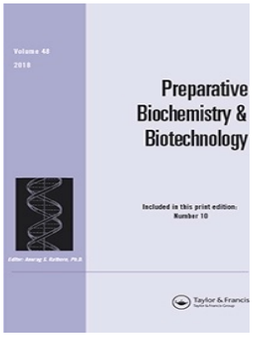

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A novel electrochemical glucose biosensor based on a poly (L-aspartic acid)-modified carbon-paste electrode

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ABSTRACT

A new amperometric biosensor was fabricated by means of electropolymerization of L-aspartic acid on a carbon-paste electrode (CPE) for the bioelectrochemical determination of glucose. The electropolymerization process was conducted via cyclic voltammetry (CV). The modified CPE with poly (L-aspartic acid) (PAA) provided free carboxyl groups so as to immobilize the glucose oxidase (GOx), and further, enhanced the electrocatalytic activity of the hydrogen peroxide (H_2O_2). The biosensor displayed both good stability and good bioactivity. The sensitivity of the prepared biosensor was 5.3 μA cm^{-2} mM^{-1}. Its linear range extended from 0.05 mM to 1.0 mM, with the low limit of detection (LOD) being 69.2 μM. The Michaelis–Menten constant was found to be 1.17 mM. Furthermore, the biosensor showed good anti-interference ability in relation to dopamine, uric acid, and ascorbic acid. Taken together, these results demonstrate that PAA/CPE is a promising material for the fabrication of glucose biosensor.

KEYWORDS

Poly-L-aspartic acid; glucose biosensor; carbon paste electrode; glucose oxidase

Introduction

The sensitive determination of glucose in biological fluids such as blood and urine is important when it comes to the diagnosis and treatment of patients with diabetes.[1] Various methods, such as colorimetric, spectroscopic and electrochemical techniques, are used to monitor glucose.[2,3] Among these methods, electrochemical sensors based on GOx are commonly employed for the detection of glucose owing to their simplicity, affordability, high sensitivity and low detection limits.[4] GOx, as a highly specific enzyme, is the model enzyme for the construction of amperometric glucose biosensors. The oxidation of β-D-glucose into D-glucono-δ-lactone is catalyzed by GOx, as in the following reaction:

$$\beta\text{-D-Glucose} + \mathrm{GOx(FAD)}^{+} \rightarrow \text{D-glucono-}\delta\text{-lactone} + \mathrm{GOx(FADH_2)} \quad (1)$$

The oxidized form of the enzyme, GOx(FAD), is regenerated via the reoxidation of the flavin in the presence of oxygen, as in the equation that follows:

$$\mathrm{GOx(FADH_2)} + \mathrm{O_2} \rightarrow \mathrm{GOx(FAD)} + \mathrm{H_2O_2} \quad (2)$$

The quantification of the glucose can be performed by means of the amperometric measurement of the H_2O_2 created during the enzymatic oxidation of the glucose.[5]

Two limiting factors are encountered in terms of the immobilization of GOx onto solid electrode surfaces. The first is enzyme leaching, while the second is poor electrical communication between the electrode and the active site of the enzyme. Identifying a suitable immobilization matrix is crucial to overcoming such issues.[6]

Electropolymerization represents a promising means by which to modify the surfaces of electrodes due to its sensitivity and selectivity to analytes, its ability to provide larger surface, its ability to promote electron transfer rate, and strong adherence of the polymer to the electrode's surface.[7,8] Recently, the electropolymerization of amino acids has become increasingly important, as they can form their own functional groups and biocompatible electroactive polymers.[9,10] Aspartic acid, which has a free carboxylic acid group, can easily be electropolymerized on the surface of an electrode. Although no prior studies have investigated electrochemical glucose biosensors based on polyaspartic-acid-coated surfaces, researchers have examined other biosensor applications. For example, Wang et al. developed a polyaspartic-acid film-based sensor for the detection of catechin in tea drinks.[11] A poly(L-aspartic-acid)-modified glassy carbon electrode was developed by Yu et al. for the determination of epinephrine.[12] Additionally, Donmez et al. fabricated a nucleic acid biosensor for the detection of the hepatitis C virus genotype 1a based on a poly(L-aspartic acid)-modified pencil-graphite electrode.[13]

To the best of our knowledge, the present study represents the first time that a poly(L-aspartic acid) film was employed in the fabrication of an amperometric glucose biosensor. First, the CPE was obtained by mixing graphite and mineral oil. Second, the PAA was formed onto the surface of the CPE via electropolymerization. Then, the GOx was immobilized onto the surface of the PAA/CPE by covalent

