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Design and Fabrication of Biosensor Based on Immobilized AchE on Modified Electrode by Graphene-multiwall Carbon Nanotubs/Beta Cyclodexterin-chitosan

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ABSTRACT

Organophosphorus (OP) forms an important class of toxic compounds. They inhibit acetyl cholinesterase (AChE, EC 3.1.1.7) that results in respiratory and myocardial malfunctions. Pesticides could be accumulated in vegetables and fruits, so detection of them is very important. The goals of this study are decreasing detection time and detection limit of methyl parathion biosensor. In this research the methyl parathion biosensor based on modified glassy carbon (GC) electrode-immobilized AChE is constructed. β-cyclodexterin (β-CD)/chitosan-multiwall carbon nanotube (CS-MCNT) composite, first by polymer wrapping method and then by layer-by-layer self-assembly technique, was prepared. Then different combination of β-CD, CS-MCNT and graphene was deposited on GC electrode. AChE solution (4 mg ml^{-1}) was deposited on the modified GC electrode and dried in air at room temperature. The electrochemical measurement is based on AChE inhibition by methyl parathion (MPT) in the presence of acetyl choline iodide (ACTI) as substrate with cyclic voltammetry method. The results show that using graphene contributes to considerable increasing in current up to 27 μA. These measurements were done in phosphate buffer (pH = 7.5) and at 25 °C. The optimum pH and temperature were 7.5 and 55 °C receptively. Detection limit for MPT was obtained 5nM.

Keywords: Biosensor, Detection limit, Organophosphorus, Cyclic voltammetry

INTRODUCTION

Organophosphorus (OP) are neurotoxic compounds that are used as pecticides. These compounds are potent inhibitors of acetylcholinesterase (AChE) [1]. OP pesticides accumulate in vegetables and fruits and decrease the quality of agricultural products and endanger the consumer health. The contamination of soil and food due to presence of pesticides is a serious problem nowadays. To protect human health from possible contaminations, it is vital to develop sensitive, fast, and reliable methods for determination of OP pesticides in water, vegetables, and fruits [2]. However, measurement of pesticides concentration in environmental, food, clinical, and forensic samples is a difficult due to the matrix complexity and low concentrations of the pesticides. High-performance liquid chromatography (HPLC) [3], mass spectrometry (MS) [4], capillary electrophoresis (CE) [5], Colorimetry [6], gas chromatography (GC) [7], thin layer chromatography [8,9] coupled with different detectors and spectral techniques, and flow injection analysis [10] are some analytical methods that are most commonly employed to measure concentration of pesticides. These methods are expensive and time-consuming and require sample preparation and sometimes do not have results and need highly specialized laboratories with very expensive equipment and trained personnel. However, biosensors overcome these limitations. Compared with various methods available for the determination of pesticides, biosensing methods provide advantages such as low cost, sensitivity, simplicity, relatively economic equipment, rapidity, specificity, and user-friendly operation.

AChE is classified into serine hydrolases enzyme group [11]. AChE acts on acetylcholine and degrades it to choline and acetic acid [1]. Inhibition of AChE will results in respiratory and myocardial malfunctions [12]. One of the common methods for OP detection in environment is AChE inhibition based biosensor. One of the most important challenge in biosensors is stabilizing enzyme by its

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immobilization [13]. The AchE immobilized on an electrode surface hydrolyses acetyl thiocholine chloride (ATCl) and produces an electro-active product of thiocholine (TCh), which shows an irreversible oxidation peak at about 0.68V [14,15] that is a marker for pesticide detection. The oxidation peak of TCh, with a high oxidation potential, is very weak. Therefore, improving the performance of the biosensors and decreasing oxidation potential of TCh have been the research focus of AChE based electrochemical biosensors.

In this research the enzyme was immobilized by noncovalent binding. Also multi wall carbon nanotubes (MWCNTs) were used as mediator for electron transferring. MWCNTs are insoluble in most organic solvents so for overcome this problem it is used in the form of MWCNTs/β-cyclodextrin composite. β-cyclodextrin (β-CD) is a polysaccharide compound that with its hydroxyl groups can bind to substrate and other functional groups. β-CD binds to MWCNTs by van der waals interactions. Moreover between β-CD, hydrogen bonds form. Besides, β-CD can interact with acetylcholine by reversible bonding, which is contribute to increase the enrichment of acetylcholine, and improve the selectivity and sensitivity of the Ops biosensor [16]. As graphene has high electron transferring ability, the effect of graphene on biosensor function was evaluated. The formed composite is fairly soluble in ethanol.

The goals of this study are decreasing detection time and detection limit of methyl parathion biosensor by construction of biosensor based on modified glassy carbon (GC) electrode-immobilized AChE.

METHODS & MATERIALS

Acetylcholinesterase, acetylthiocholine iodide, β-cyclodextrin, multiwall carbon nanotube (with 20 nm width and 15 nm length) and methyl parathion were purchased from Sigma and grapheme was purchase from Nanosa Company. Phosphate buffer and other solutions were prepared with high analytical grade and with double-distilled water.

