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Hydrogen Peroxide Biosensor Based on Hemoglobin Immobilization on Gold Nanoparticle in FFT Continuous Cyclic Voltammetry Flow Injection System

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A novel biosensor was designed for selective determination of hydrogen peroxide in flow injection analysis (FIA) system. The FIA measurement was based on fast Fourier transformation continuous cyclic voltammetry (FFTCCV) detection method in which the charge under the peak current of hydrogen peroxide calculated in a specific potential range. The constructed biosensor is a glassy carbon electrode which is modified by multiwall carbon nanotubes (MWCNTs), gold nanoparticles (AuNPs) and hemoglobin immobilization. The characterization of the modified electrode was studied by scanning electron microscopy (SEM) and electrochemical impedance spectroscopy methods (EIS). The combination of nanoparticles catalyzed electron transfer and amplifying the detection signal. The peak current of hydrogen peroxide on the enzyme activity was proportional to its concentration in the range of 0.1 to 20 µM, and a detection limit of 20 nM. Experimental parameters affected the sensitivity of the biosensors, including potential scan rate, solution flow rate and buffer pH was studied. The proposed biosensor showed good reproducibility, long-term storage stability and accuracy in analysis.

Keywords: Gold nanoparticals, multiwall carbon nanotube, hemoglobin, hydrogen peroxide, biosensor, FFT cyclic voltammetry, flow injection analysis

1. INTRODUCTION

The measurement of hydrogen peroxide (H_2O_2) is of great importance due to its significance as mediator in food, pharmaceutical, clinical, industrial and environmental analyses.

In this work, a new electrochemical biosensor for monitoring of hydrogen peroxide is introduced based on FFT continuous cyclic voltammetry (FFTCCV) technique in a flow injection

analysis system. To the best of our knowledge, this is the first application of FFTCCV method as detection method of a hydrogen peroxide biosensor.

Fast Fourier transform method provides a sensitive system when combine by electrochemical method for trace analysis of compounds [1-5]. Most of the electrochemical signals have a defined value for every possible instant in time. They are called continuous signal like analog voltage and current. To analyze these continuous signals by a computer-based system, the signal should convert to digital signal in which each sample representing a numeric value that is proportional to the measured signal at a specific instant in time. Thus, the sampling process used for electrochemical measurements creates digital signal data spaced on an even interval of time. Fourier Transform (FT) is a defined technique for converting or transforming electrochemical signals from the time domain to the frequency domain. Hence, the electrochemical signal does not suffer from environmental noises. The approach used here is designed to separate the voltammetric signal and background signal in frequency domain by using discrete Fast Fourier Transformation (FFT) method. Based on FFT information, the cutoff frequency of the analog filter is set at a certain value. Thus, some of the noises filtrate digitally and decrease the bandwidth of the measurement [6-9].

The proposed biosensor is based on hemoglobin immobilized by gold nanoparticles on modified surface of glassy carbon with multiwall carbon nanotubes (MWCNTs). The characterization of the modified electrode was studied by scanning electron microscopy (SEM) and electrochemical impedance spectroscopy methods (EIS).

Hemoglobin (Hb) is the iron containing oxygen transport metalloprotein used extensively as an ideal electron transfer molecule for in biosensors. Hb is a tetrameric protein consisting of four subunits and each subunit is a combination of a polypeptide chain and a heme. The heme consists of an iron atom (Fe^{2+} or Fe^{3+}) attached to a planar organic structure belonging to porphyrin family. Hb, the main component of red blood cells, is a protein that picks up oxygen in the lungs and delivers it to the body tissues and also offers Hb the ability to reduce different targets such as H_2O and nitrite. The mechanisms can be expressed as previously reported in the literature [10,11]:

$$Hb(Fe^{3+}) + H_2O_2 \rightarrow \text{Intermediate I } (Fe^{4+}=O) + H_2O$$

$$\text{Intermediate I } (Fe^{4+}=O) + e + H^+ \rightarrow \text{Intermediate II}$$

$$\text{Intermediate II} + e + H^+ \rightarrow Hb(Fe^{3+}) + H_2O$$

Hb is a commercial available and is a cost effective compound. However, for electron transfer with common electrode surfaces requires suitable mediators.

