BAS 100 W (Bionalytical Systems, Inc.) electrochemical analyzer. All experiments were performed by using a conventional electrochemical cell with a three-electrode system, comprising a modified platinum electrode as the working electrode, an Ag/AgCl electrode saturated with KCl as the reference electrode, and a Pt wire coil as the auxiliary electrode.

Hydrogen peroxide produced by the enzymatic reaction between glucose and glucose oxidase was determined amperometrically. Phosphate buffer solutions (PBS) used in the amperometric studies were aerated by bubbling air for about 20 min prior to use. Then, three-electrode system was immersed into 10 mL of PBS solution. The solution was stirred by using a magnetic bar to provide the convective mass transport during the experiments. A predetermined constant working potential versus Ag/AgCl was applied to the cell, and the background current was allowed to reach the steady state before glucose injections. The resulting amperometric response due to the oxidation of hydrogen peroxide formed by the enzymatic reaction was measured as a function of time, and the graphs of the current versus time were continuously recorded.

RESULTS AND DISCUSSION

Effect of pH of buffer solution

The pH of buffer solution has a very important effect on the sensitivity of the biosensor because the pH affects the bioactivity of glucose oxidase. The optimal pH for glucose oxidase is usually expressed within a pH range of 6.5-7.5, which varies with immobilization method and microenvironment around the enzyme [24,25].

To determine the optimal pH value on the responses of the biosensor, experiments were performed by measuring the current response of the biosensor to 10 mM glucose at different pH values at 700 mV. Figure 1 shows the effect of the pH of buffer solution on the amperometric responses of the glucose biosensor. As can be seen, the enzymatic electrode exhibited a maximum response to glucose injections at pH 7. This pH value agrees with the previous studies. Thus, the optimal pH value of PBS was selected to be 7 to obtain the maximum sensitivity of glucose biosensor.

Effect of working potential

The applied potential has a strong effect on the biosensor responses because it contributes to the sensitivity and selectivity of the biosensor. The effect of the applied potential on the response of glucose biosensor was investigated in the potential range from 500 to 900 mV versus Ag/AgCl reference electrode. In these experiments, glucose concentration and pH value were kept constant at 10 mM and 7, respectively. Figure 2 represents the effect of the applied potential on the responses of the enzymatic electrode. It could be seen from the results given in Figure 2 that a maximum current response to glucose was reached at a potential of 700 mV. To obtain the highest current response in the amperometric measurements, the working potential was selected to be 700 mV.

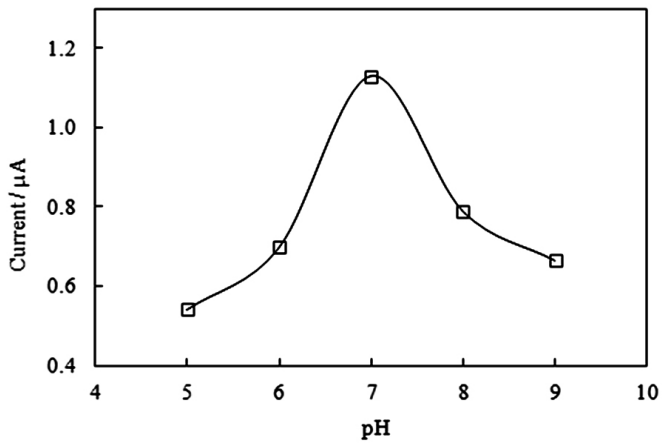

Figure 1. Effect of buffer solution pH on the sensor response.

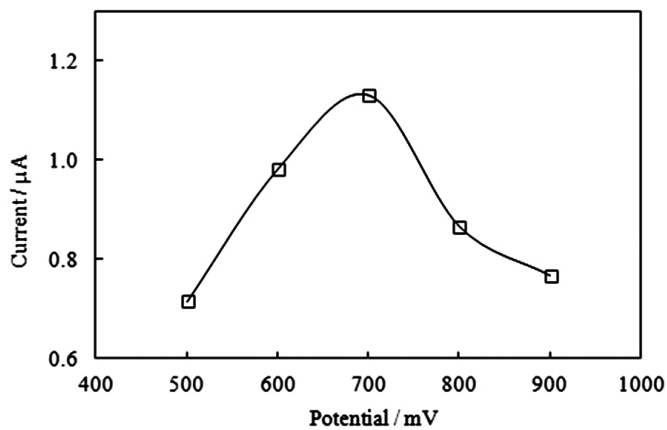

Figure 2. Effect of the working potential on the sensor response.