Apertures

The electrochemical device (Dropsens μStat200 Model), UV-Vis. devices (UNICAM UV-300 model), Atomic Force Microscopy (AFM) (EMD-Danish Micro Engineering A/S) and Sonicator (Decon device model F5200b) were used for this study.

Electrochemical studies were performed using a BIPOTENTIOSTAT μSTAT 200 electro-chemical workstation (Azar electrode Co., Iran) in a three-electrode electrochemical cell containing a platinum wire auxiliary electrode, a modified GC electrode as working electrode and a Ag/AgCl electrode as reference electrode against which all potentials were measured.

MWCNTs/β-CD Composite Preparation

MWCNTs/β-CD composite was prepared according to previous report [17]. The MWCNTs/β-CD composite was prepared with polymer wrapping and layer-by-layer self-assembly techniques. First 320 mg β-CD was added to 10 mg MWCNTs and were mix with each other in mortar. Then 1 ml ethanol was added dramatically to powder. The obtained compound was stored at room temperature for 3 h. After that it was kept in oven at 75 °C for 24 h. The obtained compound was a gray powder (Fig. 1). The MWCNTs/β-CD composite solution was prepared with adding ethanol to it and ultra-sonication for 30 min.

Biosensor Preparation

First 3 μl chitosan 0.5% was deposited on GCE working electrode (CHI/GCE). When it dried at room temperature, 4 μl MWCNTs/β-CD and chitosan solutions were added to modified GCE (MWCNTs/β-CD-CHI/GCE). In this step, 4 μl AchE enzyme solution (0.4 mg ml^{-1}) was coated on modified electrode. Moreover for studying the graphene effect on electron in another electrode the graphene was added to electrode as first layer. The deposition steps and enzyme immobilization were done according to Fig. 2. So two different depositions were done:

1- AchE-MWCNTs/β-CD-CHI/GCE

2- AchE-CHI-MWCNTs/β-CD-graphene/GCE

Electrochemical Investigation

AchE-MWCNTs/β-CD-CHI/GCE and AchE-CHI-MWCNTs/β-CD-graphene/GCE were soaked in cell containing 15 ml phosphate buffer 0.1 M. Acetylcholine

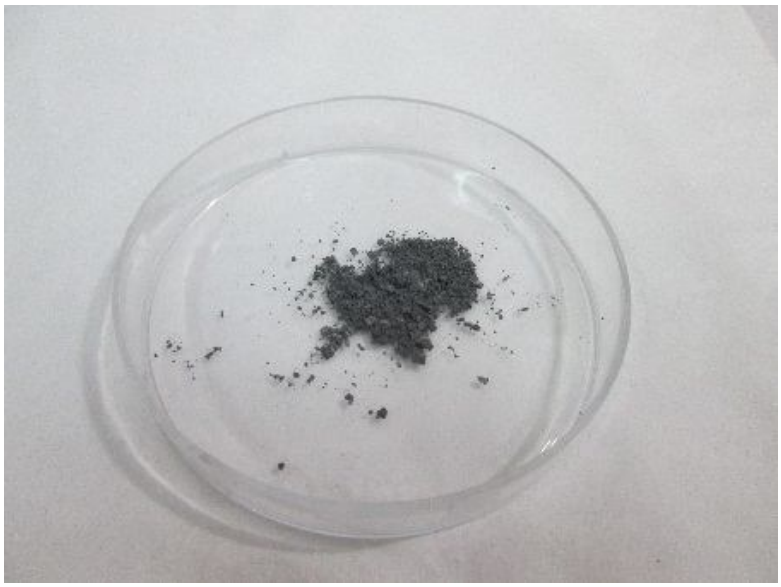

Fig. 1. Prepared composite MWCNTs/β-CD.

