Electrochemical glucose biosensor based on biosynthesized silver nanoparticles and polyvinylpyrolidone modified graphite electrode

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Abstract

In the present work, we have biosynthesized silver nanoparticles (AgNPs) using leaf extract of *Euphorbia geniculata* and successfully deposited them onto the polyvinyl pyrolidone (PVP) modified graphite electrode (Gr/PVP). The resulting electrode is used as a matrix for the immobilization of glucose oxidase (GOx) enzyme. The immobilized electrode (Gr/PVP/AgNPs/GOx) is characterized by scanning electron microscopy and its performance is evaluated and optimized using cyclic voltammetry and differential pulsevoltammetrictechniques. Under neutral pH conditions, at room temperature, the developed Gr/PVP/AgNPs/GOx sensor showed excellent electrocatalytic activity towards the oxidation of glucose. Further, it is used for the determination of glucose in the concentration range of 0.1-7 mM with a detection limit of 0.15 μM and sensitivity of 29.72 μA mM^{-1} cm^{-2}. In addition, the response of GOx biosensor is found to be uninfluenced by some common possible interferents. The findings of present work are significant and imply potential applications for biosynthesized AgNPs as effective, non-toxic biocompatible sensor fabrication materials.

Keywords: Biosynthesized silver nanoparticles, biosensor, glucose oxidase.

Introduction

Diabetes is an extreme medical condition caused by high level of glucose in blood and a high performance glucose sensor is a vital clinical requirement for its efficient detection and monitoring [1]. Over the years, many approaches based on surface plasmon resonance [2], electroluminescence [3], colorimetry [4], flow injection with spectrophotometry [5], chemiluminescence [6] and electrochemical [7, 8] techniques have been implemented to develop reliable method for the detection of glucose. Among these methods, enzyme based electrochemical method has been widely studied because of its simplicity, relative low cost, high selectivity, efficiency, intrinsic sensitivity, rapidity and high specificity towards the biological recognition element [9,10].

Amperometric electrochemical glucose biosensor based on the use of glucose oxidase (GOx) enzyme is one of the most popular biosensor which has been extensively investigated [11]. Due to high specificity of GOx enzyme, the electrochemical glucose biosensor can perform with high sensitivity and selectivity [12]. Glucose oxidase contains two flavin adenine dinucleotide (FAD) cofactors and catalyzes the oxidation of glucose in presence of oxygen which results in generation of H_2O_2.

$$GOx(FAD) + Glucose \rightarrow GOx(FADH_2) + Gluconolactone$$
$$GOx(FADH_2) + O_2 \rightarrow GOx(FAD) + H_2O_2$$

The amount of glucose oxidized is quantitatively proportional to the generated H_2O_2, which can be readily determined by measuring oxidation current generated during the electrochemical reaction [13, 14]. However, the active site of GOx, FAD which is the key factor for the electron transfer in GOx is deeply embedded with a protective protein shell [15, 16]. Thus, in order to promote efficient electron transfer between active sites of GOx and electrode surface, various nanomaterials mainly metallic nanoparticles such as gold [17,18], silver [19], platinum [20] etc., are being used. The major advantage of using nanomaterials in sensors is attributed to their high selectivity, high sensitivity, fast detection and low cost apart from their excellent optical, electrical and catalytic properties [21-24].

Among the metallic nanoparticles, silver nanoparticles (AgNPs) exhibit many excellent properties such as large surface to volume ratio, very high electrical conductivity, good adsorption ability, small particle size and high efficiency in catalysis of redox reaction of some analytes [25]. The major advantage of using AgNPs is that they enhance electron transfer between redox proteins and electrode surface [26, 27], and also facilitate the easier immobilization of biomolecules [28].

Most of the conventional methods employed in synthesis of AgNPs are either expensive or use toxic

chemicals that have adverse effects on the environment. In recent years, green synthesis is gaining importance as it is simple, cost effective and does not require high pressure, temperature and toxic chemicals. The biosynthesis produce AgNPs which are cost effective and biocompatible at low concentrations which find many medical applications [29]. However, the application of biosynthesized AgNPs in biosensor development has not been explored till date and it forms the core objective of the present work.

