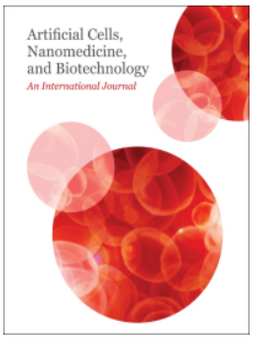

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An amperometric biosensor for L-glutamate determination prepared from L-glutamate oxidase immobilized in polypyrrole-polyvinylsulphonate film

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Abstract

In this paper, a novel amperometric L-glutamate (Glu) biosensor with immobilization of L-glutamate oxidase (L-GlOx) on polypyrrole-polyvinylsulphonate (PPy-PVS) film has been successfully developed. L-GlOx enzyme was immobilized on PPy-PVS film by cross-linking with glutaraldehyde (GA) and bovine serum albumin (BSA). Determination of Glu was carried out by oxidation of enzymatically produced H_2O_2 at 0.3 V versus Ag/AgCl. The optimum pH and temperature parameters were found to be 9.0 and 55°C, respectively. There were three linear parts in the regions between 1.0×10^{-9} and 1.0×10^{-8} M ($R^2 = 0.847$), 5.0×10^{-8} and 5.0×10^{-7} M ($R^2 = 0.997$), 5.0×10^{-7} and 5.0×10^{-5} M ($R^2 = 0.994$). Storage stability, operation stability of the enzyme electrode were also studied.

Keywords: biosensor, L-Glutamate, L-glutamate oxidase, polypyrrole, polyvinylsulphonate

Introduction

L-glutamate (Glu) is one of twenty amino acids forming proteins and found naturally in food that contain proteins. It can be synthesized in mammalian metabolism and can also be taken into metabolism from food. Glu has important functions because it is found in the central point of mammalian metabolism. First, it is a neurotransmitter in central nervous system and brain uses it as a transmitter during the transmission of nerve impulses (Ghobadi et al. 1996, Maalouf et al. 2007). Furthermore, Glu also plays an essential role in many metabolic processes. It plays a major role in detoxification of nitrogen in nitrogen metabolism by performing the deamination reaction with water. It is used by brain when glucose storage is not enough. As a result of a transamination of α-ketoglutarate which locates in TCA cycle, Glu consists (Nelson and Cox 2000). Glu is very important clinically, because increased Glu level in cerebrospinal fluid is observed in neural disorders such as epilepsy, schizophrenia, Alzheimer's and Parkinson's diseases. Its quantification is found to be useful for diagnosis of these neurological disorders. Also, it is often used in clinical laboratories for the determination of aminotransferase activities (Cooper et al., 1991; Nelson and Cox 2000). Glu has an important place in food industry as well as clinically. It is widely used as a flavor enhancer in soups, sauces, and many processed foods (Maalouf et al. 2007). Glutamate sodium salts are used as food additive (Janarthanan and Mottola 1998). E-621 is classified as monosodium glutamate (MSG) that is used in foods as a flavor enhancer. There are different views about the effects of MSG on human body. Some researchers approach cautiously to this substance, some of them expressed that it triggers tension and headache; some other researchers explain that MSG is not harmful to human body (Janarthanan and Mottola 1998).

A reliable, simple, and economic method for the determination of Glu which is important for both clinical sector and food industry would be useful. Many analytical methods have been improved until now for Glu determination such as spectrophotometry (Valero and Garcia-Carmona 1998), fluorescence (Chapman and Zhou 1999), chromatographic techniques (Swanepoel et al. 1996), and capillary electrophoresis (Tucci et al. 1998). On the other hand, biosensors are better methods in some ways. Because of their good specifications such as high sensitivity and selectivity, rapid response, low costs, etc., biosensors have become an extremely broad field which impact on many sectors including clinical, pharmaceutical, food and environmental sectors (Amine et al. 2006). So, biosensors for determination of Glu would be very useful.

