

A novel amperometric biosensor based on ZnO nanoparticles-modified carbon paste electrode for determination of glucose in human serum

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Abstract

Zinc oxide nanoparticles-(ZnONPs)modified carbon paste enzyme electrodes (ZnONPsMCPE) were developed for determination of glucose. The determination of glucose was carried out by oxidation of H₂O₂ at +0.4 V. ZnONPsMCPE provided biocompatible microenvironment for GOx and necessary pathway of electron transfer between GOx and electrode. The response of GOx/ZnONPsMCPE was proportional to glucose concentration and detection limit was 9.1×10^{-3} mM. K_m and I_{max} were calculated as 0.124 mM and 2.033 μ A. The developed biosensor exhibits high analytical performance with wide linear range (9.1×10^{-3} –14.5 mM), selectivity and reproducibility. Serum glucose results allow us to ascertain practical utility of GOx/ZnONPsMCPE biosensor.

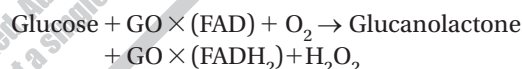
Keywords: amperometric biosensor, carbon paste electrode, glucose, human serum, ZnO nanoparticles

Introduction

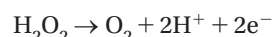
The measurement of glucose is very important in many fields such as biological analysis, environment, food industries, and clinical biochemistry especially for diagnosis of Diabetes Mellitus (DM) (Mizutani and Yabuki 1997, Wang 1999, Yoshimura and Hozumi 1996). DM, chronic and persistent carbohydrate, protein, and lipid metabolism disorder characterized with hyperglycemia may occur in childhood or in adults. The disease is associated with long-term damage, dysfunction, and failure of several organs especially eyes, kidneys, nerves, brain, and heart (Ramachandran et al. 2008). Thus, early diagnosis of diabetes can provide better management of the disease. For this reason, blood glucose levels should be determined by using very sensitive and easier methods. Many methods have been developed for determination of glucose such as chemiluminescence (Zhu et al. 2002), colorimetric (Wang et al. 2009a), electrochemical (Ye et al. 2004, Zhao et al. 2007, Comba et al. 2010), and other methods (Shafer-Peltier et al. 2002). In all these

methods, electrochemical biosensors especially enzyme-based amperometric glucose biosensors are more commonly investigated due to their simplicity, high accuracy, fast response, storage stability, miniaturization, and low cost (Wang 2007, Sadik et al. 2009).

In amperometric glucose biosensors, glucose oxidase (GOx) catalyzes the direct electron transfer from 2 glucose to oxygen to produce glucan lactone and hydrogen peroxide. An enzyme electrode for glucose determination is based on the detection of enzymatically generated hydrogen peroxide during the enzymatic reaction (Wang et al. 2007, Deyhimi and Salamat-Ahangari 2003).



The glucose can be determined by monitoring the oxidation current of liberated hydrogen peroxide at constant potential (Moreno-Bondi et al. 1990).



The oxidation of hydrogen peroxide can be achieved at high potentials. These high potentials can cause the oxidation of other electroactive compounds such as uric acid and ascorbic acid present in real samples. For this reason, redox mediators have been used in order to decrease the overpotential. In addition to different electron transfer mediators (Erden et al. 2012), nanostructured materials including carbon nanotubes (Rakhi et al. 2009), polymers (Sirkar et al. 2000), electrospun nanofibers (Manesh et al. 2008), silanes (Luong et al. 2004) and metals such as gold (Liu and Ju 2003, Anik et al. 2012), platinum (Zhou et al. 2005), nickel (Salimi et al. 2007), and copper (Zhao et al. 2006) have been used to construct biosensors. Besides metal nanostructures, metal oxides such as iron oxide (Kaushik et al. 2008), NiFe₂O₄ (Luo et al. 2010), zirconium oxide (Zhao et al. 2005), and tin oxide-chitosan nanobiocomposite (Ansari et al. 2009) have been widely used to modify electrodes and facilitate the electron transfer between the electrode surface and enzyme's active

sites (Zhang et al. 2004) in the last few years. They could keep the enzyme activity due to the covetable microenvironment (Wang et al. 2009b). These nanostructures have been widely used in optics, optoelectronics, electrics, and biosensors owing to its unique properties such as high specific surface area and nontoxicity (Wang 2005, Pumera et al. 2007).