For the present work, AgNPs are biosynthesized using leaf extract of plant *Euphorbia geniculata* and GOx biosensor is developed by co-immobilizing AgNPs with GOx enzyme onto PVP modified electrode. The performance of developed GOx sensor is evaluated.

Experimental

Chemicals and equipments

All chemicals used in our experiments have been purchased from Sigma Aldrich and Fischer Scientific. Glucose oxidase type X-S from *Aspergillusniger* lyophilized powder 50 kU is procured from Sigma Aldrich. For UV-visible spectral studies, Shimadzu – 1800 UV-visible spectrometer is used. The morphological aspects of sensor surface are investigated using ZEISS EV040EP (Germany) scanning electron microscope (SEM). Cyclic voltammetry (CV), Differential pulse voltammetry (DPV) are performed using Biologic Science Instrument SP-150. Electrochemical impedance spectroscopy (EIS) are performed using Versastat 3 (Princeton Applied Research, USA). The analysis of EIS spectral data is carried out using simulation software, Zswimp win 3.2.

Fabrication and characterization of GOx biosensor

A graphite electrode is prepared by inserting a cylindrical graphite rod (Gr) of 6 mm diameter into a teflon holder of the same internal diameter and the electrical contact is established with a copper wire running through the center of teflon bar. The Gr electrode surface is polished using PK-3 electrode polishing kit (0.05 µm aqueous polishing alumina and 1 µm polishing diamond) until a mirror shining surface is obtained. The polished electrode surface is sonicated several times to remove loosely bound particles and later rinsed with milli-Q water and dried with nitrogen gas. PVP (2 mg/ml) is dissolved in deionized water and then ultra sonicated for 10-15 minutes for the uniform dispersion of PVP. 5 µl of above PVP solution is drop casted onto graphite electrode and it is dried at room temperature to get modified Gr/PVP electrode. Further, a suspension of biosynthesized AgNPs in deionized water (w/v ratio of 10 mg of AgNPs in 1 ml) is prepared and 10 µl of the suspension is directly drop cast over the Gr/PVP electrode surface and dried at room temperature to get Gr/PVP/AgNPs electrode matrix. Finally, 5 mg/ml of GOx enzyme solution is prepared in buffer solution of pH 4.0 and 5 µl of the same solution is drop casted onto Gr/PVP/AgNPs electrode matrix to get modified Gr/PVP/AgNPs/GOx electrode. The fabricated electrode is dried in a refrigerator for 30 mins and later washed in deionized water to remove loosely bound enzyme particles from electrode surface. The fabricated Gr/PVP/AgNPs/ GOx sensor is stored at 4^0C until it is used for further studies. The developed Gr/PVP/AgNPs/GOx is subject to physical characterization using SEM. The electrochemical characterization of developed sensor was performed using CV and EIS techniques.

Electrochemical studies

All electrochemical experiments are performed using a three electrode electrochemical cell with modified Gr as working electrode. Saturated calomel electrode (SCE) and platinum wire are respectively used as reference and counter electrodes. The electrochemical characterization of bare and modified graphite electrode is performed in phosphate buffer (PBS) of pH 7.0 using 5 mM $[Fe(CN)_6]^{-3/-4}$as electrochemical probe. The charge transfer process at electrode/electrolyte interface of modified electrode is studied using EIS technique in the frequency range of 100 kHz to 0.1 Hz.

Results and discussion

Biosynthesis of AgNPs

For the synthesis of AgNPs, aqueous solution of $AgNO_3$ is mixed with leaf extract of *Euphorbia geniculata* and incubated at room temperature. The reaction mixture is initially pale yellow which gradually turned to dark brown. The visible color change from pale yellow to dark brown suggested the formation of AgNPs through reduction of Ag^+ ions to Ag^0 in presence of leaf extract. The pure $AgNO_3$ solution and leaf extract did not show any visible color changes during the same incubation period under similar conditions.