In this study, we report a new amperometric biosensor for the determination of Glu. L-GlOx was immobilized onto a PPy-PVS film surface by cross-linking with glutaraldehyde (GA) and BSA. The determination of Glu was performed by oxidation of enzymatically generated H_2O_2. Optimum working conditions of biosensor with respect to the substrate concentration, pH, and temperature were investigated. Also

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the storage stability and operation stability of the biosensor were investigated.

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{\text{L-GlOx}} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{electrode}} O_2 + 2H^+ + 2e^-$$

Material and methods

Equipment and reagents

The electrochemical studies were carried out by using Epsilon EC electrochemical analyzer with a three-electrode cell. The working electrode was a Pt plate (0.5 cm^2). Auxiliary and reference electrodes were Pt wire and Ag/AgCl electrode (3.0 M KCl), respectively. The pH values of the buffer solutions were measured with an ORION Model 720A pH/ion meter. Temperature control was achieved with Grant GD 120 thermostat. Glu oxidase (EC 1.4.3.11, purified from the *Streptomyces sp.*, activity of 1.0 unit/mL) was purchased from Sigma. L-glutamic acid monosodium salt monohydrate was also purchased from Aldrich. Pyrrole and sodium polyvinylsulphonate were supplied from Fluka and Aldrich, respectively. All other chemicals were obtained from Sigma. All of the solutions were prepared by using distilled water. Scanning electron microscopy was done by using a JEOL JEM 100 CX II scanning electron microscope (JEOL, Peabody, MA) equipped with a Link analytical system.

Preparation of Pt/PPy-PVS film electrode

The surface of the Pt plate electrode was cleaned (Gros et al. 2000) and covered with PPy-PVS film by electropolymerization of pyrrole in a medium containing sodium polyvinylsulphonate (Chaubey et al. 2000) The Pt plate electrode was immersed in 10.0 mL solution containing 0.1 M pyrrole and 2.5 mL (25.0%) of sodium polyvinylsulphonate. The solution was purged with argon in order to remove the oxygen. Pyrrole electropolymerization was carried out on the Pt electrode surface by cyclic voltammetric scans between −1.0 and 2.0 V at a scan rate of 50 mV/s [vs. Ag/AgCl electrode (3.0 M KCl)]. PPy-PVS film was obtained after 1 cycle (Figures 1 and 2). After electropolymerization,

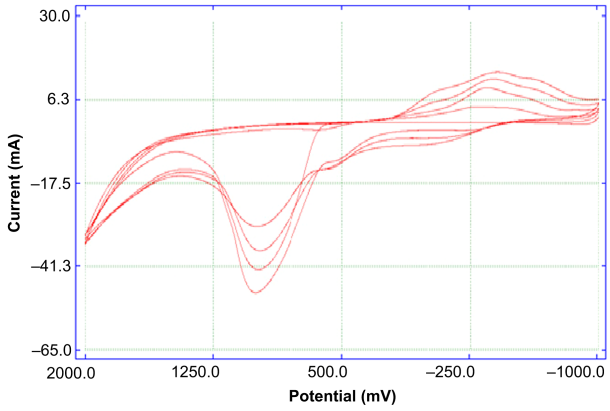

Figure 1. Growth of PPy-PVS films in aqueous solution of 0.1 M pyrrole and 2.5 mL 25.0% PVS at a scan rate of 50 mV/s versus Ag/AgCl electrode (3.0 M KCl)).