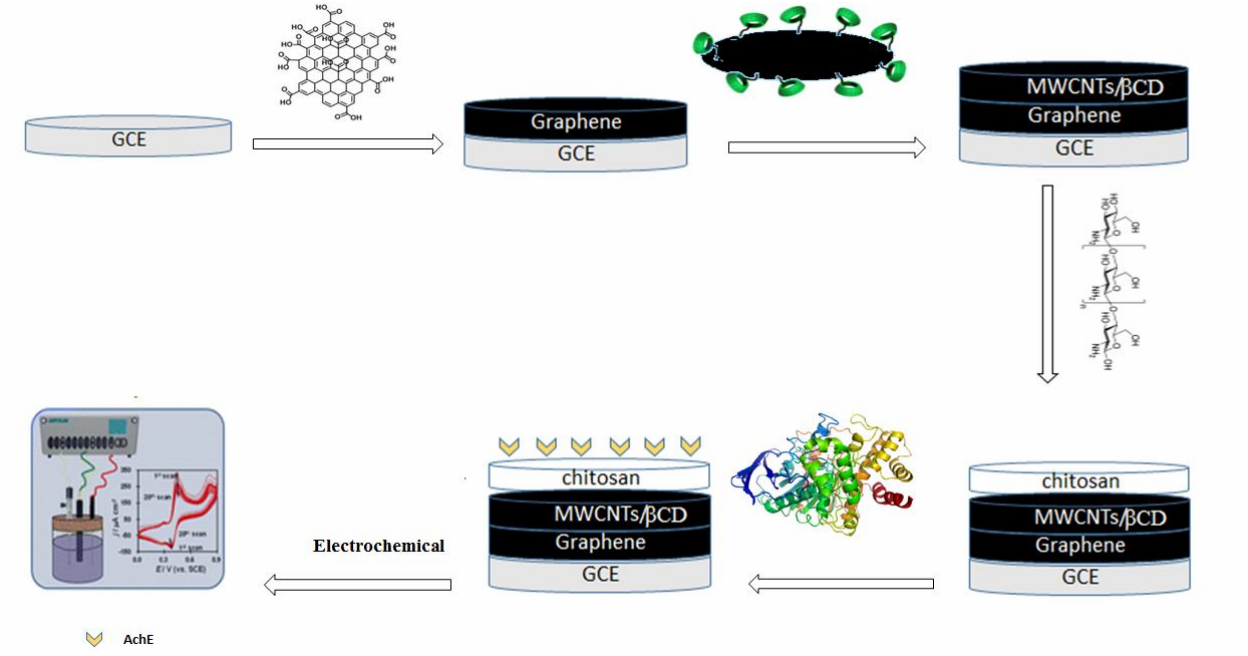

Fig. 2. Schematic diagram of preparation steps of AChE based bioprobe.

iodide as substrate with final concentration of 0.3 mM was added. The cyclic voltammetry was used for analyzing inhibition effect of MPT on immobilized AchE. So different concentrations of MPT were added to fresh phosphate buffer and incubated for 10 min. After that the electrode was soaked in cell containing 15 ml phosphate buffer 0.1%