Over the last decade, ZnONPs have been applied in oxidase-based amperometric biosensors due to excellent properties such as biocompatibility, chemical stability, and high electrocatalytic activity (Tian et al. 2002, Zang et al. 2007, Sun et al. 2008). In addition, ZnONPs provide friendly microenvironment for physical adsorption with high isoelectric point (pI 9.5) especially for acidic protein molecules such as glucose oxidase with low pI (4.2–4.5) (Saha et al. 2009).

Up to now, many studies on different ZnO nanostructures for determination of hydrogen peroxide (Gu et al. 2009), cholesterol (Umar et al. 2009), phenolic compounds (Zhao et al. 2009), hemoglobin (Ma and Tian 2010), and glucose (Ren et al. 2009, Xiao et al. 2009) have been reported. Recently, several glucose biosensor based on ZnO nanorods (Liu et al. 2009), Co-doped ZnO nanoclusters (Zhao et al. 2007), tetragonal pyramid-shaped porous ZnO (Dai et al. 2009), ZnO nanotubes (Kong et al. 2009), ZnO nanoflake (Fulati et al. 2010), ZnO nanowires (Usman Ali et al. 2010), ZnO hollow nanospheres (Meng et al. 2011), and ZnO nanoparticles (Ren et al. 2009) have been developed.

Carbon paste electrode (CPE), a mixture of graphite powder and linker (e.g paraffin/mineral oil), has been used as a matrice since 1958 by Adams for various analytical applications. Due to its wide anodic potential range, chemical stability, ease of modification, and low cost, preparation of modified carbon paste electrodes with new materials is becoming popular in electrochemical researches (Gilmarin and Hart 1995, Švancara et al. 2009, Miscoria et al. 2005, Koyuncu et al. 2007).

In this study, a simple and cost effective glucose biosensor was developed at low potential to eliminate effects of interferences. The parameters affecting the sensitivity of enzyme electrode were studied. Glucose analysis in serum samples was also performed using developed enzyme electrode. The utility of ZnONPs with carbon paste electrodes for amperometric detection was performed for the first time. In this method, the electrocatalytic properties of ZnONPs towards the oxidation of H_2O_2 via cyclic voltammetry technique were examined.

Experimental

Materials

Glucose oxidase (GOx) (from *Aspergillus niger*, EC 1.1.3.4 39680 Units/g solid) and zinc oxide (ZnO) nanoparticles (< 100 nm) were from Sigma. Uric acid (UA), ascorbic acid, creatinine, urea, glucose, glutaraldehyde, and bovine serum albumine were also purchased from Sigma. Graphite powder and parafin oil were obtained from Fluka. Hydrogen peroxide (35%, v/v aqueous solution), di-sodium monohydrogen phosphate heptahydrate, and sodium dihydrogen phosphate dihydrate were acquired from Riedel de Haën. Ultrapure water from Purelab Elga system was used for preparing all solutions. Glucose stock solutions were allowed

to mutarotate at room temperature overnight before use. Measurements were carried out in a phosphate buffer (0.1 M, pH 7.0) supporting electrolyte. All other chemicals were of analytical grade.

Apparatus and measurements

All electrochemical studies were performed on IVIUMSTAT electrochemical analyzer (Ivium Technologies, Netherlands) using a three-electrode cell stand (Bioanalytical Systems, Inc., USA). Bare and modified carbon paste electrodes were used as working electrodes. The counter electrode and reference electrode were a platinum wire (BAS MW 1034) and Ag/AgCl, 3 M KCl (BAS MF 2052), respectively. All potential values given below refer to Ag/AgCl. The pH values of the buffer solutions were measured by using ORION Model 1906 D. 720A pH/ion meter (Thermo Scientific USA). Temperature control was achieved with Grant LTD GG thermostat (Grant Instruments, UK). Amperometric measurements were carried out in a phosphate buffer (0.1 M pH 7.0, 5 ml) under stirred condition. The response current was obtained with the difference between the steady-state current and background current. All measurements were carried out at room temperature ($23 \pm 2^\circ\text{C}$).

