

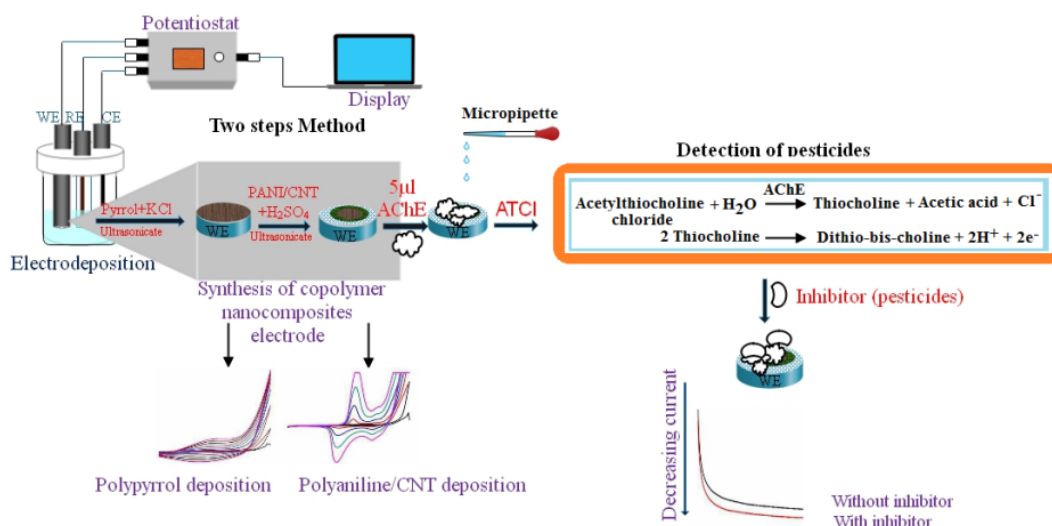
Conductive polymer nanocomposite enzyme immobilized biosensor for pesticide detection

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ABSTRACT



Conductive carbon nanotubes based polyaniline/polypyrrole polymer nanocomposite (CNT-PANI-PPy) film was electrochemically synthesized on graphite electrode using cyclic voltammetry. CNT-PANI-PPy film formed a biocompatible environment to entrap enzyme molecule and used as biosensor for pesticide detection. This study shows that enzyme based conductive polymer nanocomposite film itself acts as a mediator. Carbon nanotubes promote electron transfer reactions in presence of Acetylthiocholine chloride (ATCI) as a substrate at a lower potential and catalyzed the electrochemical oxidation of enzymatically formed thiocholine. Surface morphology was studied by scanning electron microscopy which shows a porous structure of the modified film beneficial for enzyme immobilization. Electrochemical behavior of the fabricated electrodes evaluated through cyclic voltammetry and electrochemical impedance spectroscopy. The detection of pesticide (acephate) was performed by chronoamperometry and the limit of detection (LOD) of acephate was found to be 0.007 ppm concentration which is quite low.

Keywords: Polymer, Carbon nanotubes, Acetylcholinesterase, Pesticides, Biosensors

INTRODUCTION

The research on the construction of biosensors for various fields applications such as food industry, wastewater monitoring,

medical diagnosis, forensics, environmental monitoring has been contributed to the sustainable development of society. A biosensor is an analytical device used for the detection of the analyte in the reaction. It comprises two major components, bioreceptor, which recognizes analyte, and transducer converts biorecognition event into a measurable signal.¹ The use of electrochemical biosensors is increasing now a days due to their simplicity of operation, low fabrication cost, high selectivity and short duration of analysis in comparison to conventional methods such as gas chromatography (GC), high performance liquid chromatography (HPLC), ultraviolet spectroscopy, gas-mass spectroscopy and surface plasmon resonance (SPR).^{2,3} Various techniques have been used

