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A Novel Acetylcholinesterase Biosensor Based on Chitosan-Gold Nanoparticles Film for Determination of Monocrotophos Using FFT Continuous Cyclic Voltammetry

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In this paper, a new flow injection enzymatic acetylcholinesterase (AChE) biosensor was designed for selective determination of monocrotophos (an organophosphate pesticide). The biosensor was constructed by modifying glassy carbon electrode surface with gold nanoparticles (AuNPs) and multiwall carbon nanotubes (MWCNTs) while chitosan microspheres used to immobilize AChE. The measurement method was based on fast Fourier transform continuous cyclic voltammetry (FFTCCV) in which the charge under the peak calculated in a specific potential range. The characterization of the biosensor was studied by Scanning Electron Microscopy (SEM) and electrochemical impedance spectroscopy methods (ESI). The combination of MWCNTs and AuNPs promotes the electron transfer and catalyzes the electro-oxidation of thiocholine, and causes the amplifying the detection sensitivity. The inhibition of the enzyme activity by monocrotophos was proportional to monocrotophos concentration in the range of 0.1 to 10 µM, with a detection limit of 10 nM. Experimental parameters of measurement which affect the sensitivity of the biosensors, including potential scan rate, solution flow rate and buffer pH were optimized. The proposed biosensor shows good reproducibility, long-term storage stability and accuracy in analysis.

Keywords: gold nanoparticals, carbon nanotube, acetylcholinesterase, monocrotophos, chitosan microspheres, biosensor, FFT cyclic voltammetry

1. INTRODUCTION

Nowadays, electrochemical enzyme biosensors based nano-particles offer high sensitivity; long term stability and low cost detection in biological important molecule determinations [1-6].

Monocrotophos, Fig. 1, is an organophosphate insecticide. It is acutely toxic to birds and humans. Monocrotophos is classified WHO Ib, highly hazardous, and has been responsible for deaths resulting from accidental or intentional exposure. It is highly toxic orally, as well by inhalation or absorption through the skin.

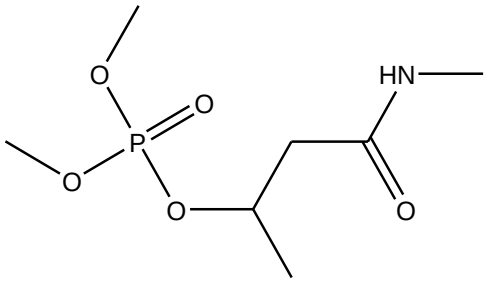

Figure 1. Chemical structure of monocrotophos

Acetylcholinesterase (AChE) is an enzyme that produces choline and an acetate group by break downing the neurotransmitter acetylcholine. It is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity serves to terminate synaptic transmission. AChE has a very high catalytic activity; each molecule of AChE degrades about 25000 molecules of acetylcholine per second. The produced choline can be back into the nerve terminals to use in synthesizing of new acetylcholine molecules [7].

An acetylcholinesterase inhibitor (AChEI) or anti-cholinesterase is a chemical that inhibits the cholinesterase enzyme from breaking down acetylcholine, increasing both the level and duration of action of the neurotransmitter acetylcholine. Monocrotophos is one of the organophosphates which can be as an AChEI. The inhibition of the enzyme activity by monocrotophos was proportional to monocrotophos concentration [8,9].

This work introduces a new flow injection electrochemical biosensor combine with FFT continuous cyclic voltammetry (FFTCCV) technique [10-14] as a sensitive method for rapid determination of monocrotophos. To the best of our knowledge, this is the first application of FFTCCV method for monocrotophos biosensor based on enzyme (AChE). In addition, the fabrication, characterization and analytical performance of the biosensor based on gold nanoparticles (NPs) and multiwall carbon nanotubes (MWCNTs) with chitosan microspheres to immobilize AChE was investigated.

Also, the experimental parameters, which can affect the sensor performance were optimized and the electrochemical characteristics of the sensor were described.