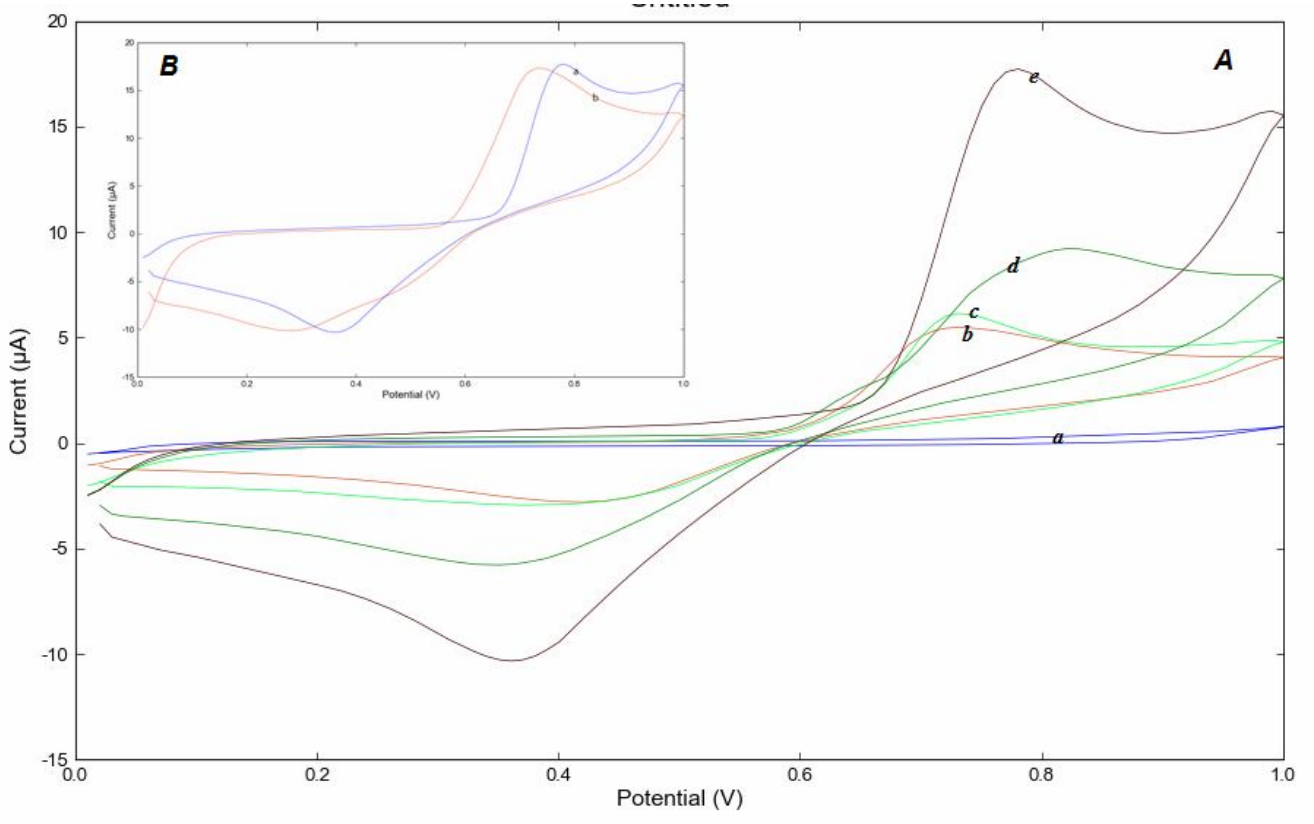

Fig. 3. A: Cyclic voltammograms of a) GCE in PBS and b) GCE c) chitosan/GCE, d) MWCNTs/β-CD/GCE, e) AchE-MWCNTs/β-CD-chitosan/GCE in the presence of 0.3 mM ATCI. B: In inset cyclic voltammograms of AchE-MWCNTs/β-CD-chitosan/GCE in PBS containing 0.3 mM ATCI before (curvea) and after (curveb) incubation in 5 nM MPT solution for 10 min. In all experiments the pH was 7.5, temperature was 25 °C and scan rate was 0.2 v s^{-1}.

and substrate. Secondary current is related to inhibition effect of MPT.

RESULTS

Electrochemical Responses of Modified GCE with AChE

Figure 3 shows the obtained cyclic voltammograms from different electrodes in phosphate buffer solution 0.1 M in presence and absence of ATCI. In figure A: 3a no peak is observed. This is related to bare GCE in phosphate buffer in absence of ATCI. Figures A: 3b, 3c and 3d show cyclic voltammograms of bare GCE, CHI/GCE and MWCNTs/β-CD/GCE in the presence of 0.3 mM ATCI respectively.

Figure B: 3a and 3b shows cyclic voltammogram of AchE-MWCNTs/β-CD-chitosan/GCE in PBS containing 0.3 mM ATCI before (curve a) and after (curve b) incubation in 5 nM MPT solution for 10 min. Oxidation peak probably is due to electron transferring characterization of chitosan. Increasing the current in figure 3d is related to electron conductance of MWCNTs/β-CD. Cyclic voltammograms related to graphene/GCE and AchE-chitosan - MWCNTs/β-CD-graphene/GCE are shown in figures A: 4a and 4b respectively. As it is shown in figure A: 4b, after adding graphene, current is increased significantly. Figure B: 4a and 4b shows cyclic voltammogram of AchE-chitosan-MWCNTs/β-CD-graphene/GCE in PBS containing 0.3 mM ATCI before (curve a) and after (curve b) incubation in 5