Metal nanoparticles have generally high effective surface area, catalysis and biocompatibility properties [12-14]. Recently, gold nanoparticles (AuNPs) have become very important in the field of electrochemical biosensors. AuNPs could adsorb redox enzymes without loss of their biological activities. AuNPs are prepared by chemical reduction of the Au salts in the presence of a suitable stabilizer, which binds to their surface [15]. The practical advantage of AuNPs is the size and surface morphology which can be controlled experimentally by adjusting the preparation conditions [15].

Small size of AuNPs (diameter about 5–50 nm) helps to a better electron transfer distance between the enzyme and the electrode [15].

MWCNTs have been recently used in various areas including biosensors and biofuel cells due to their high surface area, high surface/volume ratio, good electrical conductivity and significant mechanical strength [16].

2. MATERIALS AND METHODS

2.1. Reagents

Hemoglobin Hb (from bovine serum) was purchased from Sigma and used as received. Hydrogen peroxide (30%w/v) was obtained from Merck. Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4.3H_2O$), nafion (5 %wt.) solution in a mixture of lower aliphatic alcohols and water were purchased from Aldrich Co. MWCNTs (diameter: 10–20 nm; length: 0.5-40 nm; purity: ≥95%) were obtained from Shenzhen Nanotech Port Ltd. Co (Shengzhen, China). Phosphate buffer solutions (PBS, 0.1 M) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with H_3PO_4 or NaOH. All other chemicals were of analytical grade and were used without further purification. All solutions were made up with doubly distilled water.

2.2 .Preparation of hydrogen peroxide biosensor

A glassy carbon electrode, GCE, (3 mm in diameter) were polished well with 1.0, 0.3 and 0.05 μm alumina slurry and then it was washed thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature.

0.1-0.8 mg of MWCNTs was dispersed in dimethylformamide (DMF) with the aid of ultrasonic agitation to give a 0.1-0.8 mg/mL black suspension. Then, 5 μL of the suspension was dropped on a cleaned GC electrode and let the solvent was evaporated in the 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.

6.5 μL of 0.1 mM Hb (in PBS 0.1 M; pH=7.0) was dropped on the modified GCE surface, and allowed to dry under ambient conditions for 3 h to obtain Hb/AuNPs/MWCNTs/GCE. The modified electrode was rinsed with doubly distilled water. When not in use the Hb/AuNPs/MWCNTs/GCE stored in PBS 0.1 M, pH=7.0 at 4 °C.

To prevent loss of the enzyme molecules and to improve the anti-interfering ability of the biosensor, 5 μL of nafion (5 wt.%) was casted and used to hold the Hb/AuNPs/MWCNTs on the electrode surface stably. The process was shown in Fig. 1.

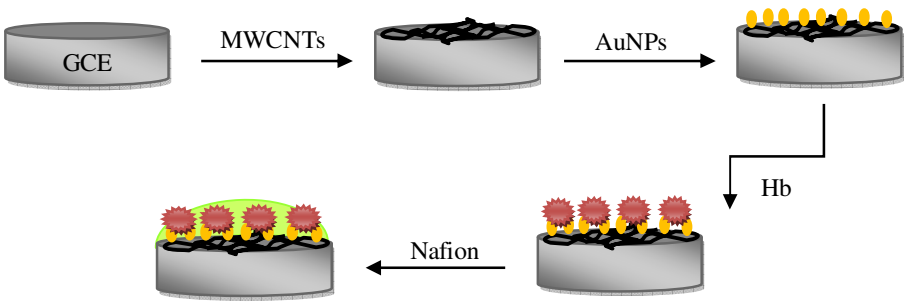

Figure 1. Schematic diagram of biosensor preparation

2.3. Instrumentation

In the electrochemical FFTCCV experiments was performed by a homemade potentiostat controlled via a PC PIV for acquiring, storing and displaying the data. An analog to digital (A/D) data acquisition board (PCL-818H, Advantech Co.) was used for generating the analog waveform and acquiring data current from the electrochemical system. In addition, 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 plotted the voltammograms. Also, for all the electrochemical the potential waveform was repeatedly applied to the biosensor.

2.4. Flow Injection Setup

An eight roller peristaltic pump (UltrateckLabs Co., Iran) was employed for the flow injection analysis. The FIA was equipped with and a four way injection valve (Supelco Rheodyne Model 5020. The analyte solutions were introduced into the sample loop (valume of300 µL) by a plastic syringe. The electrochemical cell used in flow injection analysis is shown in Fig. 2.