Preparation of carbon paste electrodes

For bare carbon paste electrode (BCPE) construction, carbon paste was prepared by mechanically hand mixing graphite powder (70.0% w/w) and paraffin oil (30.0% w/w) until homogeneous paste was obtained. ZnONPs modified CPEs were prepared by mixing ZnONPs with graphite powder in a ratio of 3:1 (w/w), followed by the incorporation of paraffin oil, and mixed regularly. In the case of the enzyme electrode, enzyme solution containing various units of GOx, 10 μL of glutaraldehyde (1.25% (v/v)) and 1.5 mg BSA was added into graphite powder-ZnONPs mixture. Finally, carbon paste was obtained by mixing with 15 μL paraffin oil for 20 min. A portion of the given pastes was placed firmly into a cavity of polyetheretherketone (PEEK) rod electrode bodies (with 3 mm internal diameter) and the surfaces of obtained electrodes were smoothed on a weighing paper before starting new experiment. Amperometric determination of glucose was performed by the oxidation of enzymatically produced hydrogen peroxide at +0.4 V versus Ag/AgCl reference electrode. To find the optimum composition, calibration graphs were plotted for each electrode and slopes of the calibration graphs (sensitivity) were compared. The obtained electrodes were stored in the refrigerator at +4°C when not in use.

Results and discussion

Electrochemical characterization of bare and ZnONPs-modified electrodes

The electrocatalytic activities of bare and ZnONPs-modified carbon paste electrodes (ZnONPsMCPE) for hydrogen peroxide oxidation were studied in the presence and absence of 10 mM hydrogen peroxide by cyclic voltammetry. The determination of H_2O_2 , a product of oxidase-based enzymatic reactions, is very important due to its easy oxidation or reduction. Cyclic voltammograms of BCPE and ZnONPsMCPEs were shown in Figure 1 in the absence H_2O_2 (a, c) and in

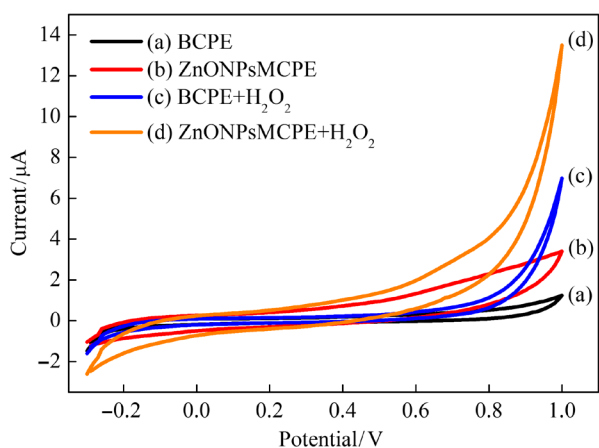


Figure 1. Cyclic voltammograms of bare and ZnONPs modified carbon paste electrodes in 0.1 M pH 7.0 PBS in absence (a, c) and presence (b, d) of 1.0 mM H_2O_2 at scan rate of 50 mV s^{-1} .

the presence H_2O_2 (b, d) in the potential range between -0.3 V to $+1.0 \text{ V}$ at scan rate 50 mV s^{-1} . As can be seen from CVs, the current response increment is observed due to hydrogen peroxide oxidation at ZnONPsMCPE. In addition, the ZnONPsMCPE show significant oxidation of hydrogen peroxide starting around $+0.4 \text{ V}$ (vs. Ag/AgCl). In contrast, the oxidation of H_2O_2 at bare carbon paste electrode begins after $+0.7 \text{ V}$. The ZnONPsMCPE provide an important decrease in working potential for determination of H_2O_2 . Thus, the determination of glucose in serum can be achieved without any interference effects at low potential.