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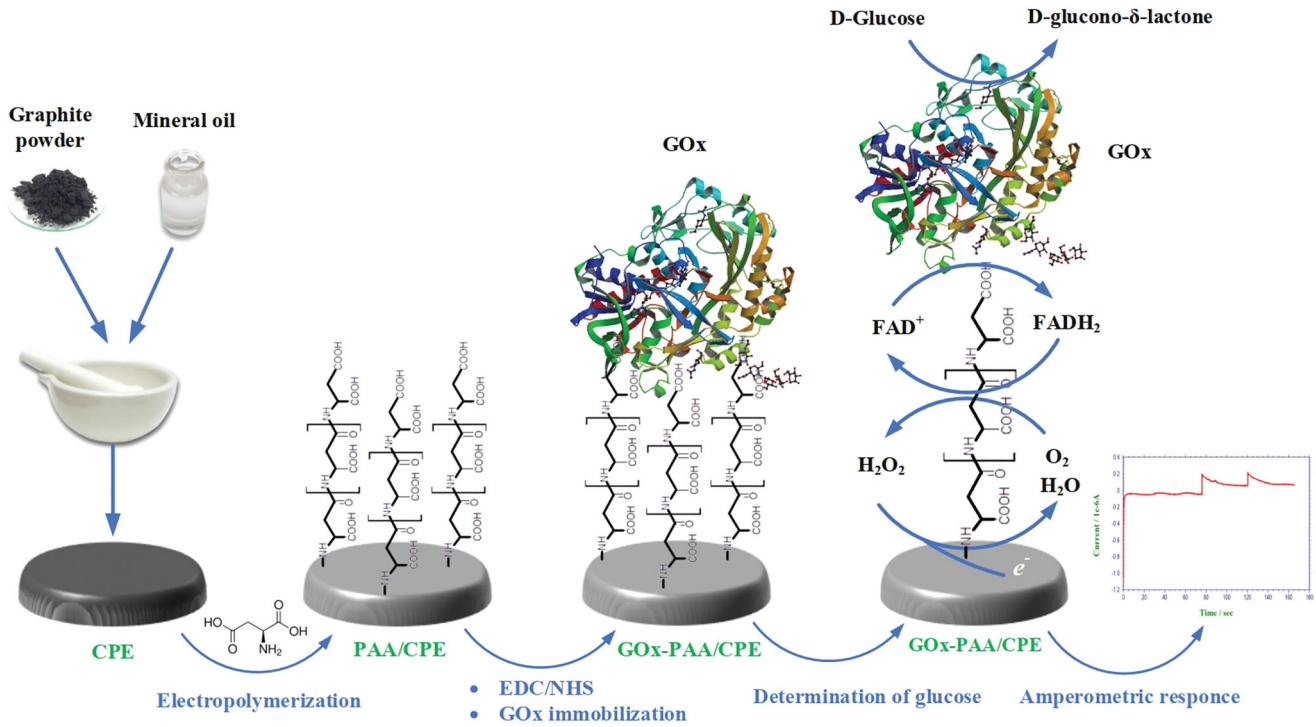

Figure 1. Schematic illustration for the fabrication of PAA-modified glucose biosensor.

attachment (Figure 1). The performance of the prepared biosensor was investigated in detail.

Materials and methods

Chemicals and apparatus

All the electrochemical experiments in this study were performed using a CHI 1230B electrochemical workstation. The utilized three-electrode systems consisted of a platinum electrode as an auxiliary electrode, a PAA-modified CPE as the working electrode, and an Ag/AgCl reference electrode.

The graphite powder was obtained from Merck, while the GOx (derived from *Aspergillus Niger*) was purchased from Sigma. The other chemicals used in the experiments were also purchased from Sigma. The glucose stock solution was left for 24 hr for mutarotation proposes prior to use in order to reach a stable ratio of the α and β forms of the D-(+)-glucose. All the experiments were conducted at room temperature. Double distilled water was used for all the experiments.

Preparation of the CPE

The carbon paste was obtained by mixing mineral oil with the graphite powder for at least 20 min. (ratio of C: Mineral oil was, 70:30, w/w). The carbon paste was finally packed into the cavity of an insulin syringe and electric contact was provided by a copper wire. The syringe plunger was pressed until a smooth surface was obtained, and then the surface was polished on a weighing paper.

Preparation of the PAA-modified electrodes (PAA/CPE)

The PAA/CPE was prepared by means of the electropolymerization of the L-aspartic acid on the surface of the CPE. First, it was vertically dipped into 0.1 M phosphate buffer solution (PBS) (pH 7.0) containing 0.02 M L-aspartic acid. Next, cyclic voltammetry (CV) was performed by applying a potential from −0.8 to 2.0 V at a scan rate of 100 mV s^{-1} continuously for 40 cycles. Following polymerization, the modified electrode was washed with deionized water to remove any unreacted aspartic acid monomer.