3. RESULTS AND DISCUSSION

The sensitivity of FFTCV measurement is related to the physical morphology of the biosensor surface. Therefore, in order to study the electrode surface, SEM of the electrode surface was done. Fig. 3 presents the typical SEM images of the bare GC and Hb/AuNPs/MWCNTs/GCE electrode surface. Significant differences in the surface structure of GCE (Fig. 3A) and Hb/AuNPs/MWCNTs/GCE (Fig. 3B) are observed. The surface of the biosensor was predominated by isolated and regularly shaped MWCNTs and AuNPs (Fig. 3B). Moreover, the SEM image of Hb/AuNPs/MWCNTs/GCE showed more uniform surface and no separated carbon layers could be observed in.

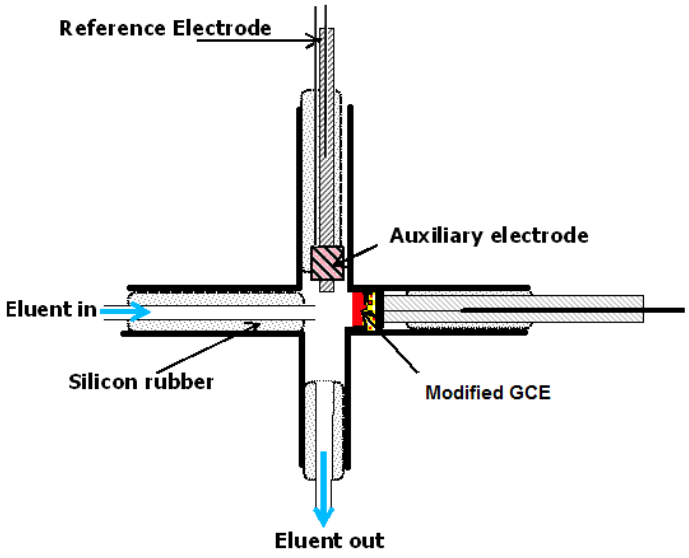

Figure 2. The diagram of the electrochemical cell used in FIA measurement

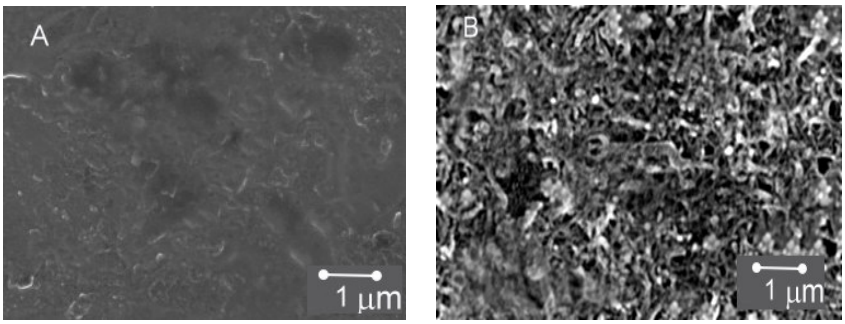

Figure 3. SEM image of the surface of A) bare GCE and B) Hb/AuNPs/MWCNTs/GC biosensor

It is well know that EIS is a valuable electrochemical method to investigate the interfacial properties of modified electrode. Also, it can be used for understanding processes associated with the conductive layers on the electrode surface. Fig. 4 illustrates the obtained results from EIS measurements on bare GCE (a), AuNPs/MWCNTs/GCE (b) Hb/AuNPs/MWCNTs/GCE (c) in the presence of equivalent 20 mM $Fe(CN)_6^{4-/3-}$ in 0.2 M KCl, which are measured at the formal potential of $Fe(CN)_6^{4-/3-}$. The figure shows that at the bare GCE, a semicircle of about 600 Ω indicating a low electron transfer resistance to the redox-probe in the solution. The diameter of the high frequency semicircle was clearly reduce to less than 200 Ω by the surface modification of the AuNPs/MWCNTs layer (curve b), suggesting that a significant acceleration of the $Fe(CN)_6^{4-/3-}$ redox reaction occurred due to the presence of AuNPs/MWCNTs layers.