Optimization of the biosensor

The sensitivities of the enzyme electrodes were determined at different GOx concentrations (4–12 U). The enzyme loading dependency on biosensor sensitivity was investigated by measuring the different GOx concentrations (4–12 U) loaded biosensors responses to sequential glucose increments from 0.02 to 0.20 mM. Other parameters were kept constant at $+0.4 \text{ V}$. As expected from the increased biocatalytic activity of the electrode, sensitivity was enhanced with the increasing of the amount of enzyme in the paste. Although the maximum sensitivity was obtained at 12 U glucose oxidase loading, 10 U was selected due to negligible increment (Figure 2).

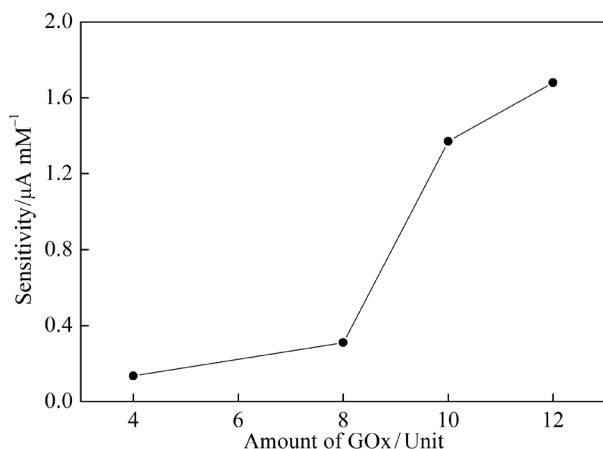


Figure 2. Effect of GOx unit on sensitivity of ZnONPs modified carbon paste enzyme electrode (0.1 M pH 7.0 PBS at $+0.4 \text{ V}$ vs. Ag/AgCl).

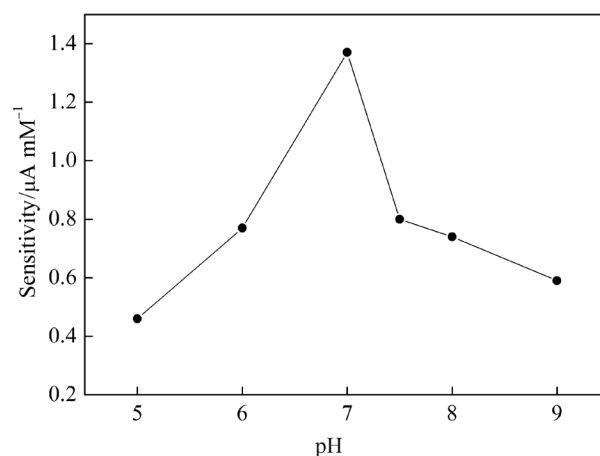


Figure 3. Effect of pH value on sensitivity of ZnONPs modified carbon paste enzyme electrode (0.1 M pH 7.0 PBS at $+0.4 \text{ V}$ vs. Ag/AgCl).

The glucose enzyme electrode sensitivity variation due to pH was obtained by measuring the electrode responses to sequential glucose increments from 0.02 to 0.20 mM at the desired pH in a stirred buffer (6.0–9.0). The maximum sensitivity was obtained at pH 7.0 (Figure 3). The same pH result was also found in related studies (Tian et al. 2002, Saha et al. 2009, Dai et al. 2009). Different pH values for glucose biosensors were also reported (Usman Ali et al. 2010, Wei et al. 2010, Lei et al. 2011). This was attributed to the amount of ZnONPs, enzyme loading, and electrode preparation procedures.

Temperature dependence of glucose enzyme electrode sensitivity was obtained by measuring the electrode responses to sequential glucose increments from 0.02 to 0.20 mM maintained at the desired temperature (10 – 50°C). As illustrated in Figure 4, the sensitivity of the biosensor increased with increasing temperature until 25°C , and then declined slowly. The increment in the response of sensors could be affected by temperature increases due to enhanced enzymatic activity, increased membrane permeability of various participating species, and decrease in dissolved O_2 concentration (Vaddiraju et al. 2011). The decline in the response of sensors could be due to denaturation of GOx. Further experiments were performed at 25°C .

