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Glucose Biosensor Based on MWCNTs-Gold Nanoparticles in a Nafion Film on the Glassy Carbon Electrode Using Flow Injection FFT Continuous Cyclic Voltammetry

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A new glucose electrochemical biosensor was designed using a flow injection system. Glucose oxidase (GOx), as a sensing material, was immobilized on to the modified surface of a glassy carbon electrode using nafion polymeric matrix. Gold nanoparticles (AuNPs) and multiwall cabon nanotubes (MWCNTs) were used to modify the surface of the glassy carbon electrode and improve the biosensor response. Fast Fourier transformation continuous cyclic voltammetry (FFTCCV) was used as detection method in which the charge under the peak calculated in a specific potential range. Ferrocene methanol in PBS buffer (pH=7.0) was used as the flowing eluent. It is a pair of well-reversible redox reagent which is used as an electron-transfer mediator. Effect of scan rate, solution flow rate and buffer pH were also studied. The results show that, the linear response range of biosensor was in the range of 0.1 to 10 µM and the detection limit was calculated 0.03 µM at a signal-to-noise ratio of 3. The proposed biosensor showed an acceptable reproducibility, good stability and low interferences.

Keywords: Glucose, glucose oxidase, biosensor, sensor, gold nanoparticles, FFT Cyclic voltammetry, flow injection system

1. INTRODUCTION

Determination of glucose is essential due to its clinical and industrial importance. Rapid determination of blood sugar for treatment and control of diabetes is essential. Development of cost-effective, simple, accurate, portable and rapid sensors for glucose are socially important due to the diabetics globally prevalence which represents about 6.4% of the world's population [1].

One of the most important sensing materials widely used in the glucose biosensor is glucose oxidase (GOx). Most of the electrochemical glucose biosensors are based on the glucose oxidase (GOx) enzyme, which catalyzes the oxidation of glucose to gluconolactone which was hydrolyzed to gluconic acid in water as seen in Fig. 1:

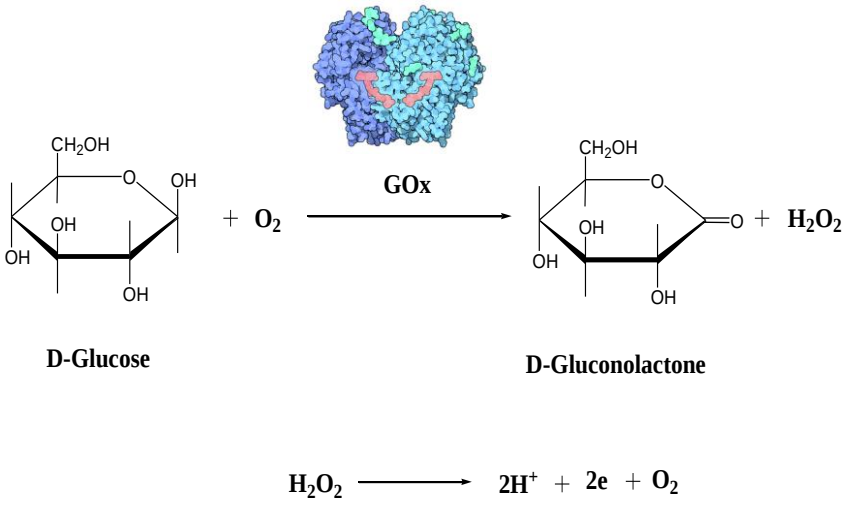

Figure 1. Schematic diagram of glucose oxidase (GOx) enzymatic reaction

The quantification of glucose can be achieved via electrochemical detection of the enzymatical released of H_2O_2.

In general, it is difficult to exchange the electron between an enzyme and solid surface of the electrodes directly. This is because of the inaccessibility of its redox center and loss of bioactivity of the enzyme due to the conformational changes by adsorption on the electrode surface. Hence, to have a stable and sensitive response, the surface of the electrode should be modified.

Carbon nanotubes (CNTs) can be used as a suitable intermediates between electrodes and enzymes. Recently, carbon nanotubes (CNTs) have been used in various areas, such as biosensors and biofuel cells, because of their high surface area, high surface/volume ratio, good electrical conductivity and significant mechanical strength [2-4]. CNTs in a suspension individually can be cytotoxic but cytotoxicity is avoided by immobilizing CNTs on surfaces or within composites [5,6]. Thus, using CNTs in the forms of paste, composite, films, thin coatings or continuous fibers, is considered a safe way to construct electrodes.