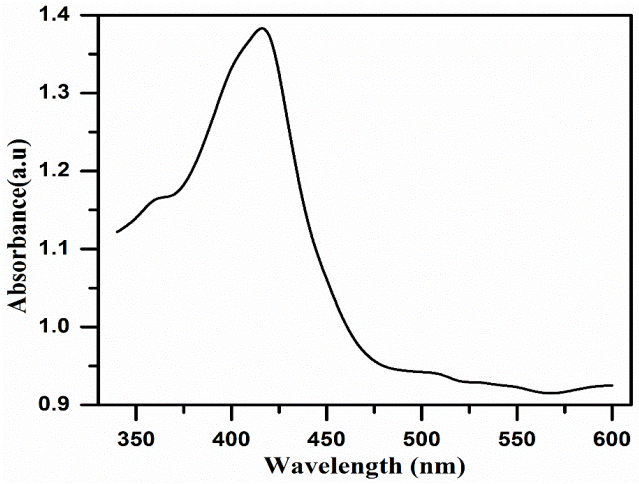

Fig..1. UV-visible spectra of biosynthesized silver nanoparticles.

UV-visible spectroscopy is used as a tool to ascertain the formation of AgNPs. The UV-visible spectral measurements of reaction mixture at regular intervals during incubation period showed a gradual increase in

absorbance. The absorbance reached a maximum and almost stabilized after 8 hours of incubation indicating the completion of reduction of silver ions to AgNPs. Further, a strong, broad peak in the UV-visible spectrum between 415 nm and 420 nm clearly confirmed the formation of AgNPs **(Fig.1)**. This peak may be attributed to surface plasmon resonance in AgNPs [30]. UV-visible study clearly confirms completion of reduction of Ag^+ and formation of AgNPs in nearly 8 hours.

Fabrication and characterization of GOx biosensor

GOx biosensor is fabricated through modification of Gr electrode surface in successive steps by drop casting PVP, AgNPs and GOx enzyme. During each modification step, SEM is used as an effective tool to analyze the surface morphology of modified electrodes. **Fig. 2** shows the SEM images of bare graphite (a), modified Gr/PVP (b), Gr/PVP/AgNPs (c) and Gr/PVP/AgNPs/GOx (d) electrodes. The branched leaf structures apparent in **Fig. 2 (b)** indicate deposition of polymer PVP onto bare Gr electrode while the powdery deposit in **Fig. 2 (c)** confirms the immobilization of AgNPs onto the Gr/PVP electrode. **Fig. 2 (d)** shows the globular morphology on the Gr/PVP/AgNPs/GOx electrode with bright spots revealing the successful immobilization of enzyme.

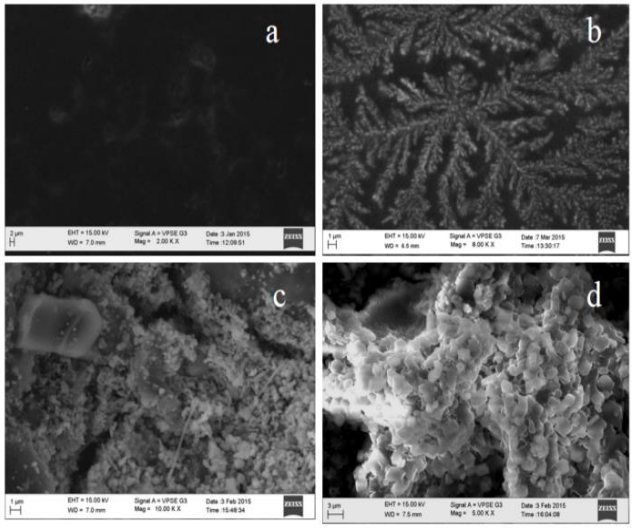

Fig.2. SEM image of a) Bare Gr, b) Gr/PVP, c) Gr/PVP/AgNPs and d) Gr/PVP/AgNPs/GOx electrode surface.