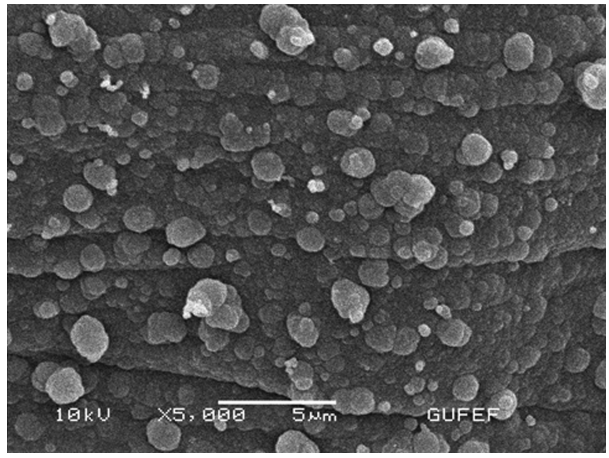

Figure 2. Scanning electron micrograph of PPy-PVS electrode surface.

PPy-PVS film was rinsed with deionized water to remove the unreacted pyrrole monomer. In Figure 1, the growth of PPy-PVS film is seen. In Figure 2, it can be seen that the surface morphology of the PPy-PVS film is a cauliflower-like structure.

Immobilization of L-GlOx on Pt/PPy-PVS film electrode

After electropolymerization, the mixture solution of 50.0 µL of L-GlOx (1.0 unit/mL), 1.0 mg of BSA, 81.25 µL of 0.1 M glycine buffer at pH of 9.0, and 7.5 µL of 2.5% GA was dropped on Pt/PPy-PVS film. Electrode was dried at room temperature and washed with buffer solution (0.1 M glycine buffer of pH 9.0) several times in order to remove the excess non-immobilized enzyme and GA. Immobilized enzyme electrode was kept in a refrigerator at 4°C in glycine buffer, when not in use.

Amperometric measurements

Quantification of Glu was achieved via electrochemical detection of enzymatically released H_2O_2.

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{\text{L-GlOx}} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{electrode}} O_2 + 2H^+ + 2e^-$$

Enzyme electrode was immersed into glycine buffer (0.1 M) at pH 9.0. The solution contained 0.1 M sodium chloride as supporting electrolyte. Electrode was brought to equilibrium by keeping it at 0.3 V [vs. Ag/AgCl electrode (3.0 M KCl)]. Steady-state current (i_a) was recorded. Glu solution was added to the cell from stock solution and the system was stirred. Current (i_b) values obtained at 0.3 V were recorded and current values ($\Delta i = i_b - i_a$) were plotted against the Glu concentration.

Results and discussion

In this study, we reported a new L-GlOx-based amperometric biosensor for the determination of Glu. L-GlOx was immobilized onto a PPy-PVS film surface by cross-linking with GA and BSA. The determination of Glu was performed by oxidation of H_2O_2, which occurs during enzymatic reactions at 0.3

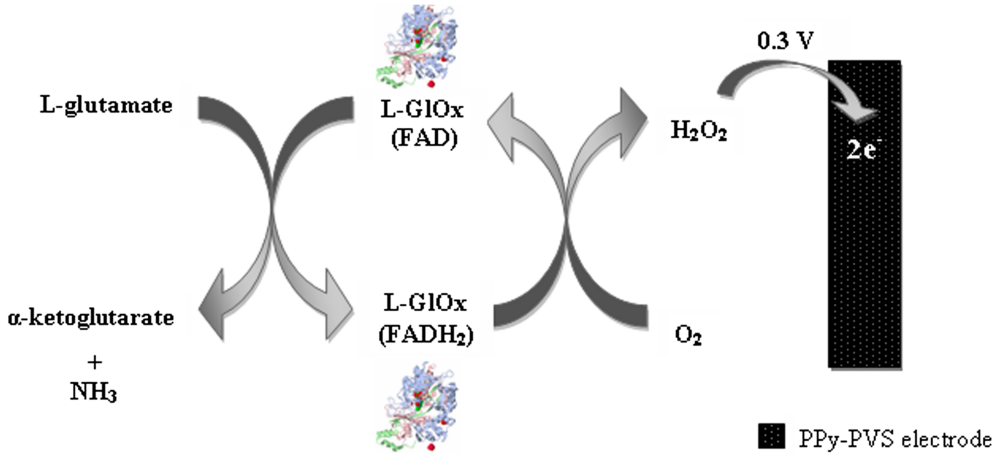

Figure 3. Reaction scheme for the Glu determination.