Metal nanoparticles (NPs) generally have high effective surface area, catalysis and biocompatibility [7-10]. It was shown that gold nanoparticles (AuNPs) could adsorb redox enzymes without loss of their biological activities. Recently, AuNPs have become very important in the field of electrochemical biosensors. AuNPs are prepared by chemical reduction of the Au salts in the presence of a suitable stabilizer, which binds to their surface to impart high stability of colloid solution [10]. The practical advantage of Au-NPs is the size and surface morphology which can be controlled

experimentally adjusting the preparation conditions [10]. AuNPs in a biosensor help to enzyme immobilization, increase retention and enzymatic activity without disordering in its biological recognition. The small size AuNPs (diameter: 5–50 nm) helps to better electron transfer distance between the enzyme and electrode [10,11].

To prevent loss of the enzyme molecules and to improve the anti interferent ability of the biosensor, Nafion films have been used extensively for the construction of biosensors. Nafion, due to its easy fabrication, good electrical conductivity, high chemical stability and good biocompatibility, has been widely used as a protective coating material and as a support for enzyme immobilization.

This work introduces a new flow injection electrochemical biosensor for determination of glucose combine with FFT continuous cyclic voltammetry (FFTCCV) technique [12-16]. To the best of our knowledge, this is the first application of FFTCCV method in glucose biosensor.

2. MATERIALS AND METHODS

2.1. Reagents

Glucose oxidase (GOx) (from *Aspergillus niger*, >100 U/mg) was purchased from Sigma-Aldrich Co. and its solution (10 mg/mL) was prepared by dissolving 5 mg GOx in 0.50 mL of 0.1 M phosphate buffer solution (PBS, pH=7.0) and stored in a refrigerator (at 4 ºC). D-(+)-glucose was purchased from Sigma-Aldrich Co. MWCNTs (diameter: 10–20 nm; length: 0.5-40 nm; purity: ≥95%) were obtained from Shenzhen Nanotech Port Ltd. Co (Shengzhen, China). Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4.3H_2O$), Nafion (5 wt.%) solution in a mixture of lower aliphatic alcohols and water and ferrocene methanol were obtained from Aldrich Co. 0.1 M phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4 and adjusting the pH values with 0.1 M H_3PO_4 or NaOH solutions. All other chemicals were of analytical grade and purchased from Merck Co. All solutions were made up with doubly distilled water.

2.2. Preparation of Nafion/GOx/AuNPs-MWCNTs/GC electrode

Glassy carbon electrode (3 mm-diameter) was polished first with 1.0, and 0.05-μm alumina slurry. After rinsing with doubly distilled water, they were sonicated in absolute ethanol and doubly distilled water for about 5 min, respectively. 0.5 mg of MWCNTs was dispersed in dimethylformamide (DMF) with the aid of ultrasonic agitation to give a 0.5 mg/mL black suspension. Then, 5 μL of the suspension was dropped on a cleaned GC electrode and let the solvent was evaporated in air. Hence, a uniform film of MWCNTs coats the surface of GC electrode. The electrochemical deposition of AuNPs was performed in 0.2 M Na_2SO_4 aqueous solution of $HAuCl_4$ (1.0 mM). The deposition time was about 200 s and the potential was -0.2 V. After that, the surface of the modified electrode was carefully washed with distilled water and dried at room temperature. Then, 15 μL of GOx solution was dropped onto the surface of the modified GC electrode with a microsyringe and allowed to dry at

ambient temperature. Finally, 5 μL of nafion (5 wt.%) was casted and used as a net to hold the GOx/AuNPs/MWCNTs on the electrode surface stably. The solvent was allowed to evaporate before use. The final electrode is taken as the Nafion/GOx/AuNPs/MWCNTs/GC electrode. All resulting electrodes were stored at 4 °C when not in use.