In addition, the further deposition of Hb/AuNPs/MWCNTs layer cause the resistance of the high frequency semicircle increase (curve c), which indicates Hb has been immobilized successfully on the modified electrode surface.

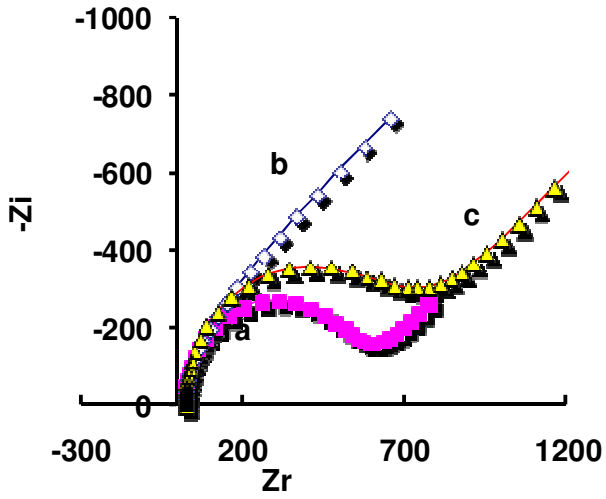

Figure 4. EIS plots in 15 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture containing 0.1 M KCl at bare GCE (a) AuNPs/MWCNTs/GCE (b) and Hb/AuNPs/MWCNTs/GCE (c) respectively

Initially, the constructed Hb/AuNPs/MWCNTs/GCE biosensor was activated in PBS (pH=7.0) by FFTCCV scans for some time in potential range of 0 V to -800 mV until stable CV curve was obtained. For the measurements of hydrogen peroxide, a FIA system was design and 400 µL of various concentrations of hydrogen peroxide were injected into the system. Fig. 5a demonstrates typical FFTCCVs of the biosensor in the potential range of 0 to -800 mV at potential sweep rate of 4 Vs^{-1}. In the Figure, the time axis represents the time passing between the beginning of the flow injection experiment and the beginning of sweeps (i.e. it represents a quantity proportional to the sweep number) [6-9].

In Fig. 5b, the typical FIA response based on signal processing, in which the biosensor signal is calculated based on the background subtraction and current integration at the range of -100 to -700 mV is shown. In this method, all the currents in CVs were subtracted from the CV that recorded in absent of hydrogen peroxide,

$$\Delta I = i - i_0 \qquad (1)$$

Where i is the electrical charge obtained analyte peak current of cyclic voltammetric, and i_0 represents i in the absence of the adsorbent. Before injection of hydrogen peroxide, the biosensor in 0.1 M PBS, showed an irreversible oxidation peak at -400 mV. In fact, this peak came from the oxidation of H_2O_2, catalyzed by the modified electrode.

In fact, a higher the peak current at -400 mV is due to the presence of Hb/AuNPs/MWCNTs which possessed natural conductive properties and catalytic behavior, which give a good conductive pathway to electron transfer. Moreover, the recorded CVs indicated that before injection (in absent of hydrogen peroxide) there is no significant peak current in the CVs, but by injection of 400 µL of 1.0×10^{-6} M hydrogen peroxide in 0.1 M buffer solution(PBS) at pH=7.0 a noticeable current changes.