V versus Ag/AgCl. Reaction in Figure 3, which shows the Glu determination, is shown below.

According to this scheme, a biochemical reaction occurs between Glu in solution and L-GlOx enzyme which is immobilized onto PPy-PVS electrode. First, Glu is oxidized to α-ketoglutarate by L-GlOx. And flavin adenine dinucleotide (FAD), which is a prosthetic group in enzyme structure, is reduced to $FADH_2$ by taking electron. $FADH_2$ in enzyme is oxidized by giving its electrons to oxygen in solution to make the reaction reversible. Thus, enzyme comes to its previous form. By taking the electron, oxygen is reduced to H_2O_2. Glu determination was made by measuring the anodic current of hydrogen peroxide on electrode surface.

The parameters affecting the performance of biosensor and optimum working conditions were investigated.

Determination of working potential

After preparing Pt/PPy-PVS electrodes, hydrogen peroxide oxidation was carried out at different potentials (0.2–0.5 V) (Figure 4). When Figure 4 was examined, it was seen that the variation in current in high potentials was higher than in low potentials. Compared with other potentials, the oxidation currents of H_2O_2 at 0.2 V were too low. Interference effects of substances presented in body fluids (e.g., ascorbic acid, uric acid) could be more in high potentials (Zhang et al. 2007). Therefore 0.3 V was used as working potential.

Effect of glutaraldehyde amount on the response of biosensor

Cross-linking is one of the suitable enzyme immobilization methods in the construction of a biosensor. GA ($CHO\text{-}CH_2\text{-}CH_2CH_2\text{-}CHO$) is a commonly used cross-linking reagent. It reacts with amine groups of enzyme molecules to make the conjugation. Because of its small size, GA can not only react with amine groups on the enzyme's surface but can diffuse into the enzyme and bind to interior amine groups (Xin and Wightman 1997). This case can affect the three-dimensional structure of an enzyme and also its catalytic function, because enzyme's active region can be destroyed by over-cross-linking (Xin and Wightman 1997). So, to improve the performance of the biosensor, amount of GA has to be investigated.

Different percentages of GA solutions (0.08, 0.13, and 0.67%) were used for immobilization of L-GlOx. The results demonstrated that the activity of the cross-linking system increased when the GA percentage was increased from 0.08% to 0.13% (Figure 5). But when the

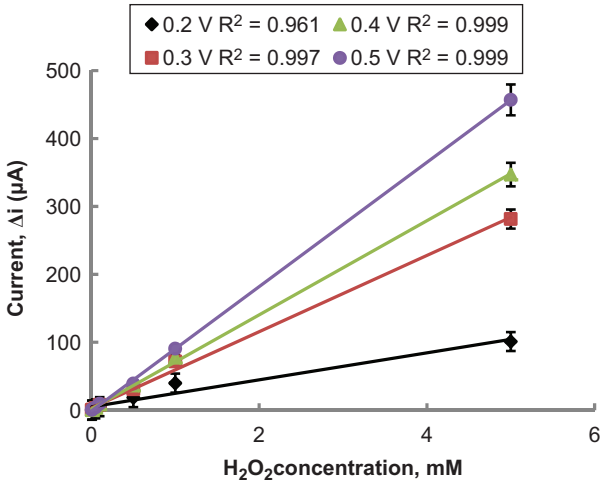

Figure 4. The effect of potential on the response of the Pt/PPy-PVS electrode to hydrogen peroxide (in the glycine buffer (pH 9.0).