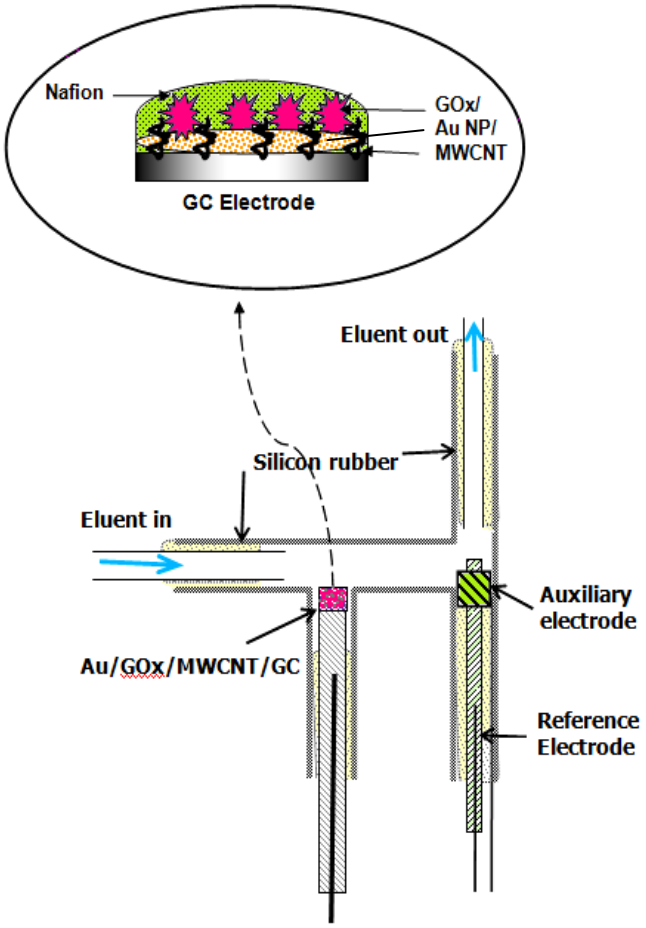

Figure 2. The diagram of glucose biosensor and the electrochemical cell used in flow injection analysis

2.3. Instrumentation

For the electrochemical FFTCV measurements a homemade potentiostat, which was connected to a PC was used. The potentiostat was constructed with an analog to digital (A/D) data acquisition board (PCL-818H, Advantech Co.). In the electrochemical system, generating the analog waveform and acquiring current was done by A/D board. The potential waveform was repeatedly applied to the working electrode and then the data was acquired, and stored by the software. In the measurements, the data acquisition requirements electrochemical software was developed using Delphi 6.0. Also, in this electrochemical setup, the data could be processed and plotted in real time, or the stored data could be loaded and reanalyzed.

2.4. Flow Injection Setup

The flow injection analysis equipment used for the measurements, was integrated with an eight roller peristaltic pump (UltratechLabs Co., Iran) and a four way injection valve (Supelco Rheodyne Model 5020) with 200 μL sample injection loop. The analyte solutions were introduced into the sample loop by means of a plastic syringe. The electrochemical cell used in flow injection analysis is shown in Fig. 2. Ferrocene methanol in PBS buffer (pH=7.0) was used as the flowing eluent. It is a pair of well-reversible redox reagent which is used as an electron-transfer mediator.

3. RESULTS AND DISCUSSION

It is well know that, the response of a biosensor electrode is related to its physical morphology. Fig. 3. shows the typical SEM images of GOx/AuNPs/MWCNTs/GC electrode surface. The diameter of these spherical AuNps and MWCNTs are in the range of 20–30 nm with a quite symmetric distribution.

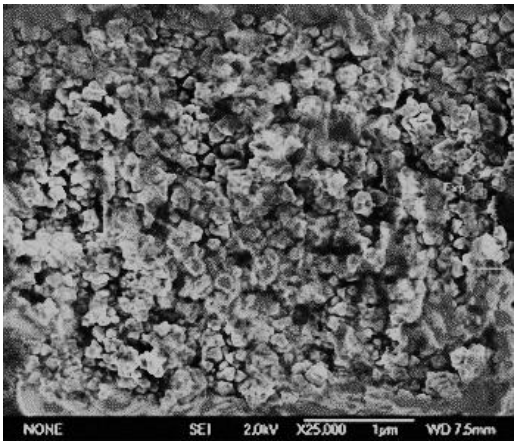

Figure 3. SEM image of the electrode GOx/AuNPs/MWCNT/GC

Fig. 4 demonstrates FFT cyclic voltammograms of the AuNPs/MWCNTs/GOx/GC electrode in the potential range of -100 to 600 mV at potential sweep rate of 5 V/s. In the three dimensional graph, the potential axis represents potential applied to the working electrode during each sweep. The time axis represents the time passing between the beginning of the flow injection experiment and the beginning of a particular sweep (i.e. it represents a quantity proportional to the sweep number) [17-20].