Immobilization of the GOx on the PAA/CPE

After the PAA films had been electro-synthesized on the CPE, the GOx was covalently immobilized. Initially, the COOH groups of the PAA were activated by dipping in a solution containing 86.8 mM NHS and 26 mM EDC for 2 hr. Then the electrode was quickly washed with PBS. 7 µL of GOx (10 mg/mL of GOx in PBS [pH 7.0]) was dropped onto the PAA/CPE and air-dried at room temperature. Finally, the biosensor was dipped into PBS (pH 7.0) for 30 min to remove non-immobilized GOx.

Amperometric measurements

The quantification of the glucose was achieved by measuring the amperometric signal of H_2O_2 released during the enzymatic oxidation of the glucose. The prepared biosensor was immersed in 0.1 M PBS (pH 7.0) containing 0.1 M KCl as a supporting electrolyte, and then its steady- state current (i_a) was measured. The electrochemical cell was stirred after the

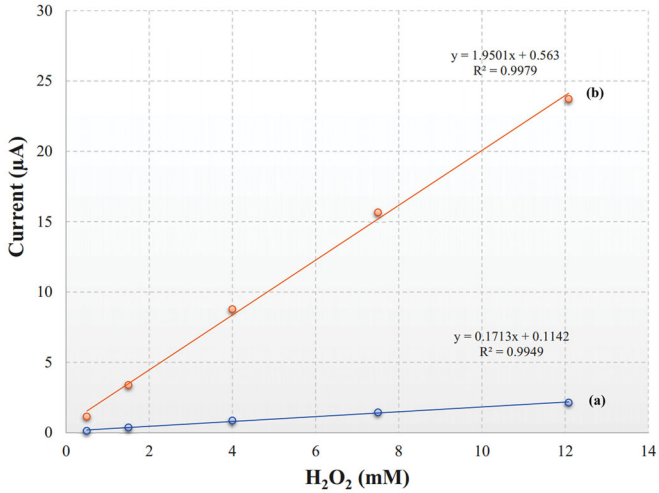

Figure 2. Amperometric responses of (a) the CPE and (b) the PAA/CPE to different concentration range of H_2O_2 (at 0.1 M phosphate buffer of pH:7.0, 25 °C, +0.7 V operating potential).

glucose had been added, and its current (i_b) was measured again. Next, the current differences ($\Delta i = i_b - i_a$) were plotted against the glucose concentrations.

Results and discussion

Amperometric responses of the CPE and the PAA/CPE to H_2O_2

To compare the sensitivities of the CPE and the PAA/CPE to H_2O_2, the amperometric responses of the electrodes to H_2O_2 were evaluated at various concentrations ranging from 0.5 to 12 mM. As can be seen in Figure 2, when the H_2O_2 concentration was increased, the amperometric responses of both electrodes (CPE and PAA/CPE) also increased. However, an almost ten-fold higher anodic current of H_2O_2 was obtained using the PAA-modified electrode when compared with the CPE. This result showed that the PAA/CPE was more sensitive to H_2O_2 than the CPE.

Electropolymerization of L-Aspartic acid

Figure 3 displays the continuous CV findings concerning the electropolymerization of L-aspartic acid on the surface of the CPE in a 0.02 M L-aspartic acid aqueous solution. The electropolymerization was carried out between the potential of −0.8 V and +2.0 V for 40 cycles at a scan rate of 100 mV/s. As shown in Figure 3, the cathodic and anodic peak currents gradually increased, thereby demonstrating the formation and growth of an electroactive layer on the surface of the CPE. The mechanism behind the electropolymerization of L-aspartic acid has previously been reported.[12] The L-Aspartic acid gives an oxidation peak at about +1.6 V during the first cycle (inset of Figure 3), which is associated with the free cation radicals of the L-aspartic acid. It is these free cation radicals that create the carbon-nitrogen covalent linkages on the surface of the CPE.[14]

To investigate the PAA film thickness, several enzyme electrodes were prepared by altering the polymerization