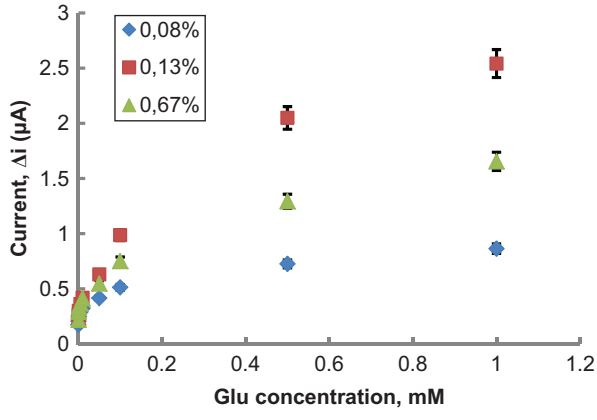

Figure 5. The effect of glutaraldehyde amounts on the response of the biosensor (at 25°C, 1.0×10^{-7}–1.0×10^{-3} M Glu at 0.3 V operating potential). (◆) 0.08% glutaraldehyde, (■) 0.13% glutaraldehyde (▲) 0.67% glutaraldehyde.

GA percentage was 0.67%, activity of the cross-linking system decreased. 0.13% was decided as suitable GA amount. Because, 0.08% GA concentration was not enough, but 0.67% was much and caused enzyme activity loss.

In this study, GA percentage was kept constant at 0.13% which was observed to give maximum activity with reasonable mechanical stability. It was also selected in the further characterization experiments.

Effect of pH on amperometric response of biosensor

pH plays an important role in maintaining the proper conformation of an enzyme, because enzyme activity is dependent on the ionization state of the amino acids in the active site. The effect of pH on the response of the Glu biosensor was tested varying between pH 6.0 and 10.0. The measurements were performed at a constant Glu concentration 1.0×10^{-3} M with 0.1 M phosphate and glycine buffers varied between pH 6.0 and 10.0. Figure 6 shows that the maximum response was obtained at pH 9.0.

pH values, greater than physiologic conditions, were also seen in biosensors which were prepared from PPy-PVS polymers (Arslan and Arslan 2011, Özdemir et al. 2012). L-GlOx enzyme kept its activity even at high pH, when they were immobilized to PPy-PVS.

The difference in pH values was attributed to the fact that the used polymer and the type of immobilization were different. Because, change in the environment of enzyme's active site can cause different interactions between enzyme and immobilization materials (Basu et al. 2006, Chang et al. 2007). Furthermore, enzymes become stronger after immobilization. Immobilized enzymes can show high activities under different pH conditions when compared with free enzyme, as the stable substrate-enzyme complex may form.

Effect of temperature

Temperature is an important factor which has a significant effect on enzyme activity. It is important to investigate the dependence of the response of the enzyme electrode to temperature. The relationship between reaction rate of an enzyme and temperature is exponential. The amperometric response of the prepared biosensor was investigated at different temperatures by using constant Glu concentration of 1.0×10^{-3} M (Figure 7). As seen from the Figure 7, the current difference increases with temperature up to 55°C and decreases afterwards. The highest current difference was obtained at 55°C. The enzyme was thought to be denaturized after this temperature. The study was carried out at 25°C due to the difficulties involved in working at 55°C. For Glu biosensor, temperature values different than 55°C were employed as found in the literature (48; 65°C) (Pan and Arnold 1996, Chang et al. 2007). This was attributed to the fact that the used polymer and the type of the immobilization were different. Additionally, immobilized enzymes can show high activity under temperatures different than physiological conditions compared to that of free enzymes. High optimum temperature was also seen in biosensors which were prepared from PPy-PVS polymers (Çolak et al. 2012, Özdemir et al. 2012, Dolmacı et al. 2012).