2. MATERIALS AND METHODS

2.1. Reagents

Acetylcholinesterase (Type VI-S; 500 U mg^{-1} from electric eel) and acetylthiocholine chloride (99%), $HAuCl_4.4H_2O$ (Au% ≥50%) monocrotophos were all purchased from Sigma–Aldrich. Chitosan

(95% deacetylation), phosphate buffer solution (PBS, pH=7.0) and other reagents used were of analytical reagent grade were purchased from Merck Co. MWCNTs (diameter: 10–20 nm; length: 0.5-40 nm; purity: ≥95%) were obtained from Shenzhen Nanotech Port Ltd. Co (Shengzhen, China). All other chemicals were of extra pure analytical grade and used without further purification. All aqueous solutions were prepared in doubly distilled water.

2.2 .Preparation of acetylcholinesterase biosensor

Since chitosan is a biocompatible polymer and its usages with MWCNTs previously had shown good electrocatalytic ability in the reduction and oxidation of hydrogen peroxide [15], it was used as a suitable matrix for enzyme stabilization.

Chitosan stock solution of 0.025%w/v was prepared by dissolving chitosan flakes in an acetic acid solution (2.0 M) and the pH was adjusted to 5.0 using NaOH solution. The stock solution of chitosan was kept in a refrigerator [15,16].

Glassy carbon electrodes (5 mm diameter) were polished first with 1.0, and 0.05-μm alumina slurry. After rinsing with doubly distilled water, and then sonicated in absolute ethanol and doubly distilled water for about 5 min, respectively.

A clean GC electrode was immersed into 2.0 mL of 0.025% w/v chitosan solution including 0.01%v/v $HAuCl_4.4H_2O$ and a -3.0 V potential were applied on the electrode. The deposition time was about 250 s. H^+ in the solution was reduced to H_2 at the cathode, and the pH near the cathode surface gradually increased. As the solubility of chitosan is pH-dependent, when the pH exceeds the pK_a of chitosan (~6.3), chitosan deposit onto the cathode electrode surface as a result [16].

The deposited electrode was dried in air at room temperature for about 3 h. Subsequently a 20 μL mixture of AChE and MWCNT (1:1) was dropped onto the deposit glassy electrode and allowed to dry at ambient temperature to obtain the AChE-MWCNTs/AuNPs-chitosan/GC modified electrode.

The modified electrode exhibited higher electroactive surface area, indicating faster electron-transfer kinetics at the electrode than at the bare electrode.

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. Carbon nanotubes (CNTs) can be used as a suitable intermediates between electrodes and enzymes.

2.3. Instrumentation

In the electrochemical FFTCCV experiments a homemade potentiostat was used, which was controlled by a PC PIV for acquiring and storing the data. In the Electrochemical system, generating the analog waveform and acquiring current was by analog to digital (A/D) data acquisition board (PCL-818H, Advantech Co.). The potential waveform was repeatedly applied to the biosensor and then. For the measurements, 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 ploted the voltammograms.

2.4. Flow Injection Setup

All of the flow injection analysis use for measurements, the equipment was integrated with an eight roller peristaltic pump (UltrateckLabs 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.