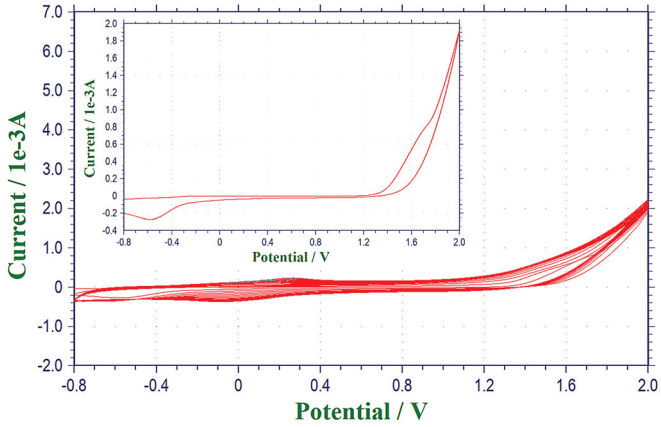

Figure 3. Cyclic voltammograms for the electropolymerization of L-aspartic acid onto the CPE. (Scan rate: 100 mV/s; potential range: −0.8 to +2.0 V; L-aspartic acid concentration: 0.02 M). Inset: First cycle of the electropolymerization of L-aspartic acid.

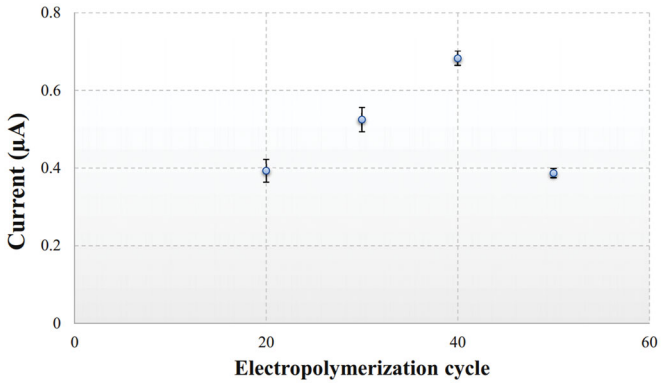

Figure 4. Effect of electropoymerization cycles on amperometric response of 1.0 mM glucose ($n = 3$) (in pH 7.0 phosphate buffer and at −0.1 V operating potential).

cycles. As shown in Figure 4, when the cycles shifted from 20 to 40, the amperometric responses to 1 mM glucose increased. This increment can be explained by the accumulation of more PAA films providing additional GOx on the surface of the CPE. Yet, when the polymerizing cycles reached 50, the amperometric responses began to decrease. This can be attributed to the fact the electron transfers were hampered by the increasing thickness of the PAA films. Therefore, the 40 cycles approach was selected for use in the future experiments.

Surface morphology of the prepared electrode

Figure 5 presents scanning electron microscope (SEM) images of the (a) unmodified and the (b) PAA-modified CPE. When the SEM images of the electrodes are compared, significant morphological differences can be observed. The surface of the unmodified CPE was formed by irregularly shaped graphite flakes (Figure 5a). Following the electropolymerization of PAA onto the CPE, PAA particles formed over the graphite flakes (Figure 5b).

Figure 5 also presents the energy-dispersive X-ray (EDX) analysis of both the CPE and the PAA/CPE. Elemental nitrogen from the aspartic acid can be detected in the PAA/CPE