The redox behavior of bare and modified Gr electrode is studied using 5 mM $Fe(CN)_6^{-3/-4}$ as electrochemical probe in PBS (pH 7.0) and compared after each modification step **(Fig. 3 A)**. The CV of Gr electrode (curve a) in 5 mM $Fe(CN)_6^{-3/-4}$ showed a pair of well-defined quasi reversible redox peaks with a peak separation (ΔE_p) of 174 mV. The CV of Gr/PVP curve (b) showed an increase in both oxidation and reduction current which is possibly due to good conducting properties of the polymer PVP. Further, when the electrode is modified with AgNPs (Gr/PVP/AgNPs) it showed an decreased ΔE_p value (155 mV) and an enhanced oxidation and reduction current (curve c). Following the immobilization of GOx enzyme onto electrode surface, the current at the Gr/PVP/AgNPs/GOx electrode decreased and the peak separation increased to 163 mV indicating that the GOx enzyme act as a weak barrier towards $Fe(CN)_6^{-3/-4}$ ion penetration (curve d). The reduced penetration of the $Fe(CN)_6^{-3/-4}$ ion may be due to the electrostatic repulsion experienced by negatively charged probe from the negatively charged or electron rich sites on GOx enzyme surface. This however confirms the successful immobilization of GOx onto the biocompatible AgNPs.

The charge transfer process occurring at electrode/electrolyte interface of modified electrode is studied by carrying out EIS measurements in a frequency range of 100 KHz to 0.1 Hz with a amplitude of 5 mV. The Nyquist plot of experimental data from EIS studies and its best fitting Randles equivalent circuit is shown in **Fig. 3 B** (Inset). Randels equivalent circuit explains the electrochemical nature of electrode solution interface. Various parameters such as charge transfer resistance (R_{ct}), solution resistance (R_s), number of electrons transferred (n) and Faradaic capacitance (C_f) calculated by fitting Randles equivalent circuit to experimental data are shown in **Table 1**. The results of EIS studies indicate that the charge transfer resistance (R_{ct}) of Gr electrode is much higher compared to Gr/PVP electrode. The reduced R_{ct} value of Gr/PVP may be attributed to increased surface area of electrode. The modified Gr/PVP/AgNPs showed a further decrease in R_{ct} value indicating that enhanced rate of electron transfer is due to incorporation of AgNPs to electrode surface. However, further modification of the electrode through incorporation of GOx enzyme resulted in increased R_{ct} value which is due to insulating property of GOx enzyme. These results are in clear agreement with CV results discussed above.

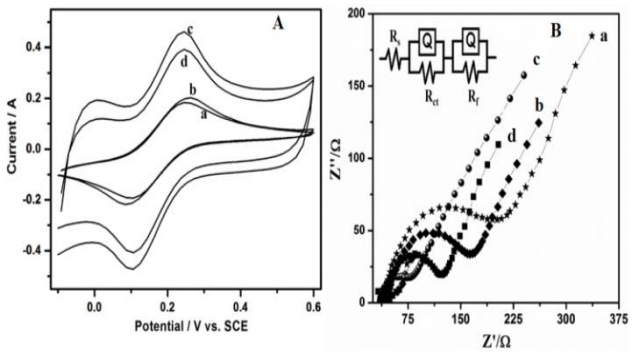

Fig. 3. (A) CVs of 5 mM $Fe(CN)_6^{-3/-4}$ solution obtained at a) Bare Gr, b) Gr/PVP, c) Gr/PVP/AgNPs and d) Gr/PVP/AgNPs/GOx. (B) Impedance spectrum of a) bare graphite (Gr), b) Gr/PVP, c) Gr/PVP/AgNPs and d) Gr/PVP/AgNPs/GOx. Inset is the Randles equivalence circuit used to fit the impedance data for the modified electrodes.