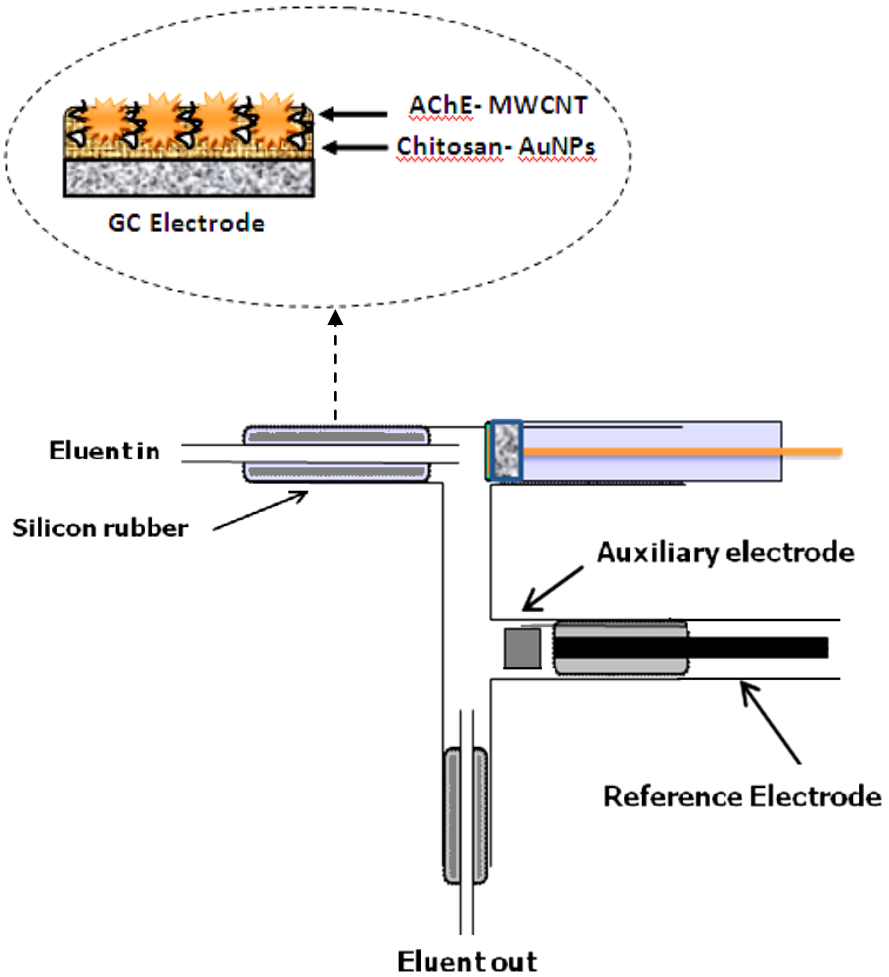

Figure 2. The diagram of monocrotophos biosensor and the electrochemical cell used in flow injection analysis

The used eluent in the measurements of monocrotophos is acetylthiocholine chloride (1.0 mM) in the PBS (pH=7) buffer. The organophosphate insecticide inhibits the following reaction:

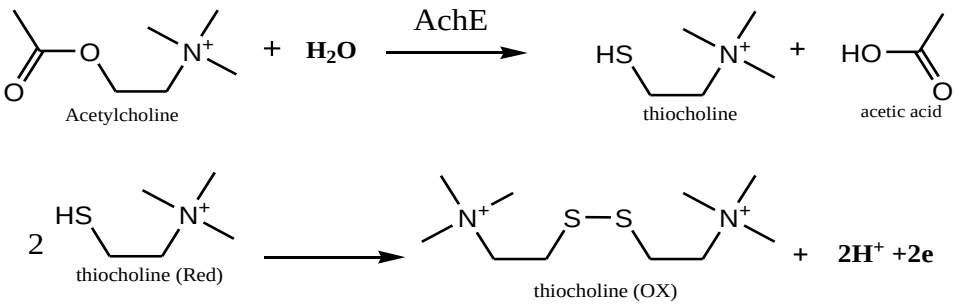

Figure 3. The biosensor reactions for determination of monocrotophos. The organophosphate insecticide inhibits the enzyme activity.

By inhibition of enzyme activity, the monocrotophos concentration can be measured indirectly.

3. RESULTS AND DISCUSSION

In FFTCV measurement, the sensitivity of the detection is related to its physical morphology of surface of the biosensor electrode. Therefore, in order to study the electrode, Scanning Electron Microscopy (SEM) of the electrode was carried out. Fig. 4 shows the typical SEM images of AChE-MWCNTs/AuNPs-Chitosan/GC electrode surface.

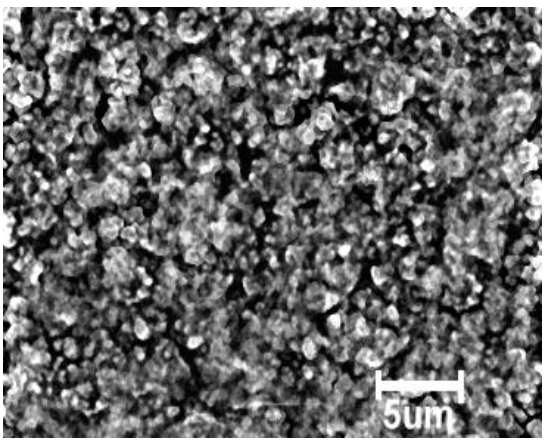

Figure 4. SEM image of the modified electrode surface (AChE-MWCNT/AuNPs-Chitosan/GC)