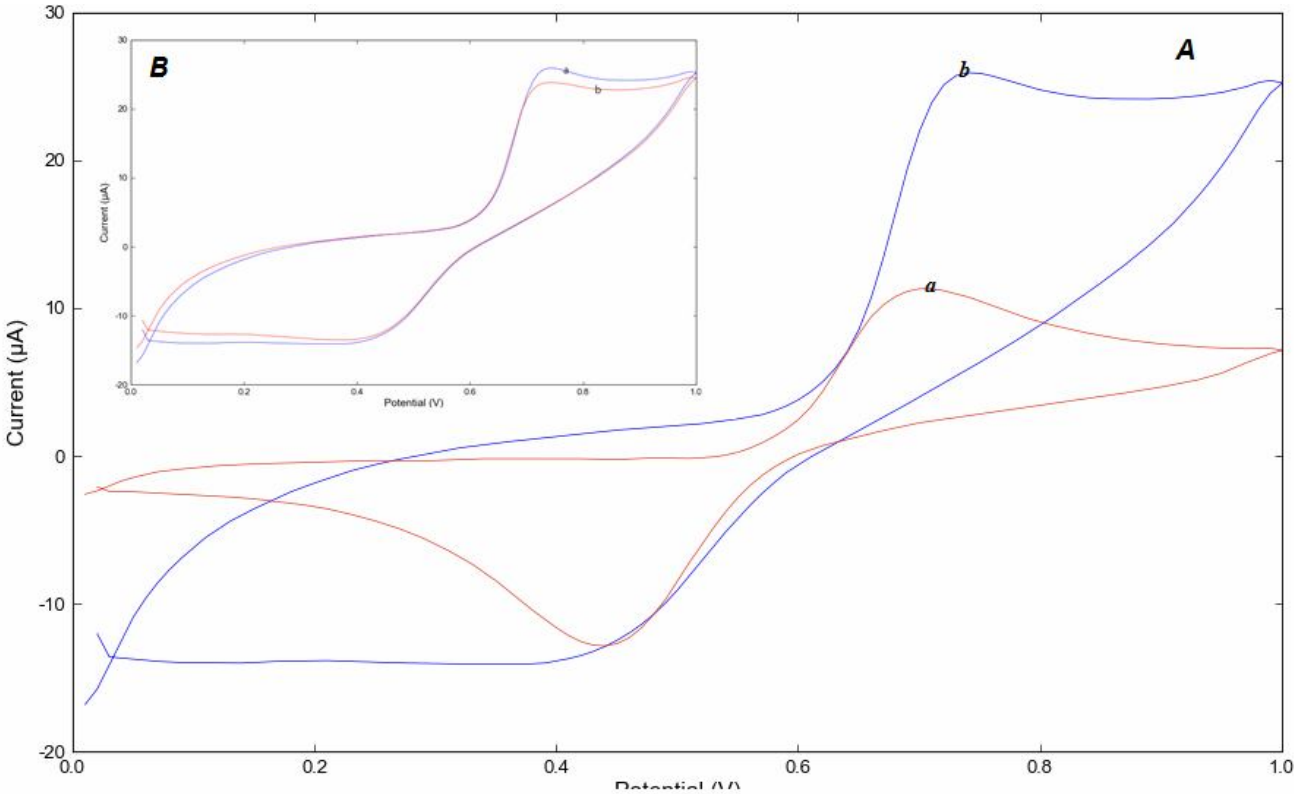

Fig. 4. A: Cyclic voltammograms of a) graphene/GCE and b) AchE-chitosan-MWCNTs/β-CD-graphene/GCE in the presence of 0.3 mM ATCI. B: In inset cyclic voltammograms of AchE-chitosan-MWCNTs/β-CD-graphene /GCE in PBS containing 0.3 mM ATCI before (curve a) and after (curve b) incubation in 5 nM MPT solution for 10 min. In all experiments the pH was 7.5, temperature was 25 °C and scan rate was 0.2 v s^{-1}.

nM MPT solution for 10 min. In figures 5a and 5b cyclic voltammograms of AchE-chitosan-MWCNTs/β-CD-graphene/GCE and AchE-MWCNTs/β-CD-CHI/GCE are depicted respectively. As it is shown by adding graphene the current is increased from 18 to 27 µA. This current increase is probably related to large surface area and good electrical conductance of graphene.

Electrochemical Investigation of Inhibition Effect of MPT on Immobilized AChE

The cyclic voltammograms of immobilized AChE (AchE-MWCNTs/β-CD-CHI/GCE and AchE-chitosan-MWCNTs/β-CD-graphene/GCE) in the presence of MPT as inhibitor were obtained. Both composites containing AChE were incubated with different concentrations of MPT for 10 min. As it is shown in both Figs. 6A and B the current is decreased in the presence of MPT in both cases. Detection limits of MPT for AchE-MWCNTs/β-CD-CHI/GCE and AchE-chitosan-MWCNTs/β-CD-graphene/GCE were obtained 10^{-8} M and 5×10^{-9} M, respectively.

Optimization of Prepared Biosensor

Figure 7 shows the effect of pH (6 to 9) on immobilized enzyme activity. Maximum current was obtained at pH = 7.5. In the other hand the effect of temperature (25 to 65 °C) on immobilized enzyme activity was investigated that the result is shown in figure 8. This study was done with AchE-chitosan-MWCNTs/β-CD-graphene /GCE in the presence of 0.3 mM ATCI. (pH was 7.5 and scan rate was 0.2 v s^{-1}). As it is shown, maximum current was obtained at 55 °C.