Table.1 EIS data of bare Gr, Gr/PVP, Gr/PVP/AgNPs and Gr/PVP/AgNPs/GOx

Electrode	R_s (Ω)	C_{dl}/F	n	R_{ct} (Ω)	C_f/F
Bare Gr	40.96	0.004724	0.8	995.3	4.941×10^{-5}
Gr/PVP	50.57	0.007303	0.9	147.1	9.783×10^{-5}
Gr/PVP/AgNPs	47.18	0.009082	0.8	69.87	7.187×10^{-5}
Gr/PVP/AgNPs/GOx	43.73	0.0001283	0.8	115.9	0.6645

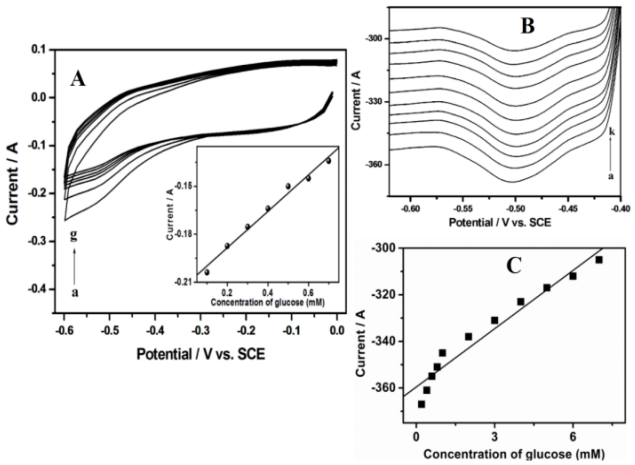

Fig. 4. (A) CVs at Gr/PVP/AgNPs/GOx electrode for different glucose concentrations (0.1 - 0.7 mM). Inset a) shows calibration plot of peak current versus glucose concentration. (B) DPVs of Gr/PVP/AgNPs/GOx electrode at varying glucose concentration (0.1 – 7 mM) in 0.1 M PBS solution. (C) shows calibration plot of peak current versus glucose concentration.

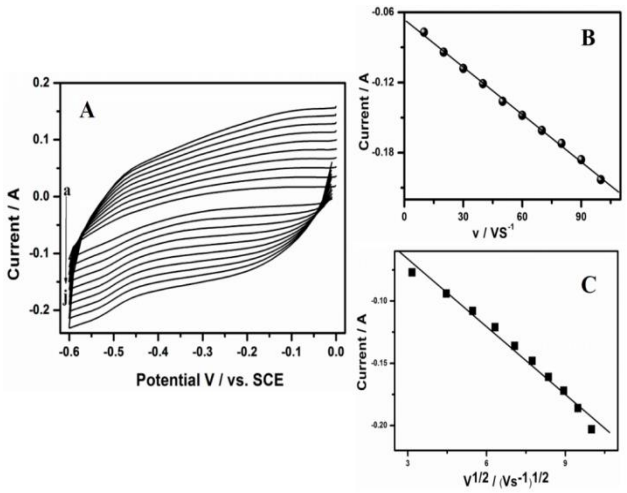

Fig. 5. (A) Cyclic voltammograms of Gr/PVP/AgNPs/GOx electrode at different scan rate (10 – 100 mVs^{-1}) in 0.1 M PBS solution, B) Peak current Vs. Scan rate, C) peak current Vs. Square root of scan rate.

Glucose sensing properties of developed Gr/PVP/AgNPs/ GOx sensor

The electrocatalytic properties of developed GOx biosensor is examined by measuring the cyclic voltametric response for different concentrations of glucose (0.1 – 0.7 mM) in oxygen saturated PBS solution. The CV results in **Fig. 4A** show decrease in reduction peak with increase in addition of glucose to oxygen saturated buffer solution. The oxidation of glucose in presence of GOx occur according to the reaction

$$Glucose + O_2 \rightarrow Gluconolactone + H_2O_2$$

When glucose is added to oxygen saturated PBS solution, the dissolved oxygen functions as an electron acceptor and mediates the oxidation of glucose in presence of GOx enzyme. As a result, there is consumption of oxygen at the electrode surface which leads to decrease in oxygen concentration. Thus, the electrochemical response of dissolved oxygen decreases after the addition of glucose into oxygen saturated PBS solution. The cathodic peak current for the reduction of oxygen decreased linearly with increase in concentration of glucose in the linear range of 0.1 – 0.7 mM **(Inset Fig. 4 A)** with a linear regression equation