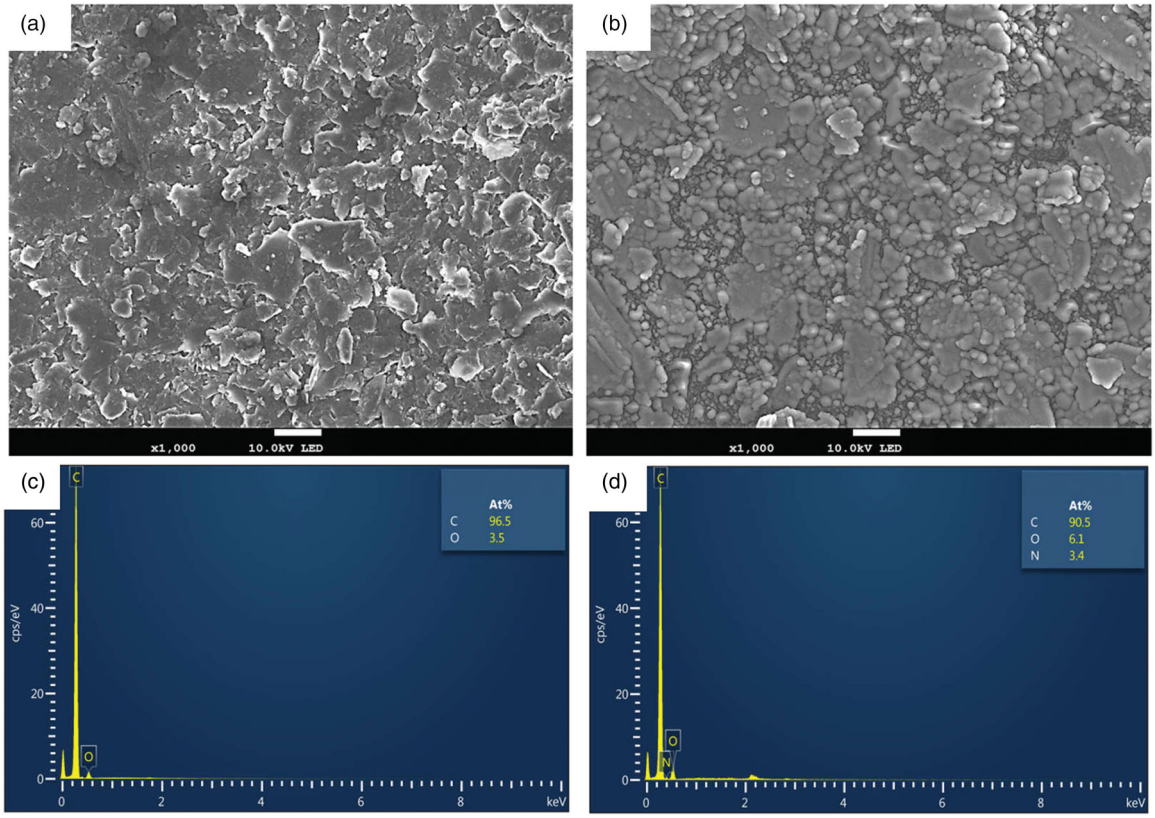

Figure 5. SEM images of (a) the CPE, (b) the PAA/CPE, EDX analyses of (c) the CPE and (d) the PAA/CPE.

(Figure 5d), although it is not present in the bare CPE (Figure 5c). This supports the notion that PAA can be successfully electropolymerized onto a CPE.

The dynamic surface area of the CPE and the PAA/CPE

The dynamic surface area of the CPE and the PAA/CPE were determined by using the Randles–Sevcik equation.[15,16]

$$i_p = (2.69 \times 10^5)\ n^{3/2} A\ D^{1/2}\ v^{1/2}\ C \qquad (3)$$

where i_p (A) refers to the peak current of electrode, n is the number of electrons involved in the reaction i.e., equal to 1, A (cm^2) is effective area of electrode, D is diffusion coefficient (for $K_3[Fe(CN)_6]$ is $7.6 \times 10^{-6}\ cm^2/s$), v (V/s) is scan rate and C (mol/cm^3) is concentration of $K_3[Fe(CN)_6]$.

The CVs at varying sweep rates (10–140 mV/s) were recorded using 5.0 mM $K_3[Fe(CN)]_6$ in 0.1 M KCl solutions (Figure 6). From the slope of the plot of i_p vs. $v^{1/2}$, the dynamic surface area was calculated to be 0.043 cm^2 for of the CPE and 0.068 cm^2 for the PAA/CPE.

Effect of the working potential

The effect of the working potentials ranging from −0.3 V to +0.5 V of the GOx-PAA/CPE was investigated in 0.1 M PBS (pH 7) containing 1 mM of glucose. As shown in Figure 7, the biosensor showed a high background current at −0.2 V and +0.5 V. It also showed low amperometric responses at 0.0 V and +0.1 V. Thus, in the subsequent experiments, the

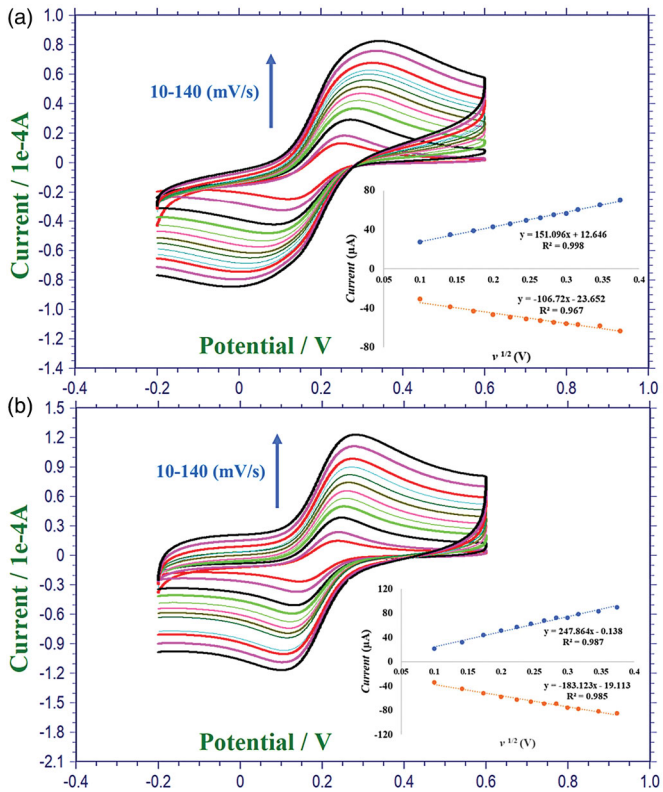

Figure 6. CVs obtained for (a) the CPE and (b) the PAA/CPE in the presence of 5.0 mM $K_3[Fe(CN)_6]$ and 0.1 mM KCl at various scan rates (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140 mV/s). The inset figures show the dependence of the redox peak currents on the square root of the scan rates.