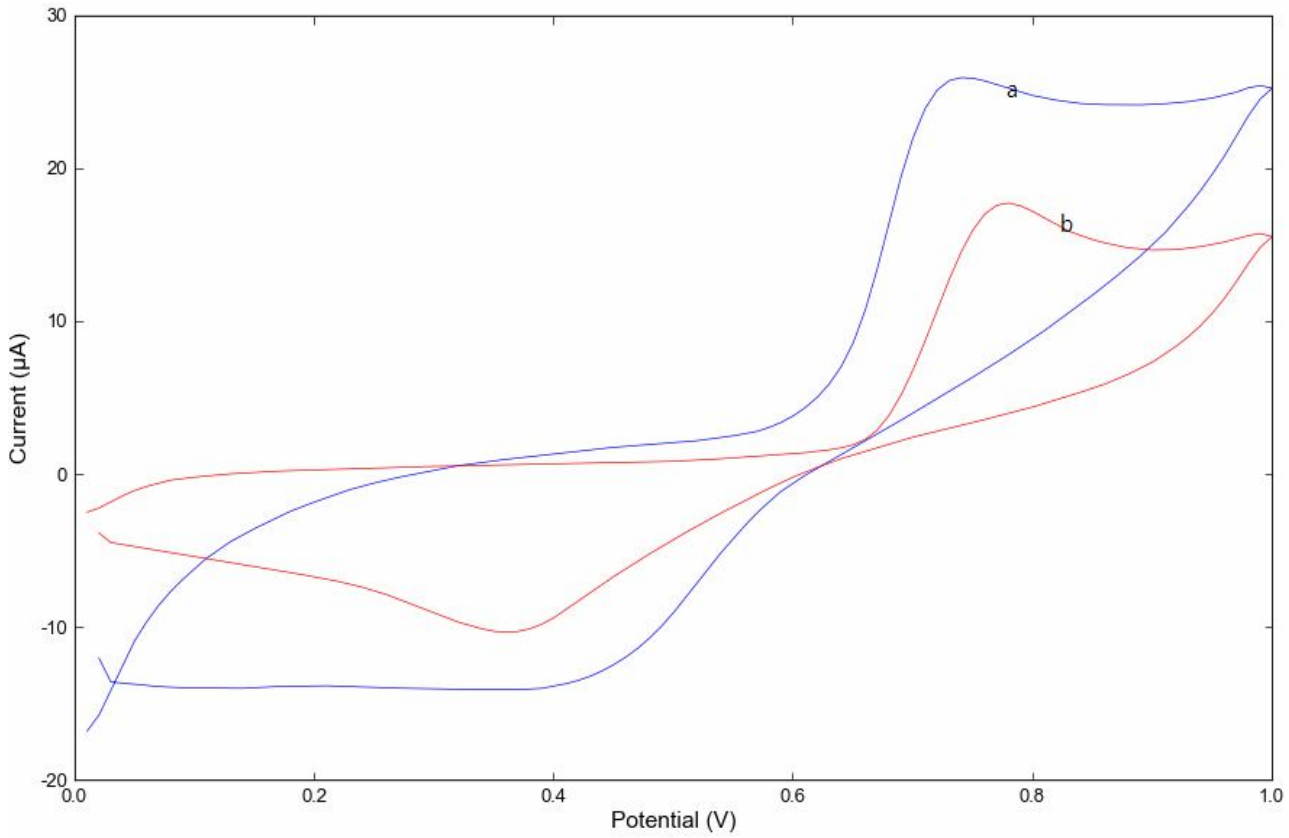

Fig. 5. Comparing cyclic voltammograms of a) AchE-MWCNTs/β-CD-CHI/GCE and b) AchE-chitosan-MWCNTs/β-CD-graphene/GCE in the presence of 0.3 mM ATCI. (pH was 7.5, temperature was 25 °C and scan rate was 0.2 v s^{-1}).

AFM Images from Modified Electrode Surface

Figures 9a, b, c and d show the AFM surface images of graphene/GCE, MWCNTs/β-CD-graphene/GCE, CHI-MWCNTs/β-CD-graphene/GCE and AChE-CHI-MWCNTs/β-CD-graphene/GCE, respectively. The images are obtained after each deposition step. As the figures show, after each deposition step, increasing the height is seen that is related to deposition of new layer.

DISCUSSIONS

The results show that chitosan-MWCNTs/β-CD-graphene/GCE composite is a good matrix for AChE based biosensor. This composite is stable in high temperature and its detection limit is better than MWCNTs/β-CD-CHI/GCE composite. As graphene and MWCNTs have good electron transferring ability so they help to transfer electron better and the current is higher than MWCNTs/β-CD-CHI/GCE. In the other hand, β-CD helps to bind substrate and chitosan helps to transfer electron from MWCNTs/β-CD composite to electrode. Also due to the good dispersibility and porous structures of MWCNTs/β-CD composite, the resulting surface provided a favorable microenvironment for acetylcholinesterase biosensor fabrication and maintained the bioactivity of AChE for screening of OPs exposure. MWCNTs promoted electron-transfer reactions at a lower potential and catalyzed the electro-oxidation of substrate, thus increasing detection sensitivity.