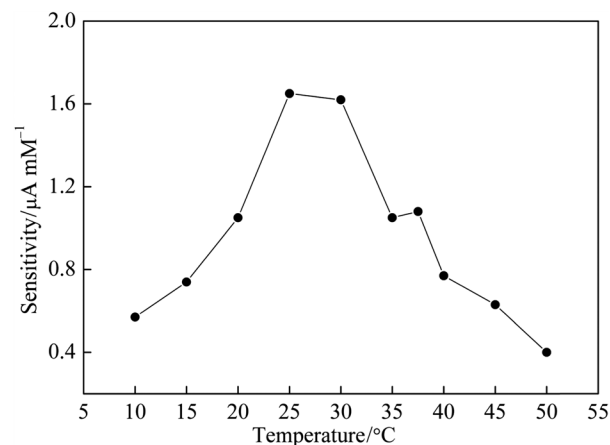


Figure 4. Effect of temperature on the sensitivity of ZnONPs modified carbon paste enzyme electrode (0.1 M pH 7.0 PBS at $+0.4 \text{ V}$ vs. Ag/AgCl).

The effect of glucose concentration

In order to determine the linear working range of the glucose biosensor, the amperometric response recorded as a function of glucose concentration. As presented in Figure 5 (inset A and B) two linear working ranges were obtained. The inset in Figure 5A shows a good linear response in the range of 9.1×10^{-3} mM–0.16 mM glucose with a correlation coefficient of 0.9800. The regression equation is $I(\mu\text{A}) = 1.73C_{\text{glucose}} + 1.29$. Also good linear response in the range of 1.0 mM–14.5 mM glucose with a correlation coefficient of 0.9853 was obtained (Figure 5B). The regression equation is $I(\mu\text{A}) = 0.026C_{\text{glucose}} + 1.72$. Relatively low detection limit (9.1×10^{-3} mM) was obtained. The linear range covers the normal serum glucose level (4.4–6.6 mM) (Wang 2007).

$$\frac{[S]}{I} = \left(\frac{1}{I_{\max}} \right) [S] + \frac{K_m}{I_{\max}}$$

K_m gives an indication of the enzyme–substrate kinetics for the glucose biosensor. Kinetic parameters, such as K_m and $I_{\max}$, are usually calculated from the well-known Lineweaver–Burk plot by plotting “1/current response vs. 1/substrate concentration”. K_m and $I_{\max}$ for developed biosensor were determined using Hanes–Wolf plot due to not linearity of Lineweaver–Burk plot. Kinetic parameters were obtained from the Hanes–Wolf plot by plotting “substrate concentration/current response vs. substrate concentration” (Figure 6). K_m and $I_{\max}$ were calculated as 0.124 mM and 2.033 μA , respectively, using following equation (Garrett and Grisham 2009).

The K_m in the present work is lower than GOx immobilized on Co-doped ZnO nanoclusters (21 mM) (Zhao et al. 2007), ZnO nanotubes (19 mM) (Kong et al. 2009), and ZnO nanorod via citric acid-assisted annealing route (1.95 mM) (Yang et al. 2010).

Repeatability, reproducibility, and response time

The sensitivities of same glucose enzyme electrode were obtained five times consecutively at +0.4 V (vs. Ag/AgCl) to evaluate of the repeatability of the enzyme electrode.

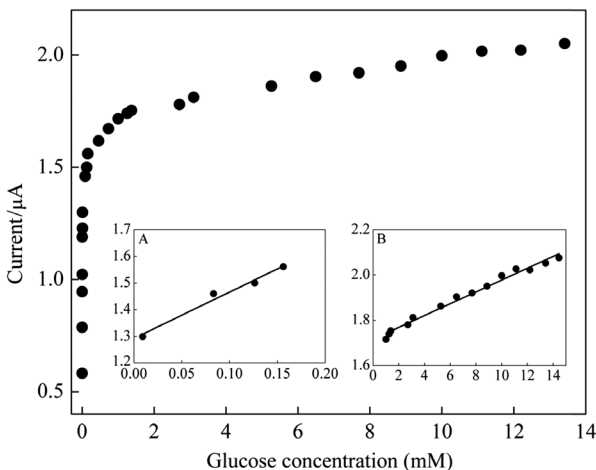


Figure 5. Effect of glucose concentration on amperometric response of ZnONPs modified carbon paste enzyme electrode, inset: linear working ranges plots (0.1 M pH 7.0 PBS at +0.4 V vs. Ag/AgCl).