Fig. 4 shows that before injection (in absent of glucose) there is no significant changes in the voltammograms, but by injection of 200 μL of 1.0×10^{-5} M of glucose in 0.01 M PBS buffer at pH=7.0 a signal appears at potential 200 mV. At the binging of the measurements, there is a small peak current due to the redox of ferrocene methanol, a pair of well-reversible redox waves with formal potential at 0.19 V (vs. Ag/AgCl), which was assigned to one-electron reversible redox reaction of Fc^{+}/Fc.

Injection of glucose sample into flowing eleuent, a well-defined catalytic peak was developed as a consequence of the GOx catalytic oxidation of glucose, and the electrocatalytic current increases with the increase of the concentration of glucose in the PBS. The increase in the current of the electrode at potential of 300 mV is due to the production of H_2O_2 by GOx enzyme during the oxidation of glucose at the biosensor surface. Nevertheless, it can be suggested that attachment of GOx to high surface area of AuNPs/MWCNTs/GC facilitates a higher rate of direct electron transfer between the active sites of immobilized GOx, which, can increase the peak current at the recorded cyclic voltammograms when the sample was injected.

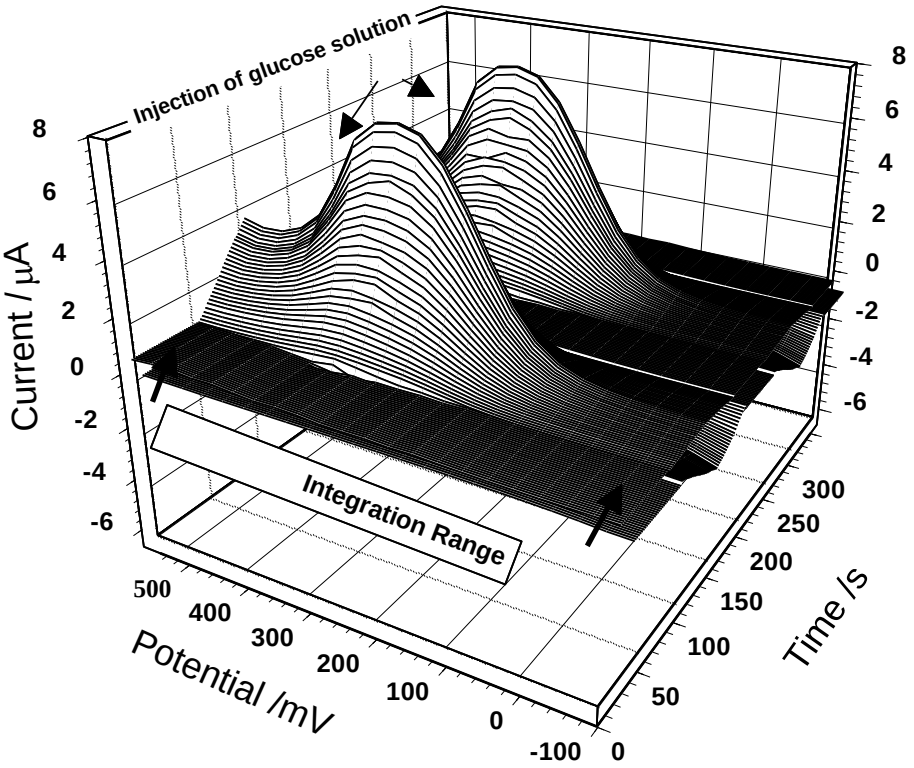

Figure 4. FFT cyclic voltamograms of the AuNPs/MWCNTs/GC electrode without (in absent) and with injection of 200 µL of 1.0×10^{-5} M glucose in 0.01 M PBS at pH=7.0 in the potential range of -100 to 600 mV at 5 V/s sweep rate and the potential integration range for the current

Once FFTCCV is used to monitor a flowing system, glucose electrochemical processes will cause a measurable change in the peak current at the voltammograms. According to Fig. 1, the electrochemical reaction for the detection of glucose in presence of GOx is proposed to the enzymatic reaction which produces H_2O_2.