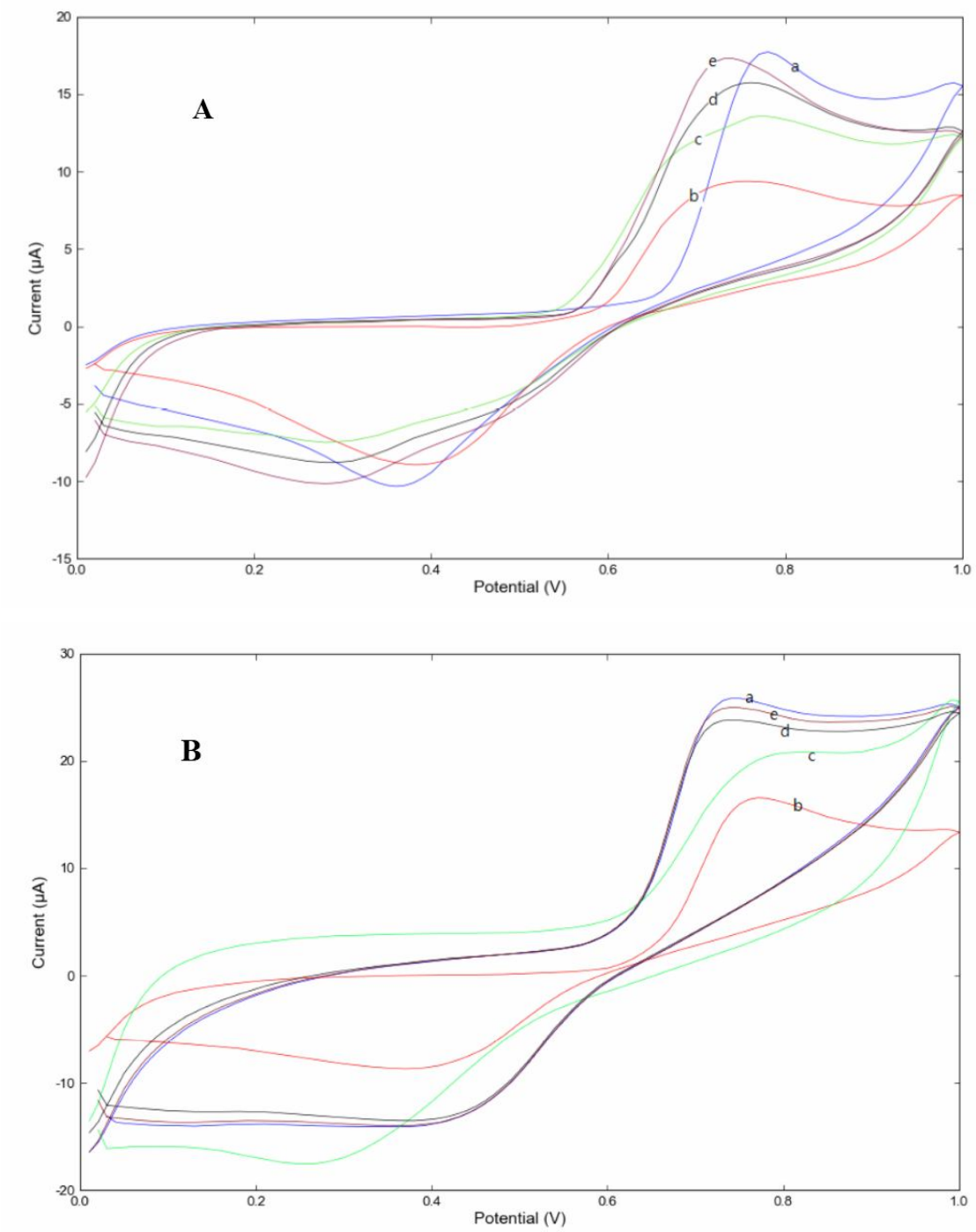

Fig. 6. Cyclic voltammograms of A) AchE-MWCNTs/β-CD-CHI/GCE in the presence of MPT as inhibitor in different concentrations of a: 0 M, b: 10^{-5} M, c: 10^{-7} M, d: 10^{-8} M and e: 10^{-9} M and B) AchE-chitosan-MWCNTs/β-CD-graphene/GCE in the presence of MPT as inhibitor in different concentrations of a: 0 M, b: 10^{-8} M, c: 7×10^{-9} M, d: 5×10^{-9} M and e: 2×10^{-9} M (ATCI concentration was 0.3 mM, pH was 7.5, temperature was 25 °C and scan rate was 0.2 v s^{-1}).

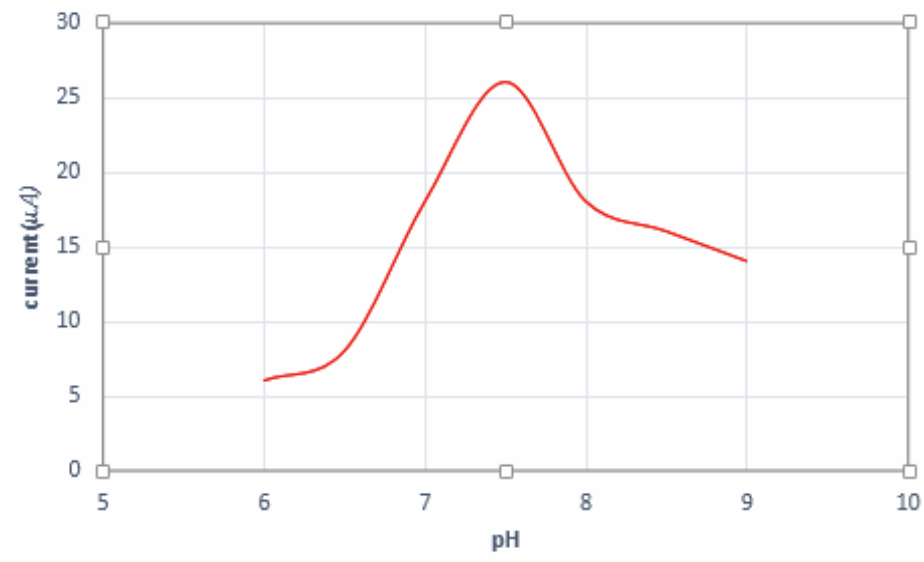

Fig. 7. Effect of pH on current of AchE-chitosan-MWCNTs/β-CD-graphene/GCE in the presence of 0.3 mM ATCI. Temperature was 25 °C and scan rate was 0.2 v s^{-1}.