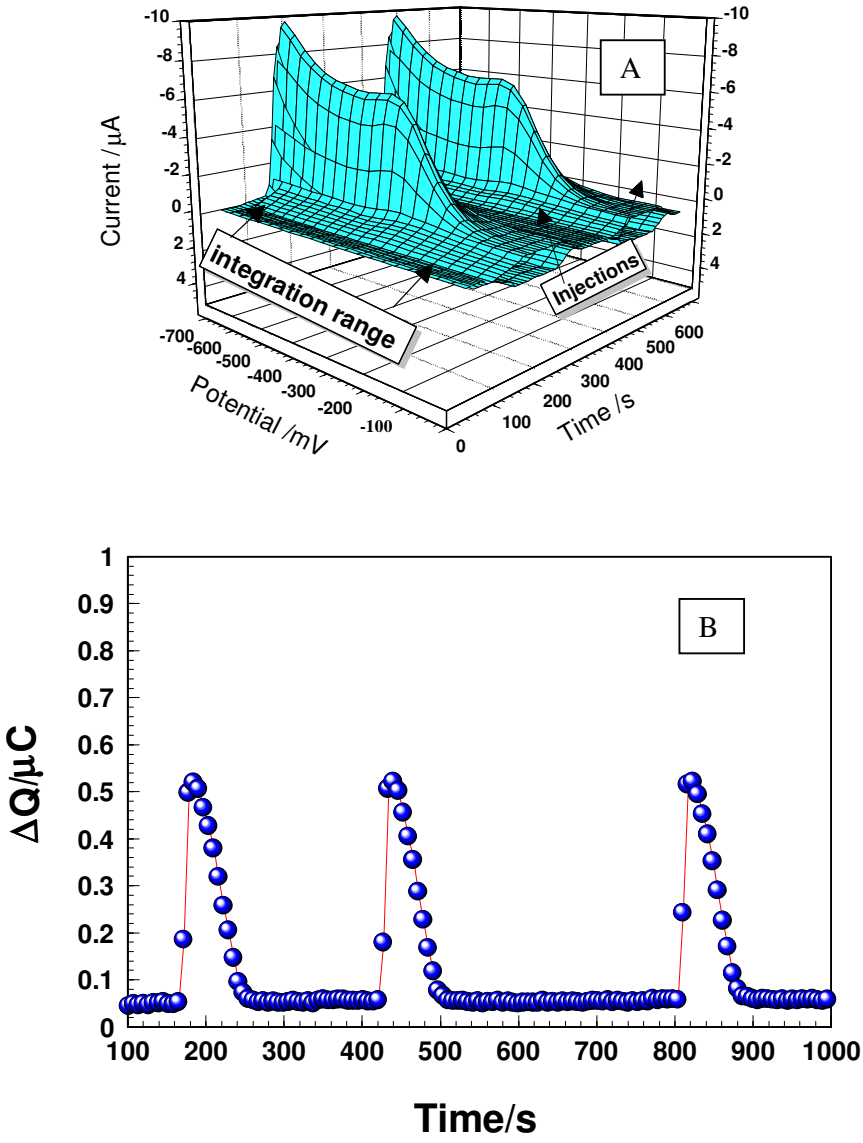

Figure 4. A) FFT cyclic voltamograms of the Hb/AuNPs/MWCNTs/GC electrode without (in absent) and with injection of 400 µL of 2.0×10^{-6} M hydrogen peroxide in 0.1 mM PBS at pH=7 in the potential range of 0 to -800 mV at 4 V/s. and the potential Integration range for the current. B) The calculated response of the biosensor from Eq.3.

FFTCCV method was used for determination of hydrogen peroxide in FIA system. The injection of the analyte creates a measurable current change in the recorded CVs. According to Eq. 2,

the electrochemical reaction for the detection of hydrogen peroxide in presence of Hb is proposed catalytic reaction. In fact, in this method the current passing through the electrode was sampled only during the potential ramp. The data processing for calculating the biosensor response was carried out simultaneously with current measurement during FIA experiments. The result of the integration is shown in Fig. 5b. The peaks are due to three consecutive injections of the same sample. The integration of net current changes is applied over the selected scanned potential range. The biosensor response (charge change under the peak) was calculated as;

$$\Delta Q = Q - Q_0 \qquad (2)$$

where Q is the electrical charge obtained by integration of cyclic voltammetric curve between 0 and -800 mV in the chatodic scan, and Q_0 represents Q in the absence of the adsorbent. The peak in this method, ΔQ is calculated based on the current changes at the CV in the integration potential range. An absolute difference charge function (ΔQ) can be calculated by using numerical calculation in software based on this equation:

$$\Delta Q\ (s\tau) = \Delta t\left[\sum_{E=E_i}^{E=E_f} \left| i(s,E) - i(s_r,E) \right|\right] \qquad (3)$$

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 CVs during the s-th scan and i (s_r, E) is the reference current of the CVs. E_i and E_f are the initial and the final potential integration range, respectively.

The selected integration range for the current is shown in Fig. 5a. The reference CV was obtained by averaging a 10 CVs, obtained at the beginning of the measurement (i.e. before injection of the analyte). Moreover, the results indicate that with increasing the concentration of hydrogen peroxide in the injected sample, ΔQ increases proportionally.