Analytical parameters of the biosensor

After determining the optimal values for the working potential and the pH of the buffer solution, analytical parameters of the prepared biosensor (response time, linearity, selectivity, and stability) were investigated under the optimized experimental conditions.

Figure 3 illustrates the typical amperometric responses of the silica/GOx electrode to the addition of aliquots of the stock glucose solution. The glucose concentration for each injection was 2 mM. It can be seen that the enzymatic electrode exhibited a rapid and sensitive response to changes in the glucose concentration. It was observed that the biosensor responded rapidly to the glucose and achieved a steady-state current value within 50 s. Such a fast reply can be attributed to the fast diffusion of the glucose molecules through the pores of the resulting sol-gel film.

To check whether the enzyme under the sol-gel film was responsible from the steady-state responses for glucose, the amperometric behavior of the enzyme-free sol-gel film was tested with the successive glucose injections. When no glucose oxidase was encapsulated under the silica sol-gel film, glucose did not create a detectable signal on the electrode (in Figure 4). This test indicates that the enzyme layer under the sol-gel film is responsible from the resulting amperometric responses by means of the oxidation of H_2O_2 produced by the enzymatic reaction in the presence of glucose oxidase.

Figure 5 depicts the calibration graph constructed for the enzymatic electrode by using the steady-state amperometric responses shown in Figure 3. It is clear that the biosensor produces a linear response up to a concentration of 18 mM glucose. The sensitivity of the biosensor was determined to be 0.1067 μA/mM. The limit of detection (LOD) and limit of quantification (LOQ) for the enzymatic electrode were calculated to be 0.055 mM and 0.184 mM at a signal-to-noise ratio of 3, respectively.

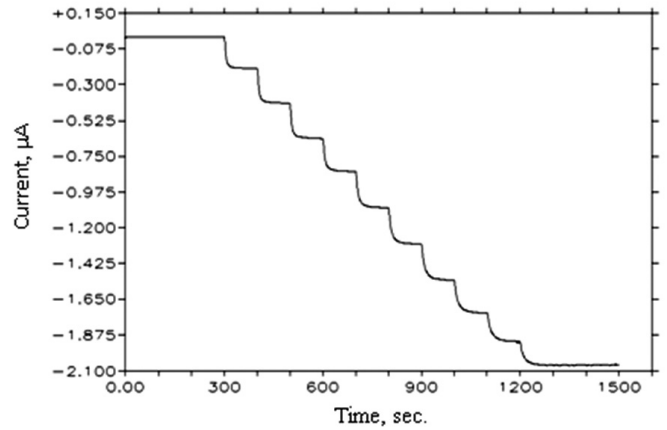

Figure 3. The amperometric responses of enzymatic electrode to successive glucose injections.

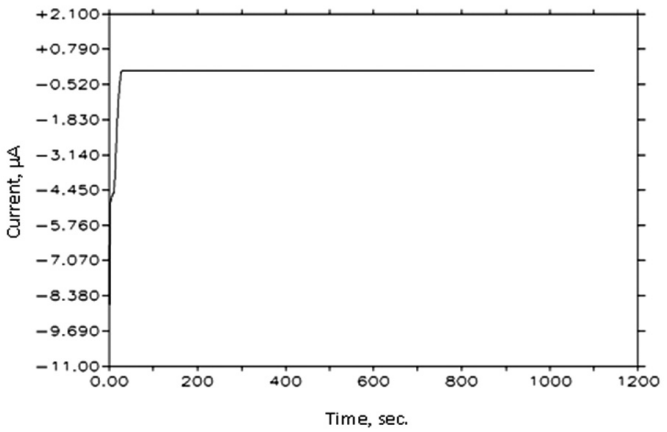

Figure 4. The amperometric responses of enzyme-free electrode to successive glucose injections.