$$I_{pc}(A) = -0.211 - 0.113C_{glucose}(mM); R = 0.992$$

The DPV experiments is performed in the potential range of -0.4 to -0.65 V showed a stable and well defined reduction peak for the varying concentration of glucose in air saturated PBS buffer. The voltammagram **(Fig. 4 B)** showed decreased reduction peak current with increase in concentration of glucose. The calibration plot of I_{pc} vs. glucose concentration that the peak current is in linear relationship with glucose concentration in the range of 0.1 to 7 mM (Fig 4 C) with a linear regression equation

$$I_{pc}(A) = -359.54 - 8.334\, C_{glucose}(mM); R = 0.974$$

Effect of scan rate

The effect of scan rate on the voltammetric response of Gr/PVP/AgNPs/GOx electrode in 0.1 M PBS solution at pH 7.0 is investigated in the range of 10-100 mVs^{-1} to determine the kinetics of electrode reaction. **Fig. 5 A** shows that the increase in scan rate from 10 to 100 mVs^{-1} increases the reduction peak current and it also shifts the cathodic peak potential to more negative value. **Fig. 5 B** shows that the cathodic peak current decreases in the linear range from 10–100 mVs^{-1} with a linear regression equation.

$$I_{pc}(A) = -6.626 \times 10^{-2} - 1.35 \times 10^{-3}\, v(Vs^{-1}); R = 0.999$$

The plot in inset **Fig. 5 C** indicate that the cathodic peak current varies proportional to square root of scan rate with a linear regression.

$$I_{pc}(A) = -1.84 \times 10^{-2} - (-1.813 \times 10^{-2}) v^{1/2}\, (vs)^{1/2}; R = -0.991$$

These results demonstrate that oxidation of glucose in presence of GOx is an adsorption controlled reaction.

Effect of pH

The effect of pH on biosensor performance is of great practical importance as the enzyme activity is strongly dependent on pH conditions. The effect of pH on GOx biosensor performance is examined by measuring current response of 2 mM glucose in the pH range of 2 to 10. **Fig. 6 A** shows the cathodic current response to glucose in different pH conditions. The maximum cathodic peak current is observed at pH 5.0. It is previously reported that for the free GOx, when oxygen is used as an electron acceptor, the optimum pH varied between 4.8 to 6.0 [31]. However, the optimum pH for immobilized GOx may slightly vary depending on type of electrode material used and on the immobilization technique employed. For our experiments, we have selected pH 7.0 as most biological samples have pH around this value.

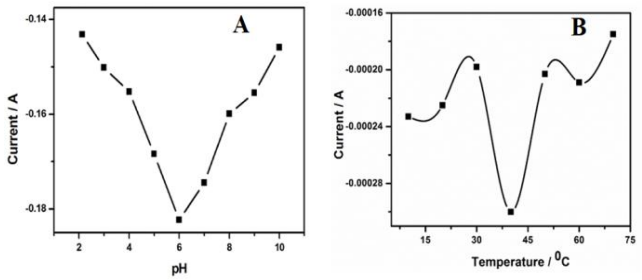

Fig. 6. (A) Effect of pH on Gr/PVP/AgNPs/GOx modified electrode in a varying pH of 2 to 10, (B) Effect of temperature on Gr/PVP/AgNPs/GOx modified electrode in a varying temperature of 10 to 70 ^{0}C.

Effect of temperature

Effect of temperature on the response of developed Gr/PVP/AgNP/GOx electrode, is examined over the temperature range of 10 ^{0}C to 70 ^{0}C in 2 mM glucose. The obtained results are shown in **Fig. 6 B.** The optimum temperature that generated maximum current response is 40 ^{0}C. Above this temperature partial denaturation of enzyme takes place. However, for practical reasons all our experiments are performed at room temperature.