Fig. 5 illustrates the results of electrochemical impedance spectroscopy (EIS) on bare GCE (a), AuNPs-Chitosan/GC (b) AChE-MWCNTs/AuNPs-Chitosan/GC (c) in the presence of equivalent 15 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl, which are measured at the formal potential of $Fe(CN)_6^{4-/3-}$. This figure shows that at the bare GC, a semicircle of about 1100 Ω indicating a low electron transfer

resistance to the redox-probe dissolved in the electrolyte solution. The diameter of the high frequency semicircle was obviously reduce to 900 Ω by the surface modification of the AuNPs/chitosan layer (curve b), suggesting that a significant acceleration of the $Fe(CN)_6^{4-/3-}$ redox reaction occurred due to the presence of AuNPs-Chitosan assembly.

Also, the further deposition of AChE-MWCNTs layer cause the resistance of the high frequency semicircle (400 Ω) reduced (curve c), which indicates a decreased resistance to the anion redox reaction at the AChE-MWCNT/AuNPs-Chitosan/GC. This may be attributed to the well conductivity of the surface deposited of MWCNTs.

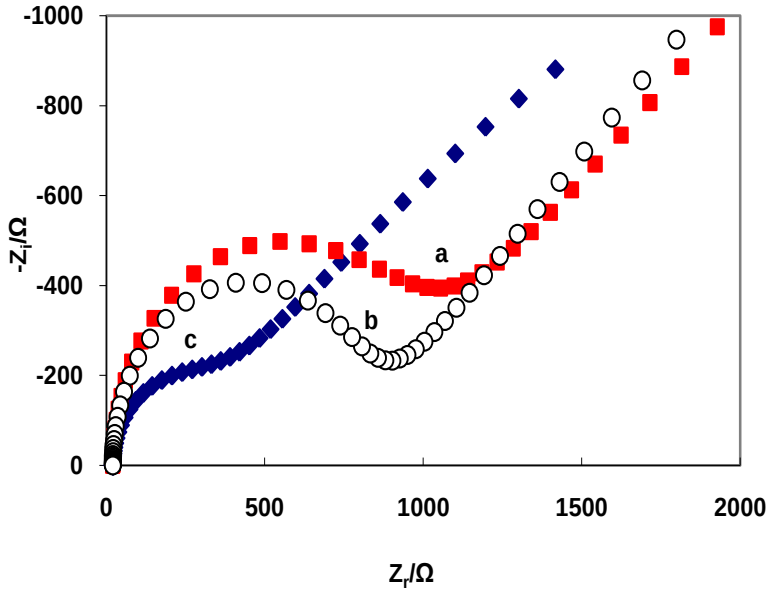

Figure 5. EIS plots in 15 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture containing 0.1 M KCl at bare GC (a) AuNPs-Chitosan/GC (b) and AChE-MWCNTs/AuNPs- Chitosan/GC (c)

The prepared AChE-MWCNTs/AuNPs-Chitosan/GC was first activated in pH of 7.0 PBS by FFTCCV scans from 0 to 1000 mV until stable baseline curve was obtained. For the measurements of monocrotophos, a FIA system was design (the used eluent is acetylthiocholine chloride (1.0 mM in the PBS buffer) and 200 µL of various concentrations of monocrotophos solutions were injected into the system. The organophosphate insecticide inhibits the enzymatic reaction, thus the redox signal of acetylthiocholine chloride which is proportional to the monocrotophos concentration are reduced.

Fig. 6a demonstrates typical FFTCCVs of the AChE-MWCNTs/AuNPs-Chitosan/GC electrode in the potential range of 0 to 1000 mV at potential sweep rate of 5 Vs^{-1}. In the three dimensional graph, 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) [13-21]. In Fig. 6b, 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 200-900 mV is shown.