In this detection method the current passing through the electrode was sampled only during the potential ramp. This data processing operation was carried out simultaneously with data acquisition

during flow injection experiments. The result of the integration is shown in Fig. 5. The response was calculated as follow:

$$\Delta Q = Q - Q_0 \quad (1)$$

where Q is the electrical charge obtained by integration of cyclic voltammetric curves between 200 and 500 mV in the chatodic scan, and Q_0 represents Q in the absence of the adsorbent. The peaks in Fig. 5 are due to five consecutive injections of the same sample. The integration of net current changes is applied over the selected scanned potential range. In this method, ΔQ is calculated based on the all-current changes at the CV. A total absolute difference function (ΔQ) can be calculated by using the following equation:

$$\Delta Q\ (s\tau) = \Delta t\left[\sum_{E=E_i}^{E=E_f} \left| i(s,E) - i(s_r,E) \right|\right]$$

Where, s is the sweep number, τ is the time period between subsequent potential scan, Δt is the time difference between two subsequent points on the cyclic voltammograms, i (s, E) represents the current of the cyclic voltammograms recorded during the s-th scan and i (s_r, E) is the reference current of the cyclic voltammograms. E_i and E_f are the initial and the final potential, respectively, for integrating of current. This integration range for the current is shown in Fig. 4. The reference cyclic voltammogram was obtained by averaging a 5 to 10 cyclic voltammograms, recorded at the beginning of the experiment (i.e. before injection of the analyte). In addition, the results show that with increasing the concentration of glucose in the injected sample, ΔQ increases. This confirms a fast electrocatalytic and electron exchange behavior of modified electrode at high potential sweep rates.

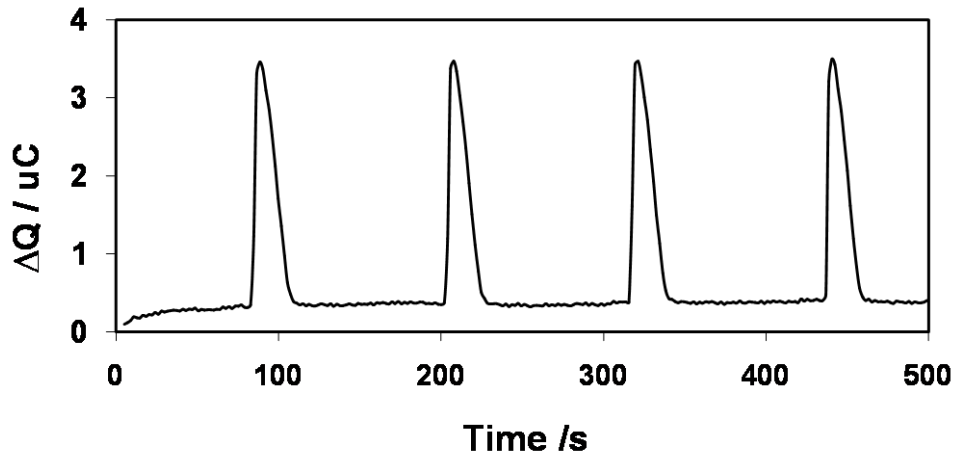

Figure 5. Response of the GOx/AuNPs/MWCNTs/GC electrode to the consecutive injections of 200 μL of 1.0×10^{-5} M glucose in 0.01 M PBS Based on experimental data shown in Fig. 4.

3.1. Optimizing the experimental parameters

In this method, sensitivity of the measurement mainly depends on the potential sweep rate and eluent solution flow rate, which is mainly due to kinetic factors of the electrode processes, and instrumental limitations [21-24]. From this point of view, it is necessary to exam, the sensitivity of the biosensor to the applied potential sweep rate. In this direction, the influence of the scan rates and the elunet flow rate on the sensitivity of the detector response, at scan rates (from 0.5 to 10 V/s) and the eluent flow rate (0.5 to 4 mL/min), were investigated and the responses of the detector were recorded. The acquired results from injecting solutions of 1.0×10^{-5} M of glucose are presented in Fig. 6.

As it is shown in this figure, the biosensor exhibits the maximum sensitivity (or signal) at 5 V/s of scan rate and 1.5 mL/min of the eluent flow rate. The effects of the sweep rate on the detection performance can be taken into consideration from three different characteristics; speed of data acquisition, kinetic factors of electrochemical processes at the electrode surface, and the flow rate of the eluent, which controls the time retention of the solution sample zone in the electrochemical cell [25,26]. The main reason for lower sensitivity of the biosensor at higher scan rates is limitation in the rate of electron transfer of electrochemical processes of GOx with glucose molecule.