Substrate concentration and calibration curves

The effect of the substrate concentration on the reaction rate, catalyzed by immobilized enzyme, was studied by using varying concentrations $1.0\times10^{-9}-1.0\times10^{-3}$ M Glu (Figure 8A). There were three linear parts in the region between 1.0×10^{-9} and 1.0×10^{-8} M ($R^2=0.847$) (Figure 8B), 5.0×10^{-8} and 5.0×10^{-7} M ($R^2=0.997$) (Figure 8C), and 5.0×10^{-7} and 5.0×10^{-5} M ($R^2=0.994$) (Figure 8D). When compared with the literature ($1.0\times10^{-7}-1.0\times10^{-4}$ M and $8.0\times10^{-6}-8.0\times10^{-4}$ M, respectively, for Karyakin et al. 2000 and Ghobadi et al. 1996), linear working range of biosensor is wider and biosensor is sensitive to low Glu concentration. It has been shown that the linearity of these graphs is highly satisfactory and can be used for the quantitative determination of Glu.

The low detection limit of the biosensor was found to be 5.0×10^{-9} M and response time for the biosensor was 200 s. Detection limit of biosensor is lower than detection limit in literature (2.0×10^{-5} M and 1.0×10^{-7} M, respectively, for Maalouf et al. 2007 and Karyakin et al. 2000). Because of the reasons that prepared Glu biosensor has a wide linear working range which includes low concentrations and determination limit, Glu determination can be made with a variety of different samples with our biosensor. $K_{m(app)}$ and $I_{max(app)}$ values were found to be 1.1×10^{-6} mM and 0.268 μA, respectively. The $K_{m(app)}$ value that shows the affinity of the biosensor was 1.1×10^{-6} mM and the values, cited in the literature, were 1.92 mM (Basu et al. 2006) and 2.84 mM (Maalouf et al. 2007). PPy-PVS film, used in this study, increased the affinity of enzymes

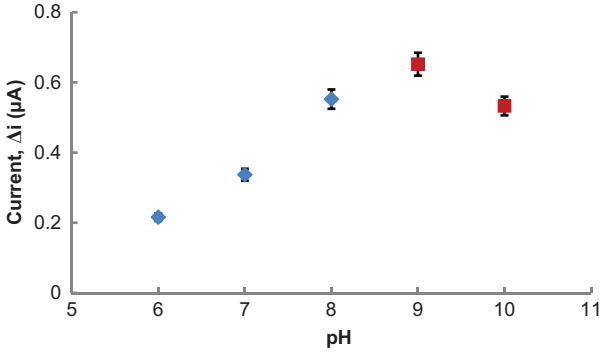

Figure 6. The effect of pH on the response of the biosensor (at 25°C, 1.0×10^{-3} M Glu, at 0.3 V operating potential).). (◆) 0.1 M phosphate buffer, (■) 0.1 M glycine buffer.

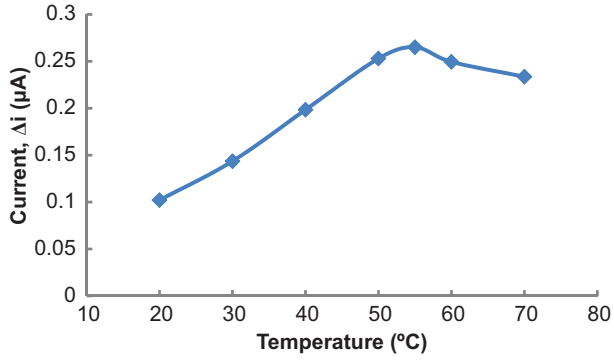

Figure 7. The effect of temperature on the response of the biosensor (at pH 9.0, 1.0×10^{-3} M Glu at 0.3 V operating potential).