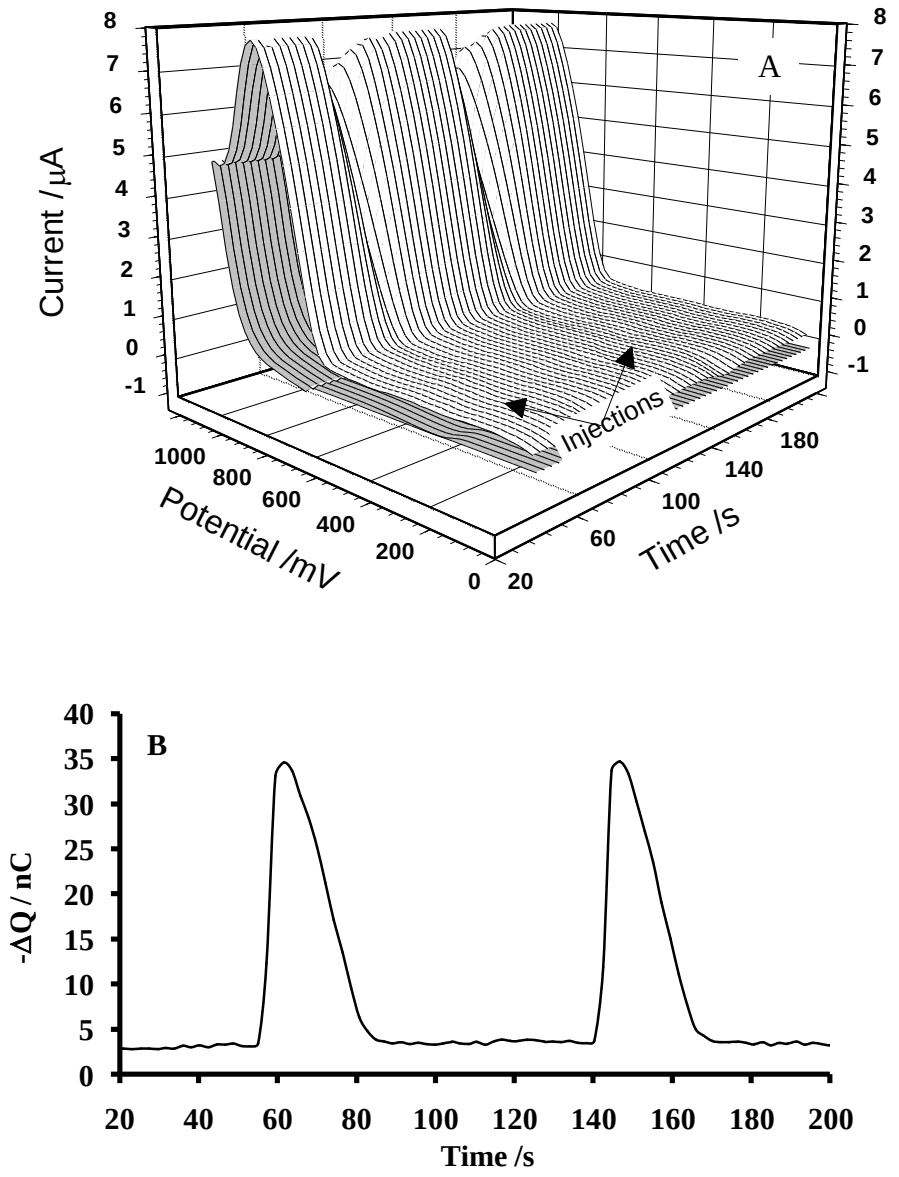

Figure 6. A) FFT cyclic voltamograms of the AChE-MWCNT/AuNPs-Chitosan/GC electrode without (in absent) and with injection of 200µL of 4.0 µM monocrotophos in 1.0 mM acetylthiocholine chloride in PBS, pH=7. 0 in the potential range of 0 to 1000 mV at 5 V/s scan rate. B) the calculated response of the biosensor based on Eq.2.

Before injection of the analyte, AChE-MWCNTs/AuNPs-Chitosan/GC electrode in presence of 1.0 mM acetylthiocholine chloride in PBS, showed an irreversible oxidation peak at 800 mV. In fact, this peak came from the oxidation of thiocholine, hydrolysis product of acetylthiocholine chloride catalyzed by the immobilized AChE.

The figure shows that before injection (in absent of analyte) there is no significant changes in the voltammograms, but by injection of 200 µL of 4.0 µM monocrotophos in 1.0 mM

acetylthiocholine chloride buffer solution (PBS) at pH=7.0 a signal appears at potential 800 mV, due to inhibition of the oxidation of thiocholine.