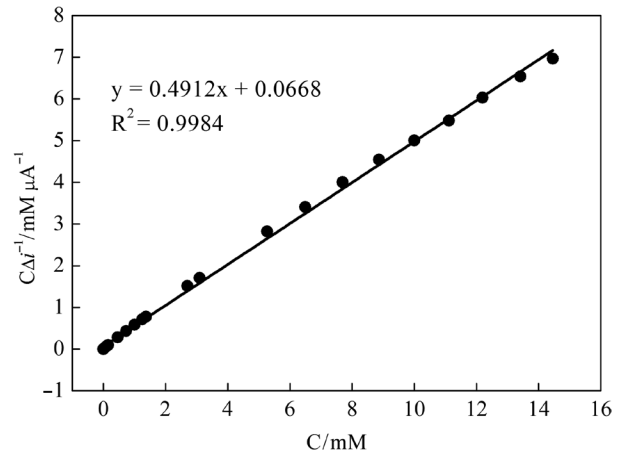


Figure 6. Substrate concentration/current response vs. substrate concentration plot (Hanes–Wolf plot).

The relative standard deviation of the obtained sensitivities was found as 3.307%. This result was the indication of good stability. The reproducibility of enzyme electrode was also investigated by measuring the biosensor responses to sequential glucose increments from 0.02 to 0.20 mM in the same day. Four different enzyme electrodes, constructed independently, had a relative standard deviation of 4.273% (Table I). These results indicate that the repeatability and reproducibility of the enzyme electrode were highly satisfactory and obtained electrode can be used in many analyses. The response time is important for the biosensor applications. The response time of ZnONPs modified carbon paste enzyme electrode which defines as the time it takes for the electrode to reach 95% of the steady-state current. The response time was found as 20 s.

Storage stability

The storage stability of the enzyme electrode was investigated by measuring the biosensor responses to sequential glucose increments from 0.02 to 0.20 mM in a period of 150 days (Figure 7). The optimized biosensor was retained about 85%, 60%, 45%, 40% of its response after 1 week, 3 weeks, 3 months, and 5 months, respectively. Good long-term stability can be attributed to the mild immobilization procedure and a beneficial environment for preventing enzyme leakage. Immobilization of GOx into carbon paste provides a biocompatible microenvironment around the enzyme, maintaining its biological activity.

Table I. Reusability and reproducibility for ZnONPs modified carbon paste enzyme electrode.

Sensitivities ($\mu\text{A}/\text{mM}$)	
For reusability*	For reproducibility*
1.402	1.308
1.421	1.441
1.339	1.393
1.368	1.426
1.306	—
%RSD = 3.307	%RSD = 4.273

*The results have been given sensitivities of five measurements for reusability and four different electrodes for reproducibility. Relative standard deviations were calculated for both of them.

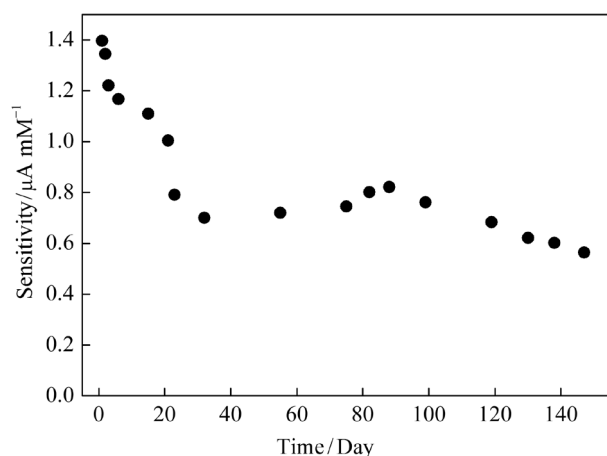


Figure 7. Storage stability of ZnONPs modified carbon paste enzyme electrode stored at 4°C (The applied potential +0.4 V vs. Ag/AgCl).