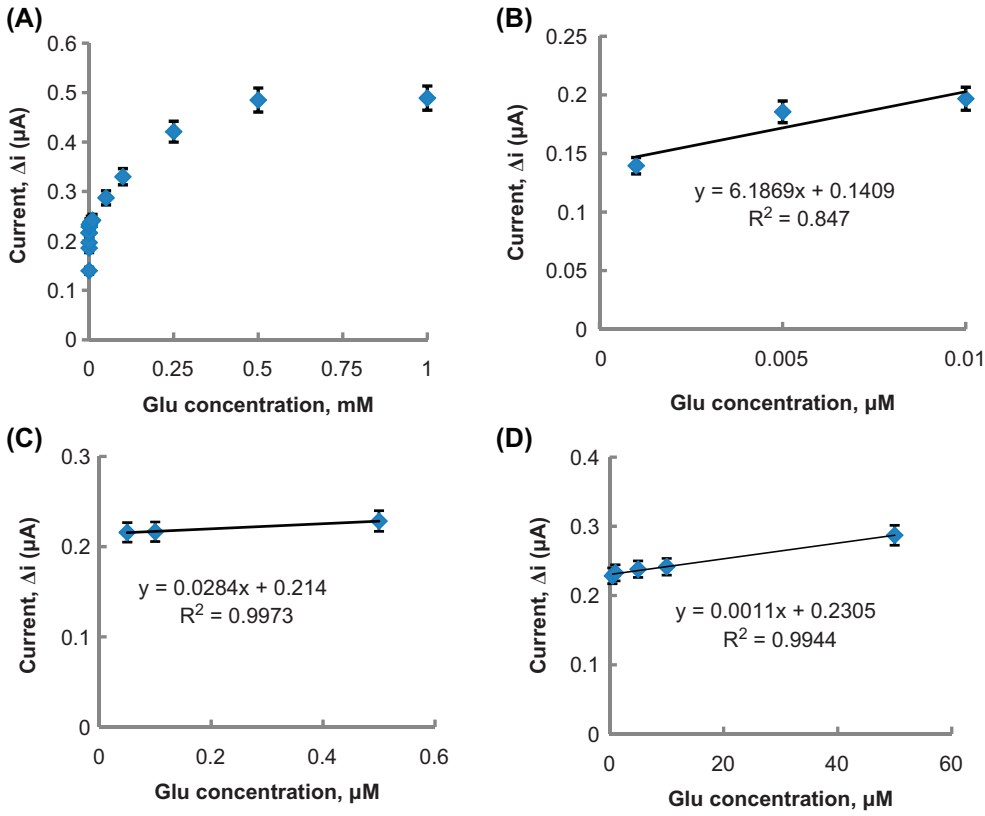

Figure 8. (A) The effect of Glu concentration on the response of the biosensor (at 0.1 M pH 9.0 glycine buffer, 25°C, 0.3 V operating potential). (B) The calibration curve of Glu biosensor (at 0.1 M, pH 9.0 glycine buffer, 25°C). (C) The calibration curve of Glu biosensor (at 0.1 M, pH 9.0 glycine buffer, 25°C). (D) The calibration curve of Glu biosensor (at 0.1 M, pH 9.0 glycine buffer, 25°C).

to Glu. It can be understood from the $K_{m(app)}$ value which is lower than the $K_{m(app)}$ values in literature.

Operational stability of the Glu biosensor

In order to test the operational stability of the prepared enzyme electrode, the current changes, obtained after subsequent usage, were plotted against the number of measurements. At the end of the 20 measurements, the biosensor had lost 4.0% of its initial activity. The relative standard deviation, obtained after 20 measurements at a constant Glu concentration of 5.0×10^{-6} M, was found to be 4.4% (Figure 9).

Storage stabilization of the enzyme electrode

Response of the enzyme electrode, prepared under optimum conditions, was measured for a period of 42 days at constant Glu concentration (1.0×10^{-3} M). Results of 13 measurements during this period are plotted in Figure 10. There was no noticeable decrease in the response during the first 3 days. There was a rapid decrease in current values between the third and twenty-fourth days and there was not a significant change after the twenty-fourth day. The electrode showed 25.5% of the initial amperometric response at the end of the 42 days.