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. 6b. The response was calculated as;

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

where Q is the electrical charge obtained by integration of cyclic voltammetric curve between 200 and 900 mV in the chatodic scan, and Q_0 represents Q in the absence of the adsorbent. The peaks in Fig 6b are due to two 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:

$$\Delta Q\ (s\tau) = \Delta t\left[\sum_{E=E_i}^{E=E_f} \left| i(s,E) - i(s_r,E) \right|\right] \quad (2)$$

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. 6. 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 monocrotophos in the injected sample, ΔQ increases. This confirms a fast electrocatalytic and electron exchange behavior of modified electrode at high potential sweep rates.

Once FFTCCV is used to monitor a flowing system, monocrotophos electrochemical processes will cause a measurable change in the peak current at the voltammograms. According to eq. 2, the electrochemical reaction for the detection of monocrotophos in presence of AChE is proposed to the enzymatic reaction.

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 0.5 to 10 V/s) and the eluent flow rate (0.2 to 3 $mLmin^{-1}$) from injecting solutions of 4.0 µM of monocrotophos. In fact, the measurement method, sensitivity of the biosensor depends on the potential sweep rate and eluent flow rate, which is mainly due to kinetic

factors of the electrode processes, and instrumental limitations [22-27]. As it is show in Fig. 6, the biosensor exhibits the maximum sensitivity (or signal) at 5 V/s of scan rate and 0.7 mL min^{-1} of the eluent flow rate.

However, the effects of the sweep rate on the biosensor performance 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. This controls the time retention of the solution sample zone in the electrochemical cell [28,29]. 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 AChE with the electrode surface.

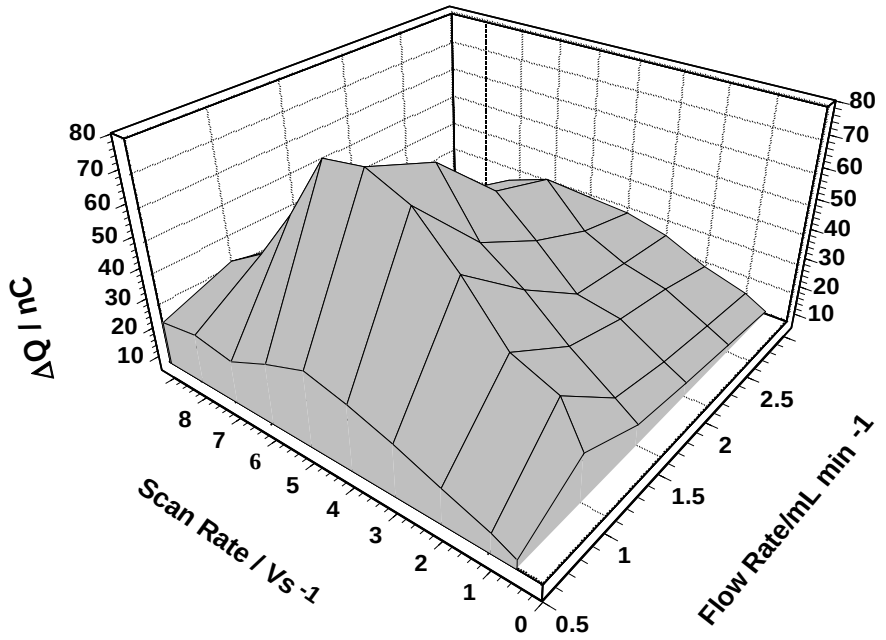

Figure 7. Effect of the sweep rate and effect of flow rate on the response of AChE-MWCNTs/AuNPs-Chitosan/GC electrode to injections of 4.0 µM monocrotophos in 1.0 mM acetylthiocholine chloride in PBS at pH=7.0

3.2. Effect of pH on AChE biosensor

Investigation of the effect of the pH value on the performance of the biosensor is very importance, because the activity of the immobilized AChE is pH dependent. The results of measurements of the electrode response ΔQ in the pH range of 5.0–8.0 (see Fig. 8), indicate that ΔQ response reaches to the maximum at pH of 7.0, where the activity of the enzyme is the highest.