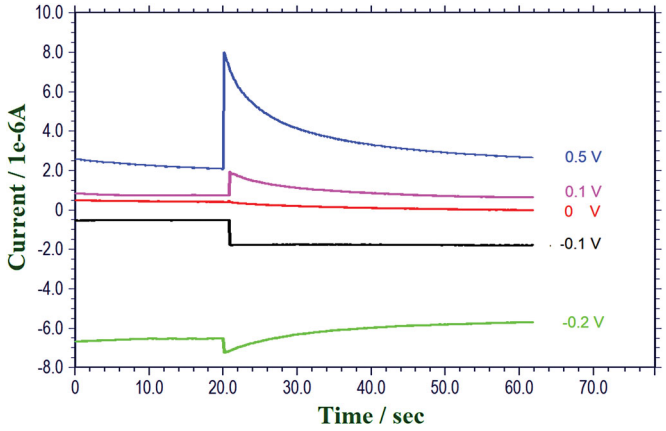

Figure 7. Effect of applied potential on the amperometric response of the biosensor to 1.0 mM glucose in 0.1 M PBS (pH 7.0).

amperometric detection of glucose was performed at a working potential of −0.1 V.

Interference, stability, repeatability and reproducibility

Some of the electroactive species found in serum are known to may affect the performance of a glucose biosensor. Therefore, the effects of species capable of potentially interfering, such as dopamine (DA), ascorbic acid (AA), and uric acid (UA) were investigated. AA and UA commonly coexist with glucose in the blood or fermentation system.[17] For the propose of the investigation, 0.1 mM of each interfering species was consecutively added into the cell. As can be seen in Figure 8, no appreciable change in the current change was monitored following the addition of DA, AA, and UA; however, after the addition of 1.0 mM of glucose, an increase in the current response of the PAA-modified biosensor was noted. The result indicated that the prepared biosensor exhibited good selectivity to glucose.

The storage stability of the fabricated biosensor was examined using the same electrode by monitoring the current response of 1 mM of glucose over a period of 60 days. the biosensor was stored in a PBS (pH 7.0) solution at a temperature of 4 °C in a refrigerator, when not in use. During the first five days, the activity of the biosensor decreased drastically by up to 60% of its initial activity. In the following days, there was almost no change in the activity of the biosensor. A loss of activity of about 45% was observed on the 60th day (Figure 9). This result is similar to results previously reported in a number of glucose biosensor studies.[18–20]

Both the repeatability and the reproducibility are significant parameters in relation to the assessment of a biosensor's performance.[1] The repeatability of the prepared biosensor was evaluated by examining the current response of the same electrode to 1.0 mM of glucose. After ten sequential measurements, the standard deviation (SD), and relative standard deviation (RSD) of the prepared biosensor were 0.015 and, 3.189%, respectively, which indicated the good repeatability of the biosensor. In addition, the limit of detection (LOD) of the biosensor was estimated to be 69.2 µM at a signal/noise of 3. It also exhibited good

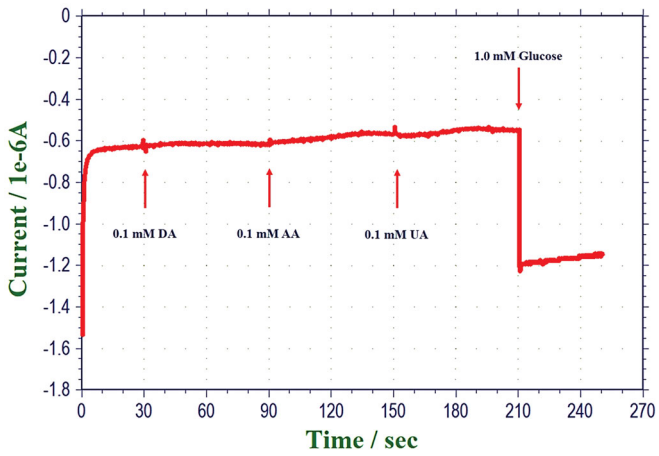

Figure 8. Amperometric response of the biosensor with addition of 1 mM glucose, 0.1 mM uric acid, 0.1 mM ascorbic acid and 0.1 mM dopamine in 0.1 M PBS (pH 7) at the applied potential of −0.1 V.