3.1. Optimizing the experimental parameters

Fig. 6 demonstrates the influence of the scan rates and the eluent flow rate on the sensitivity of the detector response, at scan rates (from 1 to 8 V/s) and the eluent flow rate (0.2 to 4 $mLmin^{-1}$) from injecting solutions of 2.0×10^{-6} M of hydrogen peroxide. In the measurement method, the sensitivity of the biosensor toward hydrogen peroxide depends on the potential sweep rate and eluent flow rate, which is principally because of the kinetic factors of the electrode processes at the electrode surface, and instrumental limitations [17-21].

As is show in Fig. 6, the biosensor exhibits the maximum sensitivity (or signal) at 4 V/s of scan rate and 1.5 mL min^{-1} of the eluent flow rate. However, the effects of the sweep rate on the biosensor

operation can be taken into consideration from three different factors; speed of data acquisition, kinetic factors of electrochemical processes at the electrode surface, and the flow rate of the eluent.

The eluent flow rate controls the time retention of the solution sample zone in the electrochemical cell. 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 hydrogen peroxide.

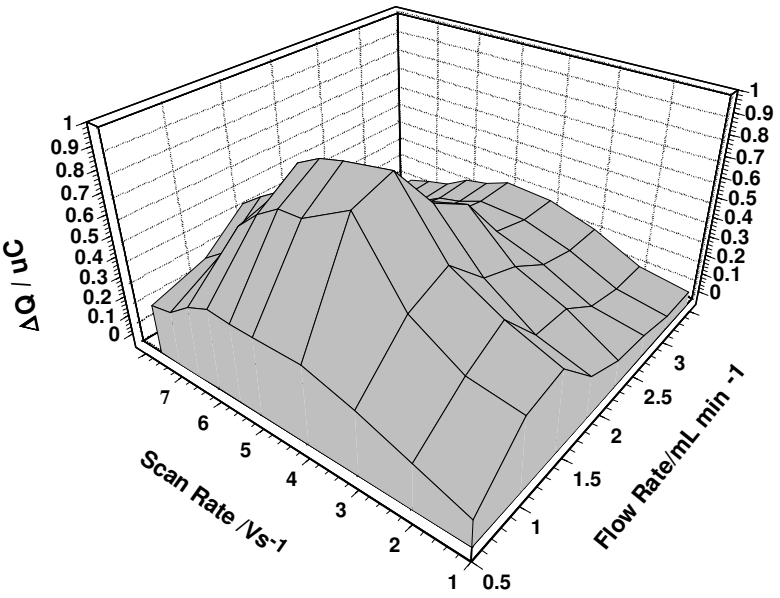

Figure 6. Effect of the sweep rate and effect of flow rate on the response of Hb/ AuNPs/MWCNTs/GC electrode to injections of 2.0×10^{-6} M hydrogen peroxide in 0.1 M PBS at pH=7.0

3.2. Effect of pH, AuNPs and Hb concentration on the biosensor response

Investigation of the effect of the pH value on the performance of the biosensor is very importance, because the activity of the immobilized Hb is pH dependent. The results of measurements of the electrode response ΔQ in the pH range of 5.0–8.0 (see Fig. 7), indicate that ΔQ response is maximum at pH 7.0, where the activity of the Hb is the highest.

Fig. 8 shows the effect of time of AuNPs deposition, and amount of MWCNTs on the biosensor response to 2.0×10^{-6} M hydrogen peroxide in 0.1M PBS at pH=7.0. As shown in the graph, the value of the biosensor response increase with increasing the deposition time of AuNPs which is corresponds to amount of AuNPs on the electrode surface, and reaches to a maximum up to 200 s.

On the other hand, at the higher deposition time up to 200 s the value of ΔQ decrease, which can be due to the increase of the resistance of the surface electrode. Moreover, Fig 8 illustrates that the change of the biosensor sensitivity with the weight of MWCNTs added to the content of the modifier at the surface. The results specify that the biosensor response increase with the adding weight of MWCNTs up to 0.4 mg and then reaches to almost a constant value at higher weights.