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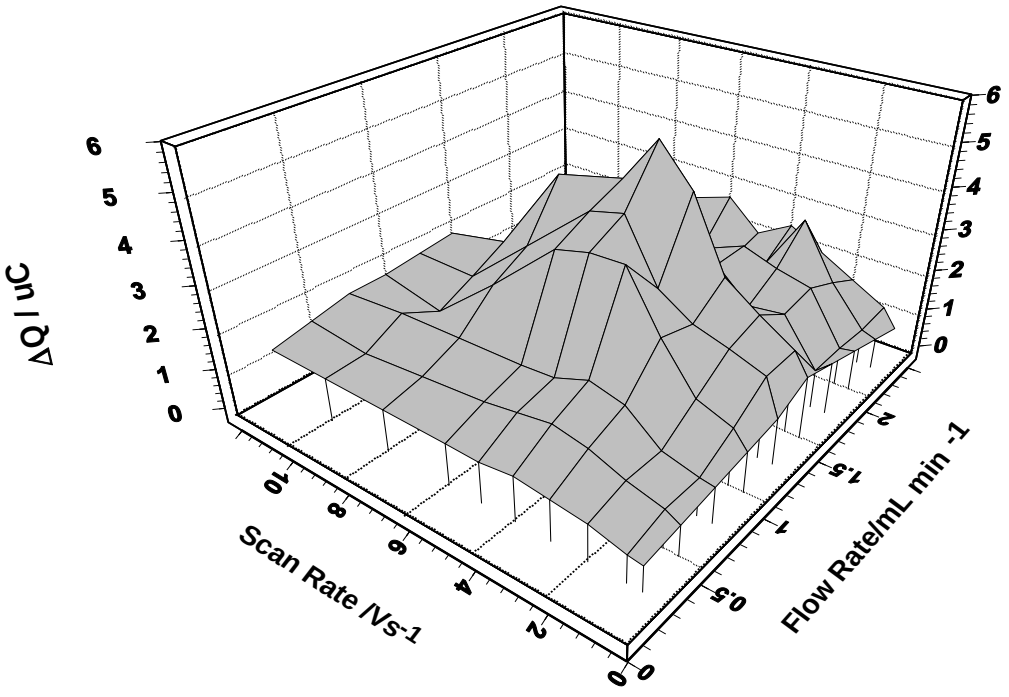

Figure 6. Effect of the sweep rate and effect of flow rate on the response of the GOx/AuNPs/MWCNTs modified GC electrode to injections of 1.0×10^{-5} M glucose

3.2. pH Effect on the current response of the biosensor

Investigation of the pH effect on the performance of the biosensor is of great importance, because the activity of the immobilized GOx is pH dependent. The current response of the enzyme

electrode were studied in the pH range of 5.0–9.0 (Fig. 7), the current response decreases and the background current increased at higher pH values. The signal/noise shows the maximum value at pH of 7.0.

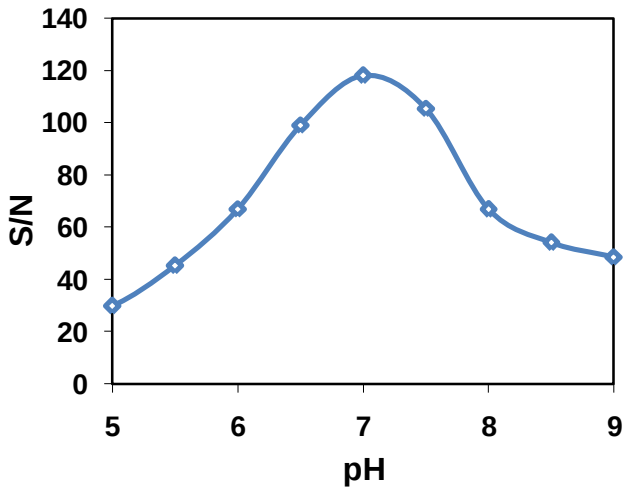

Figure 7. The effect of pH on response of the biosensor

3.3. Calibration curve and biosensor characterization

Fig. 8 shows typical FFTCV response curves of glucose in 0.01 M PBS by the proposed biosensor. The regression equation is $\Delta Q(\mu C) = 0.0649\ c(\mu M)+0.0194$ with $R = 0.993$. The ΔQ responses were obtained for standard solution of glucose (from 1.0 to 200.0 µM in 0.01 M PBS, pH=7.0). The results shown in this figure represent the integrated signal for 3 to 5 consecutive flow injections of the standard solution. A correlation coefficient of R=0.993 with %R.S.D. values ranging from 0.24–3.5% across the concentration range studied were obtained following linear regression analysis.