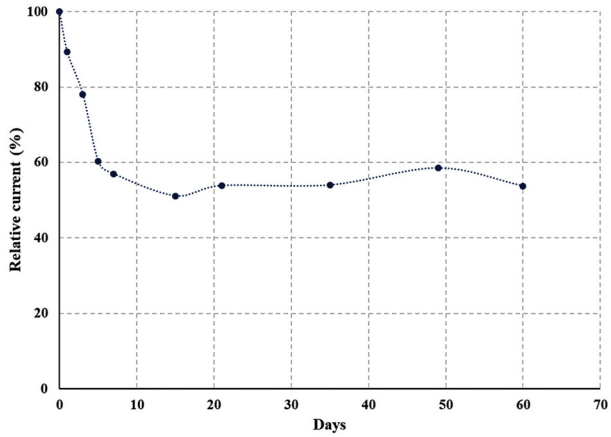

Figure 9. Storage stability of the biosensor at the applied potential of −0.1 V.

reproducibility (three individual measurements) with an SD of 0.014 and an RSD of 3.69%.

The calibration curve of the prepared biosensor is characteristic of the Michaelis–Menten kinetic mechanism (Figure 10A). As shown in Figure 10B(a), the amperometric responses are linearly increased within the glucose concentration range of 0.01–1.0 mM ($R = 0.9942$). The biosensor possessed a sensitivity of 5.3 µA $mM^{-1}\,cm^{-2}$ to glucose. The time required to reach 95% of steady-state current was less than 4 s.

The apparent Michaelis-Menten constant (K_m^{app}) reflecting enzymatic affinity can be calculated using the Lineweaver–Burk equation, where i_{max} is the maximum current measured under a saturated glucose condition, i_{ss} is the steady-state current following the addition of glucose, and C is the concentration of glucose.[21]

$$\frac{1}{i_{ss}} = \frac{K_m^{app}}{i_{max}}\,\frac{1}{C} + \frac{1}{i_{max}} \tag{4}$$

The K_m^{app} and i_{max} values for the fabricated biosensor were calculated to be 1.17 mM and 0.83 µA, respectively (Figure 10B(b)). The low K_m^{app} value can be attributed to the excellent affinity between the electrode and the enzyme.