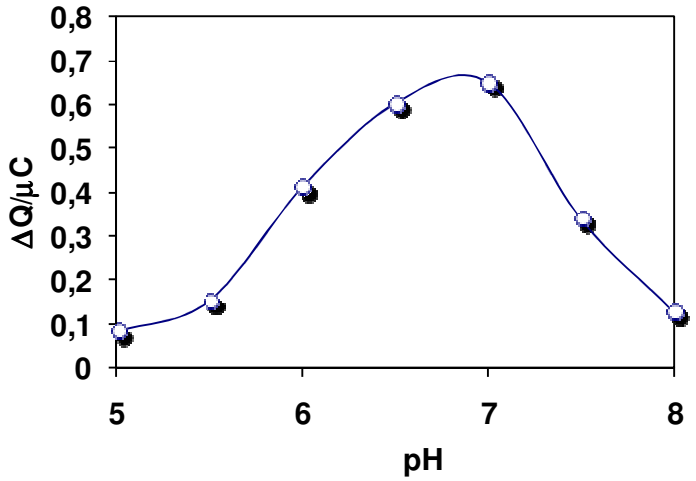

Figure 7. The effect of pH on response of the biosensor of the biosensor to 2.0×10^{-6} M hydrogen peroxide in 0.1M PBS at pH=7.0

Fig. 9 shows the change of the biosensor sensitivity with the amount of Hb added to the content of the electrode surface. The graph indicates that the value of ΔQ increase with the adding volume of Hb and then reaches to a constant value. It is appears that more than 6.5 μL Hb the electrode goes to a saturation state.

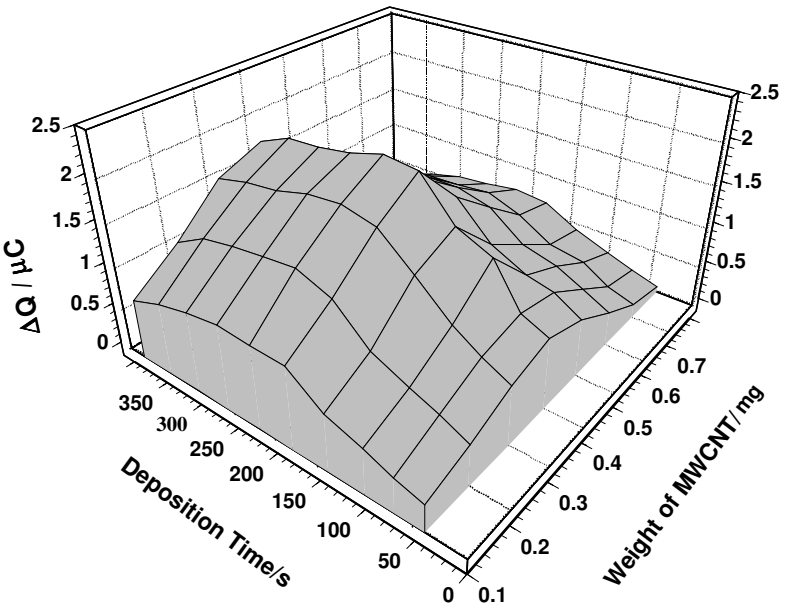

Figure 8. The effect of amount of AuNPs and MWCNTs on the biosensor response to 5.0×10^{-6} M of hydrogen peroxide in 0.1M PBS at pH=7.0

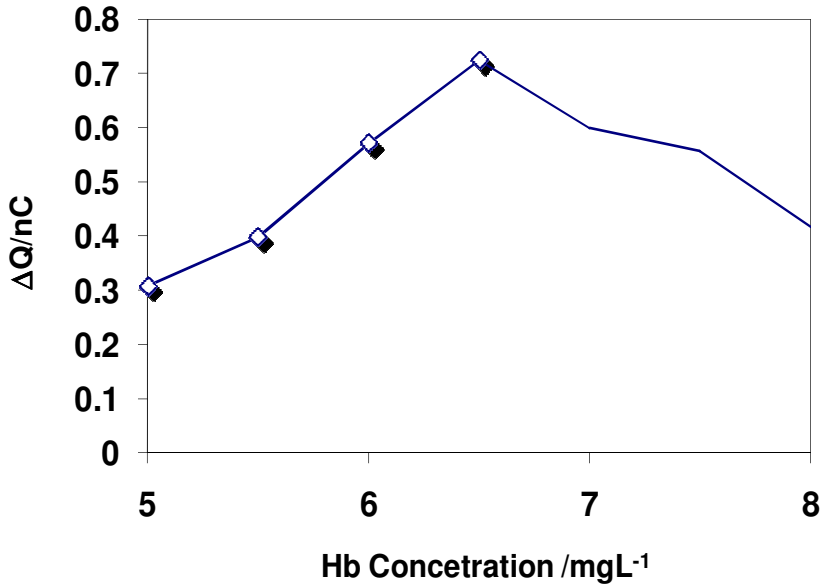

Figure 9. The effect of Hb concentration on the biosensor response to 2.0×10^{-6} M of hydrogen peroxide in 0.1M PBS at pH=7.0