Effects of interferences

The real sample contains other common species that could potentially interfere with the glucose detection. In the light of low concentrations of these ascorbic acid, creatinine, urea, uric acid in the blood, the interference needs to be seriously considered because they could be easily oxidized at a relatively high over potential, and a lower working potential helps to avoid the electrochemical reaction of the interferences.

The selectivity of developed biosensor was evaluated in the presence of common interfering species such as ascorbic acid (0.1 mM), creatinine (0.1 mM), urea (0.8 mM), and uric acid (0.05 mM) in oxygenated PBS. The percentages of interference were determined in terms of the difference in percentage of response between the buffer solution containing glucose and buffer solution containing interferences and glucose. The biosensor shows quick response for each addition of 0.1 mM glucose. However, as can be seen from Table II no noteworthy response was observed for the addition of creatinine, urea, and uric acid into buffer solution containing 0.1 mM glucose at +0.4 V (vs. Ag/AgCl). Although the ascorbic acid caused a small increase in the current, these results can be overseen for the determination of glucose in serum samples due to maximum concentration of L-ascorbic acid in human body is about 0.1 mM (Xiao et al. 2000). The results of interference study were shown in Table II. Thus, the developed biosensor is highly selective for glucose and can be applied for glucose determination in human serum samples under physiological conditions.

Table II. Effects of interferences on glucose response.

Interfering species	Concentration ^a (mM)	Interference ^b (%)
L-Ascorbic acid	0.1	8.94
Creatinine	0.1	4.05
Urea	0.8	4.83
Uric acid	0.05	5.69

^aThe electrochemical cell concentration of interfering species.

^b% interference = $(I_x - I_0)/I_0 \times 100$; I_0 : The amperometric response of buffer solution containing 0.1 mM glucose solution, I_x : The amperometric response of buffer solution containing 0.1 mM glucose and interfering species with above concentration.

Table III. The determination of glucose levels in human serum samples.

Serum samples	Glucose concentration (mg/dL)	
	Determined by proposed biosensor*	Provided by the hospital
1	87.8 ± 0.6	88
2	104.7 ± 3.0	101
3	386.4 ± 7.5	388

*The results have been given average of three measurements.

Determination of glucose in serum

Human serum samples were assayed in order to show the practical utility of this glucose biosensor. Serum samples were provided by a local hospital, which were firstly analyzed with Beckmann Coulter Olympus AU2700 analyzer which is based on hexokinase method. Then serum samples of 10 μL were successively added into 5 mL phosphate buffered (0.1 M pH 7.0), and the response of the developed biosensor was obtained at 0.4 V. The accuracy of the developed biosensor was checked by t-test. The t value was found as 0.408 at 95% confidence level, for t_{critic} 4.30. As shown in Table III, good agreement between the two methods results allows us to ascertain the practical utility of the developed biosensor at a confidence level of 95%.

The developed biosensor have demonstrated better performance in terms of working range, detection limit and K_m compared to other studies based on various synthesized ZnO nanostructures. ZnO nanostructures created a biocompatible environment for GOx, the obtained biosensor has higher storage stability compare to other modified electrodes. Moreover, real sample application for the determination of glucose couldn't find in most of developed biosensors (Saha et al. 2009, Ren et al. 2009, Kong et al. 2009, Yang et al. 2010).

Conclusion

The development of highly sensitive electrochemical glucose oxidase biosensor for the determination of glucose was performed by immobilization of GOx within a ZnONPsMCPE. The ZnONPs modified carbon paste provided a biocompatible microenvironment for GOx and a necessary pathway of electron transfer between GOx and the electrode. ZnO nanoparticles tend to act like nanoscale electrodes. The response of the GOx biosensor was proportional to glucose concentration and the detection limit was 9.1×10^{-3} mM. Kinetic parameters, K_m and I_{max} , were calculated as 0.124 mM and 2.033 μA, respectively. The developed biosensor exhibits high analytical performance with a wide linear range (from 9.1×10^{-3} to 14.5 mM), high sensitivity, selectivity, reproducibility, reusability, and storage stability. The serum glucose results allow us to ascertain the practical utility of the ZnONPs-modified GOx biosensor at a confidence level of 95%.

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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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