Interference effects

A few common substances, found in serum, were investigated with Pt/PPy-PVS film electrode (without immobilizing enzyme) for any interfering effect. The response current for a constant concentration of H_2O_2 (1.9×10^{-4} M) was compared with the current value, obtained in the presence of

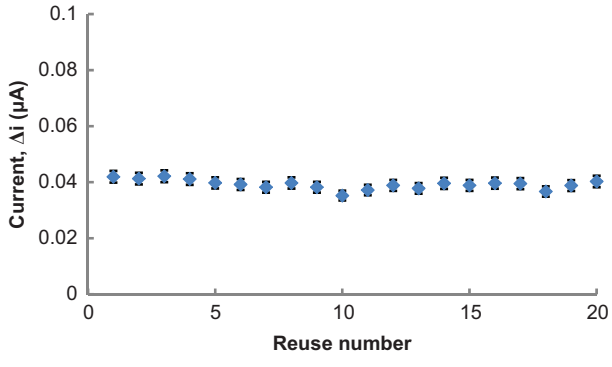

Figure 9. Operational stability of the biosensor in pH 9.0 glycine buffer, at a 0.3-V operating potential, 25°C.

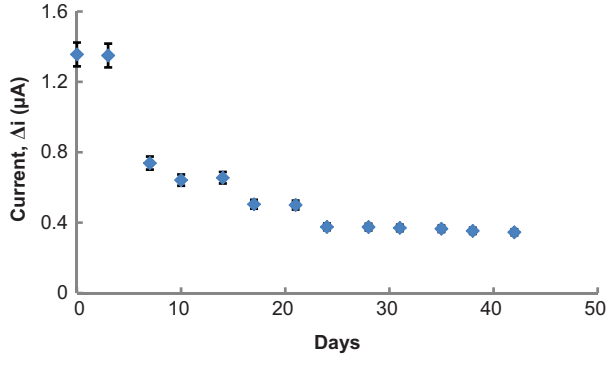

Figure 10. Storage stabilization of the biosensor in pH 9.0 glycine buffer, at a 0.3-V operating potential, 25°C.

the variable concentrations of the interfering species. These interferants and their concentrations were ascorbic acid (1.0×10^{-4} M), uric acid (3.0×10^{-4} M), paracetamol (1.0×10^{-4} M), and fructose (8.0×10^{-5} M). The results indicated that in the presence of ascorbic acid, uric acid, paracetamol, and fructose, the response current was increased by 40.0, 70.0, 10.0, and 3.0%, respectively. These interferences were almost removed by dilution of solution in cell. There was not any interference effect of these compounds with 6-fold dilution. The aim of diluting was to eliminate interference (Perez et al. 2003, Çete et al. 2006).

Glu determination in synthetic blood sample

A synthetic blood sample that contains body concentrations of some interferants was prepared. These interferants (and their concentrations) were ascorbic acid (1.0×10^{-4} M), uric acid (3.0×10^{-4} M), paracetamol (1.0×10^{-4} M), and fructose (8.0×10^{-5} M). Glu concentration was kept as 1.9×10^{-4} M. The synthetic blood sample was added into a cell and diluted 6-fold. By using the calibration curve, Glu amount determined after three experiments was $(1.90 \pm 0.024) \times 10^{-4}$ M.

Conclusions

In this study, we report a new amperometric biosensor for the determination of Glu. L-GlOx was immobilized onto a PPy-PVS film surface by cross-linking with GA and BSA. In this study, prepared Glu biosensor can be used in a wide concentration range. It has a very low detection limit and an acceptable response time for a biosensor. The interference effects of different interferants were not observed after dilution. Therefore, Glu determination can be made in a variety of different samples with our biosensor. It was seen that the Glu biosensor was highly sensitive, selective and its operational stability were found to be very good. Thus, Glu determination can be made with our biosensor for many times. In this study, it is found that Glu biosensor is easy to prepare and is highly cost effective. This composite (PPy-PVS) can supply a biocompatible and electrochemical microenvironment for immobilization of the enzyme, making this material a good candidate for the fabrication of highly sensitive and selective Glu biosensors.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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