As mentioned above the electrode response could be expressed in various ways as peak heights or peak areas in the FFTCV. For this reason, the magnitude of the flow-injection response depends on the choice of the data processing methods.

The linearity was evaluated by linear regression analysis, which calculated by the least square regression method. The detection limit, estimated based on signal to noise ratio (S/N=3), was found to be 0.03 µM. The long-term storage stability of the sensor was tested for 30 days. The sensitivity retained 92.3% of initial sensitivity up to 50 days which gradually decreases afterwards might be due to the loss of the catalytic activity.

In assessment, the performances of the fabricated biosensor is compared with some of the best recently reported glucose biosensors based on the utilization of different materials as the working electrode and different detection techniques (Table 1), it was confirmed that the presented

GOx/AuNPs/MWCNTs/GC electrode combined with FFT continuous cyclic voltammetry method exhibits an outstanding and reproducible sensitivity for the glucose biosensor [8,27–30].

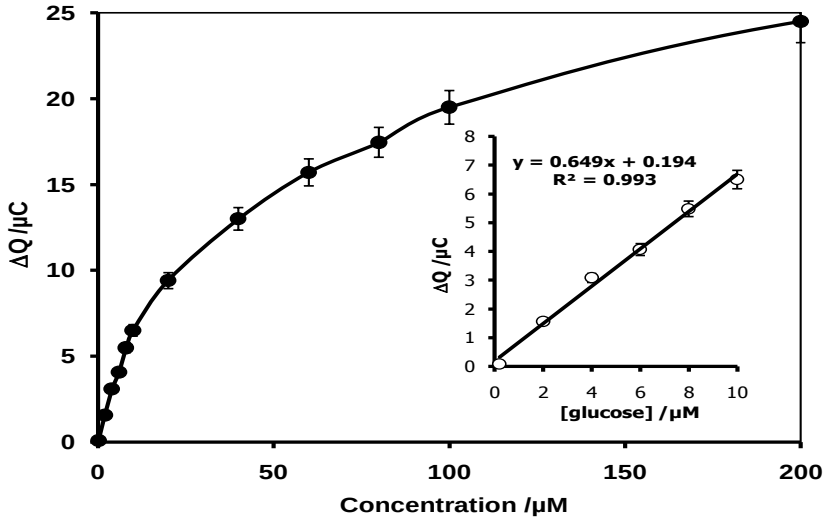

Fig. 6. The calibration curve for glucose determination in 0.01 M PBS

Table 1. The comparison of the proposed biosensor with the best recently reported ones based on the utilization of different materials

Ref.	Detection Method	Materials	Electrode	DL
8	Amperometry	AuPt NPs-CNTs-ionic liquids	GC	10 μM
27	Amperometry	chitosan/$NiFe_2O_4$ NPs	GC	100 μM
28	Amperometry	Fe3O4–Au–poly(HDT) (PHDT)–GOx magnetic polymeric bionanocomposites	Au	0.3 μM
29	Amperometry	ferrocene-carbonyl-beta-cyclodextrin/MWCNTs	GC	2.2 μM
30	Capacitance Measurement	concanavalin A-labeled nanogold colloids	Au	1 μM
This work	FFTCCV	Au NPs/MWCNTs	GC	0.03 μM

4. CONCLUSIONS

In this work, a highly sensitive glucose biosensor has been fabricated by modifying the GC electrode surface with AuNPs and MWCNTs. Fast Fourier transformation continuous cyclic voltammetry (FFTCCV) was used as detection technique in which the charge under the peak calculated in a specific potential range. The flow injection system was coupled with the electrochemical method. Ferrocene methanol in PBS buffer (pH=7.0) was used as the flowing eluent. To the best of our knowledge, it is the first time that FFTCV flow injection analysis, a very high-sensitivity and low detection limit, is used for glucose biosensors. The sensitivity retained 92.3% of initial sensitivity up to 50 days which gradually decreases afterwards might be due to the loss of the catalytic activity. The long-term storage stability of the sensor was tested for 30 days.

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