Effect of interferents

The metabolites such as ascorbic acid, uric acid and acetaminophen present in biological samples may often interfere in the estimation of glucose. The effect of these interferents in detection of glucose is examined by measuring reduction current on subsequent addition of uric acid (0.5 mM), ascorbic acid (0.1 mM) and acetaminophen (0.1 mM) as interferents. At -0.5 V addition of 2 mM glucose causes an immediate current response while subsequent addition of interferents did not cause any apparent change in current response as the potential is too low for the oxidation of interferents. This indicates that the developed GOx sensor is highly selective to glucose and it needs no mediator.

Sensitivity and detection limit

The detection limit was determined according to the equation

$$\frac{3\sigma}{S}$$

where σ is the standard deviation of blank and S is the slope of the calibration plot [32]. The detection limit of sensor is found to be 0.15 µM with a sensitivity of 29.72 $\mu AmM^{-1}\ cm^{-2}$. The results of present work are compared with those of previous works is given in **Table 2**. The data indicate that our GOx sensor in the present work has shown higher linearity range and improved detection limit compared to other GOx sensors. On the basis of this study, it may be concluded that the biosynthesized AgNPs can be effectively employed in fabrication of glucose biosensor.

Storage stability of GOx sensor

The storage stability is a critical issue for the application of the developed GOx sensor. Storage stability study is carried out for a period of 4 weeks using CV in the presence of 2 mM glucose in 0.1 M PBS (pH 7.0). The recorded current response of developed electrode is 94% during the first week and later the current response decreased further to 89% and 84% respectively for the third and fourth week. The decrease in current may be attributed to the partial denaturation of the enzyme. The electrode is kept stored at 4 ^{0}C when not in use.

Analysis of real samples

Developed GOx sensor is examined for its practical applicability in blood serum sample analysis. The developed Gr/PVP/AgNPs/GOx electrodes were used for the determination of glucose in blood serum sample. The serum sample is diluted in such a way that the glucose concentration fall in the working range of above mentioned electrodes. The current response from each serum sample is measured. The glucose concentration of serum sample is then determined by interpolation on the linear range of calibration curves of standard glucose sample. The detected concentrations are close to the actual concentration in real samples which indicate that the developed sensor shows good practical applicability with relative standard deviation ranging from 3 to 5.5%.

Table 2. Comparison of proposed glucose biosensor with other GOx immobilized enzyme**.**

Electrode	Response time	Linear range (mM)	Detection limit (µM)	Sensitivity/µM mM^{-1} cm^{-2}	Reference
GOx/AgNP-A/F-Sio_2/GO/GCE	-	2-12	310	-	[33]
Gr/AuNPs/GOx/chitosan	-	2-10	180	-	[34]
Gr/f-MWCNTs/(Rd-GDH)/GOx	>5	0.5–28.5	0.16	15	[35]
Gr/PVP/AgNPs/GOx	3 s	0.1 – 7	0.15	29.72	present work

Conclusions

In the present work, we have synthesized AgNPs using leaf extract of *Euphorbia geniculata* and successfully deposited those onto PVP modified Gr electrode (Gr/PVP). The CV and EIS studies using $Fe(CN)_6^{-3/-4}$ as electrochemical probe showed that the Gr/PVP electrode modified with AgNPs exhibited higher anodic to cathodic peak separation, higher peak currents, lower charge transfer rate than Gr/PVP electrode without modification. This suggests that the biosynthesized AgNPs can be effectively used as electrode fabrication material. Further, Gr/PVP/AgNPs electrode is immobilized with glucose oxidase enzyme and glucose biosensor is developed. The developed Gr/PVP/AgNPs/GOx biosensor showed high electrocatalytic response towards oxygen reduction reaction which can be used for detection of glucose. The

current response is uninfluenced by interferents and varied linearly with glucose concentration suggesting that the GOx sensor developed using biosynthesized AgNPs can possibly find application as a mediator free glucose biosensor in medical applications.

Acknowledgements

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