3.3. Effect of nanoparticles amounts on AChE biosensor

Fig. 9 shows the effect of amount of AuNPs and AChE on the biosensor response. As shown, the value of ΔQ increase with increasing the amount of AuNPs and reaches a maximum up to17%

(V_{Au}/V), however, at higher amount of AuNPs up to 35% (V_{Au}/V) the value of ΔQ decrease, which can be due to the increase of the resistance of the surface electrode.

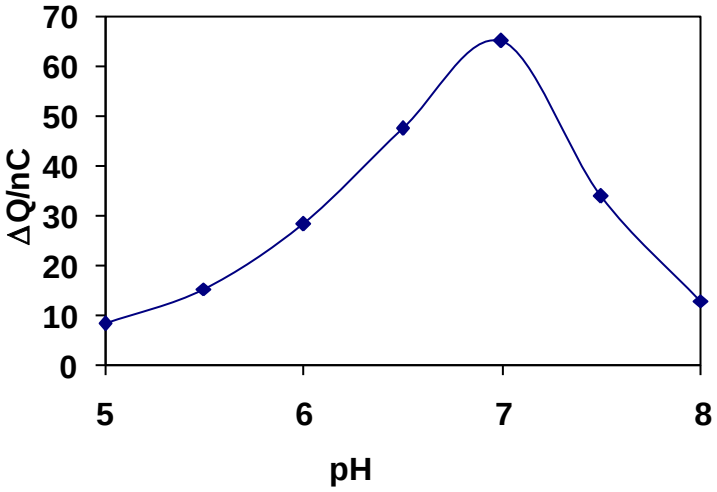

Figure 8. The effect of pH on response of the biosensor

Also, Fig. 9 shows the change of the biosensor sensitivity with the amount of AChE added to the content of the electrode surface. The graph indicates that the value of ΔQ increase with the adding volume of AChE and then reaches to a constant value. It is appears that more than 5.0 μL enzyme the electrode reaches to a saturation state.

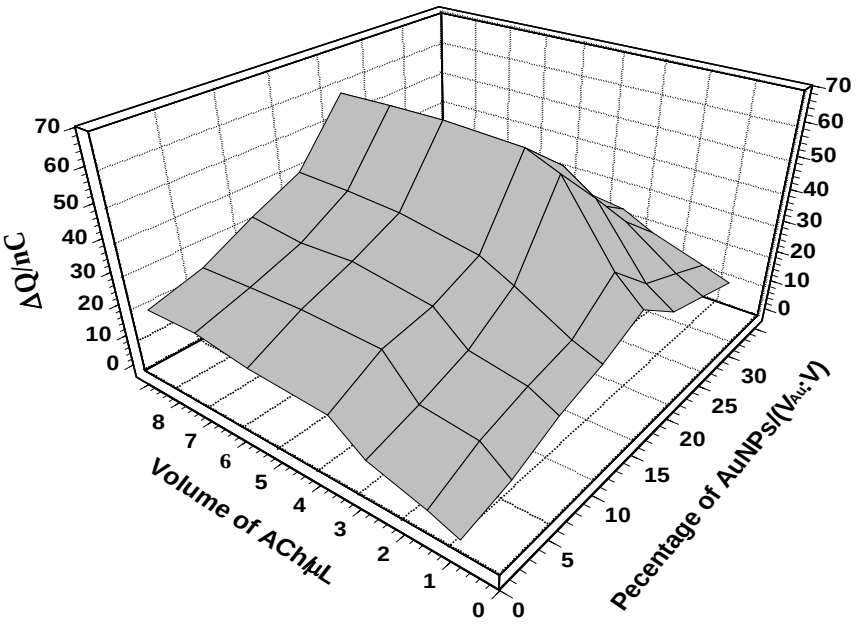

Figure 9. The effect of amount of AuNPs and AChE volume on response of the biosensor to 4.0 μM monocrotophos in 1.0 mM acetylthiocholine chloride in PBS at pH=7.0

3.4. Calibration curve and biosensor characterization

Fig. 10 illustrates that the peak current decrease in present of monocrotophos in 1.0 mM acetylthiocholine chloride in PBS at pH=7.0, and the current decline increase with an increasing monocrotophos concentration, which is proportional to concentration monocrotophos. Fig. 9 shows typical FFTCCV response curves of in 1.0 mM acetylthiocholine chloride of the biosensor electrodes. The regression equation is ΔQ (nC) = 18.383 C(μM) +0.77. The ΔQ responses were obtained for standard solution of monocrotophos (from 0.1 to 100 μM in 1.0 mM acetylthiocholine chloride in PBS at 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=0.992 with %R.S.D. values ranging from 0.22–3.3% across the concentration range studied were obtained following linear regression analysis.