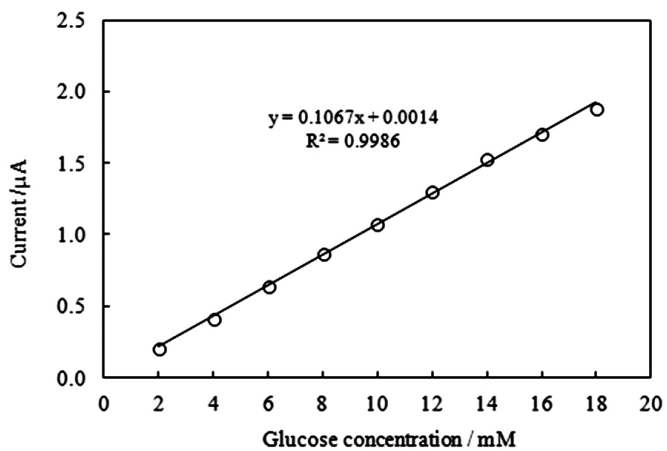

Figure 5. The calibration graph of the enzymatic electrode.

To see the repeatability of the construction method of the glucose biosensor, the sensor-to-sensor reproducibility was investigated by measuring the current responses of three different enzymatic electrodes. Each sensor was tested with successive addition of 2 mM glucose solution (n=10). The results showed that the sensor had an acceptable reproducibility with a standard deviation of 2.05%. It can be said that the biosensor fabrication procedure described here is reliable for different sensors to be constructed in the same manner.

One of the major characteristics of an amperometric glucose biosensor is the selectivity of the sensor for the determination of glucose in the presence of interfering species. Some electroactive and non-electroactive species coexisting with glucose in the real samples behave as interfering substances. The electroactive species (e.g., ascorbic acid, uric acid, and oxalic acid) are oxidized at the electrode surface and can affect the current responses of hydrogen peroxide produced by the enzymatic reaction, while the nonelectroactive species (e.g., lactose, sucrose, and urea) can foul the electrode surface or block the pores of the sol-gel film. Therefore, they can cause a decrease in the current responses. To determine the anti-interference ability of the developed sol-gel film, the amperometric responses of enzymatic electrode to glucose injections in the presence of interfering substances were determined. Figure 6 shows the effect of the interfering species on the steady-state amperometric current responses of the biosensor. Each injection indicated in Figure 6 corresponds to 2 mM of the relevant substance. Among the injected electroactive substances, only for ascorbic acid was observed a small response. The current response of ascorbic acid is caused from its electro-oxidation on the surface of electrode by passing through the sol-gel film. The amperometric response for a total concentration of 4 mM ascorbic acid was 10.9% of the determined net current. The interfering effect of ascorbic acid has been excluded by using the thicker sol-gel film. But, the amperometric responses of glucose decreased reasonably when the thicker film was used because of decrease in glucose diffusion through the thick sol-gel film. The other species did not cause any observable signal. However, it was observed that the responses of biosensor to glucose reduced slightly in the presence of fouling substances. This decrease in current responses of glucose may be probably related to the blockage of the pores due to contaminants. Nevertheless, it can be seen in Figure 6 that the enzymatic electrode responds successfully to glucose injections in the presence of various electroactive and nonelectroactive interfering molecules. Consequently, it can be said that the developed sol-gel film effectively protects the electrode surface from interfering substances.

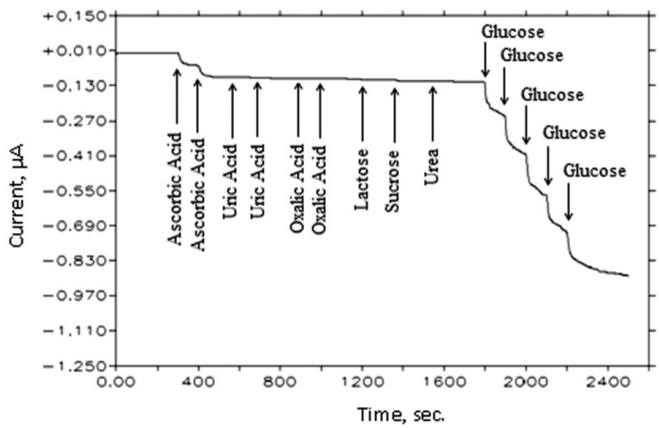

Figure 6. The selectivity of enzymatic electrode to glucose in presence of interfering species.