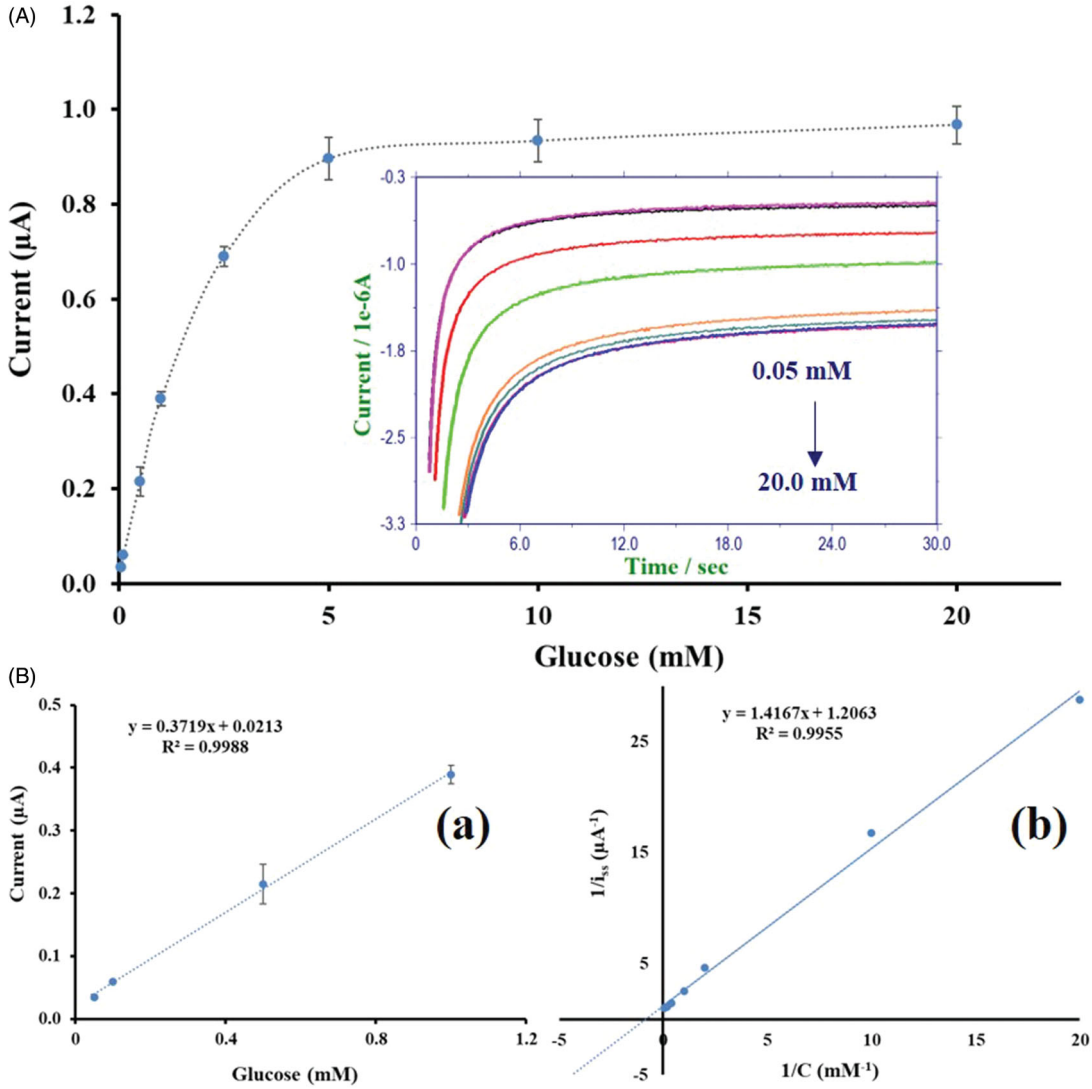

Figure 10. (A) Effect of the glucose concentration on the amperometric response of the biosensor (at pH 7.0 PBS and at applied potential of −0.1 V, ($n = 3$)). Inset: Amperograms of the biosensor in 0.1 M PBS (pH 7.0) containing various concentrations of glucose (0.05–20 mM). (B) (a) The calibration curve of the biosensor, (b) Lineweaver–Burk plot of the biosensor.

Table 1. Comparison of the analytical performance of different enzymatic glucose biosensors.

Immobilization matrix	Applied potential (V)	Response Time (s)	Km (mM)	Linear range (mM)	LOD (μM)	Sensitivity (μA cm^{-2} mM^{-1})	Ref.
GOD/MAA/AuNPs/TiO_2NT/Ti	0.25	<10	7.2	0.40-8	310	–	[22]
GOx – PoPD/AuNPs – GO/Pt	0.45	10	2.29	0.1 – 3.8	75	17.68	[23]
GOx/P-L-Arg/*f*-MWCNTs/GCE	−0.45	<5	2.2	0.004–6	0.1	48.86	[24]
Cs/GOx/PGA/GCE	−0.35	–	–	0.5-5.5	120	2.3	[25]
GOx/PAA/CPE	−0.1	4	1.17	0.05-1	69.2	5.3	This work

GOD, GOx: glucose oxidase; MAA: mercaptoacetic acid; AuNPs: Gold nanoparticles; TiO_2NT: TiO_2 nanotube array electrode; Ti: Titanium foil; PoPD: poly-o-phenylenediamine; GO: graphene oxide; P-L-Arg: poly(L-arginine); *f*-MWCNTs: functionalized multiwalled carbon nanotubes; GCE: glassy carbon electrode; Cs: chitosan; PGA: poly(glutamic acid); PAA: poly(L-glutamic acid); CPE: carbon paste electrode.

Thus, the immobilized GOx was carried by the high enzymatic activity, thereby showing that the immobilization technique was biocompatible in the present study. To demonstrate the advantages offered by the prepared biosensor, its performance was compared with the performance of certain previously reported glucose biosensors (Table 1). When compared with the references included in the table, the GOx/PAA/CPE exhibited relatively improved characteristics in terms of the fast response time, K_m and LOD.

Conclusion

In this study, the fabrication of a PAA-modified glucose biosensor was successfully performed for the first time by means of the electropolymerization of L-aspartic acid on the surface of a CPE. The experimental data show that the prepared biosensor is much better than many previously reported glucose biosensors in terms of its response time, linear range, detection limit and cost-effectiveness.

In addition, the prepared biosensor exhibited satisfactory anti-interference ability in the presence of DA, UA, and AA. PAA exhibits good biocompatibility, and therefore, it demonstrates promise in relation to the fabrication of glucose biosensors. As the free carboxylic acid groups of PAA contribute to the covalent immobilization of GOx, it can also be easily adapted so as to immobilize other enzymes for the fabrication of enzyme-based biosensors.

Disclosure statement

The authors declare no conflict of interest. Neither ethical approval nor informed consent was required for this study.

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