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 10.0±0.1 nM. The long-term storage stability of the sensor was tested for 30 days. The sensitivity retained 92.3% of initial sensitivity up to 54 days which gradually decreases afterwards might be due to the loss of the catalytic activity.

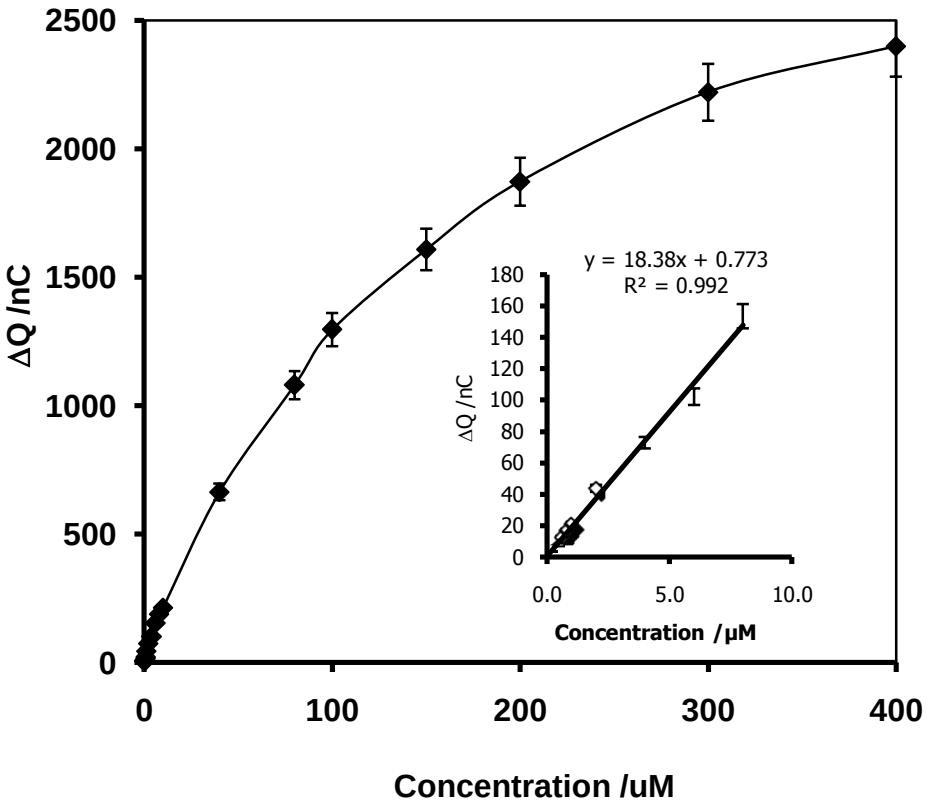

Figure 10. The calibration curve for monocrotophos determination in 1.0 mM acetylthiocholine chloride in PBS at pH=7.0

4. CONCLUSIONS

In this work, a fast and sensitive monocrotophos biosensor is fabricated by modifying the GC electrode surface with AChE-MWCNTs/AuNPs-Chitosan/GC, and based on the inherent properties of AuNPs and MWCNTs, the immobilized AChE exhibited a higher affinity to it substrate and produced detectable and fast response in FFTCCV method for determination of monocrotophos. To the best of our knowledge, this is the first time that a very high-sensitivity and low detection limit flow injection analysis is used for monocrotophos biosensors based on AChE-MWCNT/AuNPs-Chitosan/GC electrode. A good producible sensitivity, response time less than 70 s and detection limit of 10 nM was observed from the fabricated biosensor. The long-term storage stability of the sensor was tested for 50 days.

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