The stability of the glucose sensor was tested by means of the amperometric measurements over period of one month using 10 mM glucose. The results of these experiments were given in Figure 7. The enzymatic electrode was stored in dry condition at 4 °C to prevent the enzyme leaching through the sol-gel matrix when it was not in use. It was determined that the glucose sensor response decreased by 59% of its initial response over a period of one month. This result shows that the activity of the enzyme under the sol-gel layer is protected for a long time.

The reusability of the prepared biosensor was examined by doing 20 consecutive amperometric experiments on the same day for a concentration of 10 mM glucose. These tests were performed at the same optimal conditions as the previous amperometric measurements. The enzymatic electrode was rinsed by distilled water before each measurement. The experimental findings showed that the current response was decreased by 10% of the initial response after the first five measurements. The current response was gradually decreased in the subsequent measurements, and it was determined that the response of the enzymatic electrode diminished by 70 % of the initial response at the end of the twentieth measurement.

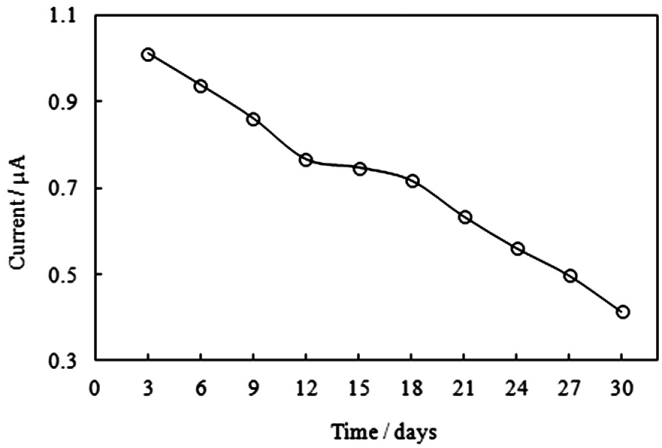

Figure 7. Stability of biosensor.

The performance of the enzymatic electrode developed in this study is compared with earlier reported glucose sensors based on the sol-gel process. This comparison is given in Table 1. As can be seen, the present biosensor has a higher linear range than the others.

Table 1. Comparison of electrochemical glucose biosensor based on the sol-gel process.

Reference	Response time (s)	Linear range (mM)	Detection limit (mM)	Sensitivity (μA/mM)
9	10	1.3	0.0075	5.94
10	-	0.5	0.0158	0.117
23	-	1	0.071	0.0197
26	2	7	-	15
27	12	4.75	0.02	1.182
28	30	14	0.01	0.274
Present study	50	18	0.055	0.1067

CONCLUSIONS

In this paper, a glucose biosensor has been developed by using the sol-gel method. Glucose oxidase adsorbed on the platinum electrode surface was immobilized under a silica sol-gel film, which has been prepared by using GLYMO, TEOS, and MTEOS. The resulting biosensor was used to detect glucose in a PBS buffer solution with amperometric method. It was determined that the prepared glucose biosensor exhibited high sensitivity, selectivity, fast response time, good stability, and wide linear range. LOD and LOQ for the enzymatic electrode were calculated to be 0.055 mM and 0.184 mM, respectively. It was determined that the developed biosensor had an acceptable reproducibility. The lifetime of the glucose sensor indicates that a silica sol-gel matrix is a good immobilization medium for GOx. The results obtained from the experiments demonstrate that the sol-gel organic-inorganic hybrid materials are an excellent matrix for the immobilization of glucose oxidase enzyme to develop the glucose biosensor due to their biocompatibility. The immobilization technique used in this work is simple and low cost. This method can be also employed for other enzyme systems to develop the electrochemical biosensor.

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