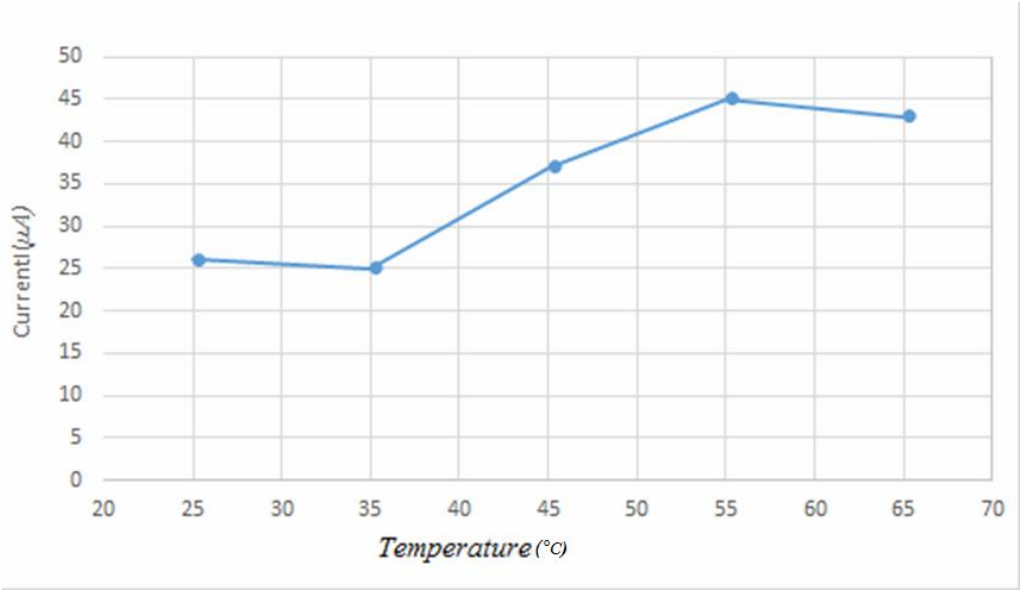

Fig. 8. Effect of temperature on current of AchE-chitosan-MWCNTs/β-CD-graphene/GCE in the presence of 0.3 mM ATCI. (pH was 7.5 and scan rate was 0.2 v s^{-1}).

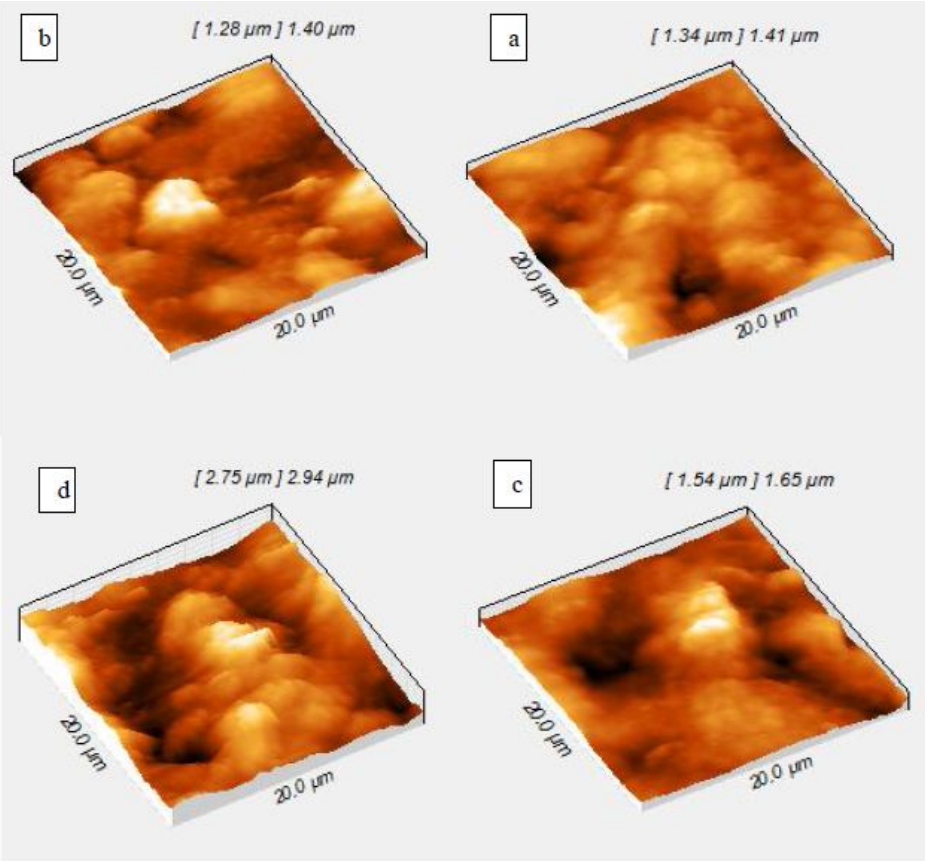

Fig. 9. AFM surface images of a) graphene/SP, b) MWCNTs/β-CD-graphene/GCE, c) CHI- MWCNTs/β-CD-graphene/GCE and d) AChE- CHI- MWCNTs/β-CD-graphene/GCE.

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