3.3. Calibration curve and biosensor characterization

Fig. 10 illustrates that the peak current decrease in present of hydrogen peroxide in 0.1M PBS at pH=7.0 solution, and the biosensor response increase with an increasing hydrogen peroxide concentration, which is proportional to concentration hydrogen peroxide in solution.

Fig. 10 shows FFTCCV response curves of the biosensor. The regression equation is ΔQ (μC)=0.181C(μM),+0.3575. The biosensor responses were obtained for standard solution of hydrogen peroxide (from 0.1 to 300 μM in 0.1 M PBS at pH=7.0 solution). The linearity was evaluated by linear regression analysis, which calculated by the least square regression method

The figure represents the response of the biosensor for 3 consecutive flow injections of the standard solution of hydrogen peroxide. A correlation coefficient of R^2=0.9947 with %R.S.D. values ranging from 0.3–4.5% across the concentration range studied were obtained following linear regression analysis.

The detection limit, calculated based on signal to noise ratio (S/N=3), was found to be 20±0.8 nM. The long-term storage stability examination of the sensor was tested for 30 days. The sensitivity retained 96.2% of initial sensitivity up to 40 days, and then slowly decreases afterwards might be owing to the defeat of the catalytic activity of the enzyme surface.

3.5. Interference

In negative potential, no significant interference affects the detection of H_2O_2 except the dissolved oxygen. Thus, all solutions in this work were deoxygenated by bubbling nitrogen for at least 15 min and maintained under nitrogen atmosphere during the experiments.

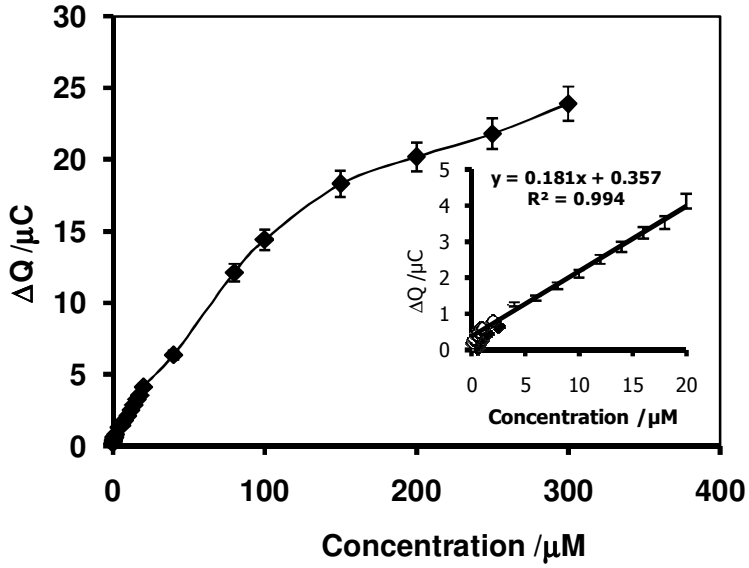

Figure 10. The calibration curve for hydrogen peroxide biosensor determination in 0.1M PBS at pH=7.0

4. CONCLUSIONS

In this work, a fast and sensitive hydrogen peroxide biosensor is fabricated by modifying the GC electrode surface with Hb/AuNPs/MWCNTs/GCE, and based on the inherent conductive properties of MWCNTs and AuNPs, the immobilized HB exhibited a higher affinity to the substrate and produced detectable and significant response in FFTCCV method for determination of hydrogen peroxide. To the best of our knowledge, the method is the first time that a very high-sensitivity and low detection limit flow injection analysis that is used for hydrogen peroxide biosensors based on Hb/AuNPs/MWCNTs/GCE. A good producible sensitivity of 10 μCυM/cm^{-2}, response time less than 30 s and detection limit of 20 nM was observed from the fabricated biosensor. The long-term storage stability of 40 days was observed.

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