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for the preparation of biosensors.⁴⁻⁷ The electrochemical polymerization allows to a better control of the synthesis parameters such as final electrode potential, number of cycles, scan rate, thickness, electrical conductivities, and rapid polymerisation time.⁸⁻¹¹ In recent years, electrochemical biosensors based on polymer modified nanocomposites have received great attention for the development of highly sensitive, selective, reliable and low cost biosensors.¹²⁻¹⁵ Electrochemical polymerization of conducting polymer is very easiest and simple process to construct biosensor. Electrochemically modified conducting polymers provide suitable environment for immobilization of biomolecules and act as a mediator.¹⁶⁻²⁰ Among various conducting polymers, polypyrrole, polyaniline, and their nanocomposites have been extensively studied due to its ease of synthesis and good redox properties. For fabrication of biosensor, immobilization of enzyme on the surface of the electrode material is very challenging task. The activity of immobilized molecules depends on the surface area, porosity, and the hydrophilic character of immobilizing matrix.²¹ Various immobilization techniques have been reported in the literature included encapsulation,²² cross-linking,²³ covalent binding,²⁴ physical adsorption onto a solid support.²⁵ Nanomaterials such as carbon nanotubes (CNTs) are electrochemically inert materials. They also have gathered great attention due to their unique physical, chemical and electrical properties, which allows for their use in biosensor.²⁶ They have large length to diameter (aspect) ratio provide high surface-to-volume ratio and fast electron transfer rate due to their unique sp^2 electronic structure.²⁷ In the recent years, polymers incorporated with nanomaterials have become one of the most popular electrodes in biosensor field.²⁸

Pesticides have drawn much attention due to their wide use in agricultural and environmental applications, which can cause significant contamination of soil and groundwater, which then transfers the contaminants to foods.²⁹ They have raised serious public concern regarding the healthiness, environment and food safety due to its high toxicity because of accumulation in human body.^{30, 31} There are many conventional methods which have been used nowadays for the detection of various pesticides.^{2,3} Unfortunately, many of these methods are time consuming, expensive and are no longer morally acceptable.^{32,33} Therefore, the development of rapid and field monitoring tools including high performance liquid chromatography, gas chromatography, liquid chromatography, mass spectroscopy, etc needs to be replaceable by modified biosensors which are highly desirable.³²

This paper demonstrates a facile approach for developing conducting polymer based graphite electrode for pesticide detection. We have synthesized CNT-PANI-PPy modified graphite electrode by using electrochemical technique for immobilization of enzyme. Conducting polymer form a host matrix that provided an excellent environmental and chemical stability around the enzyme molecule to stabilize its biological activity to a large extent.²⁰ This electrode not only promoted electron-transfer reactions but also increased the surface area to capture a large amount of enzymes, thus increasing detection sensitivity. Typical pesticide acephate was selected to evaluate the proposed biosensor. In this work, we have modified the graphite electrode with two layers of different polymers. We have used

first layer of polymer as it is but in second layer, we have doped CNTs with Polyaniline, which is on the top of the surface of graphite electrode. In this, CNTs maintained electrocatalytic activity of AChE enzyme; Also, CNTs influences the adsorption and activity of immobilized Enzymes. This process is reversed as earlier reported.²⁰ In this work, for the first time we have reported the detection of acephate with AChE/CNT/PANI/PPY modified graphite electrode. Acephate is very toxic pesticide and there is only few literature available based on such type of biosensors for the detection.

EXPERIMENTAL

Materials and methods

Acetylcholinesterase (AChE) (Type C3389, 500 U/mg from electric eel), Acetylthiocholine chloride (ATCl) and acephate were purchased from Sigma–Aldrich and used as received. Pyrrole and aniline were obtained from Aldrich, purified twice by distillation, and then kept in refrigerator before use. Other reagents, including Tris Base, Potassium chloride and phosphate buffer solution (PBS, pH 7.2) were of analytical reagent grade. Sulfuric acid (Aldrich 98%) was used as received. All solutions were prepared using water deionised water purified in Millipore Milli-Q system (resistivity 18.2 MΩcm). All electropolymerisation and electrochemical measurements were performed in a standard three-electrode system. (Platinum wire as an auxiliary electrode, (Ag/AgCl) (3M KCl) for aqueous media as a reference electrode and working electrode was graphite electrode) using an electrochemical workstation Palmsense Epsilon Potentiostat/Galvanostat in phosphate buffer solution as an electrolyte. Electrochemical impedance measurements (EIS) were carried out in phosphate buffer solution in frequency range from 0.01 Hz to 1 MHz at open-circuit potential using small excitation amplitude of 10 mV. Scanning electron microscopy (SEM) images were performed on Zeiss Model EVO-18.

Fabrication of AChE-CNT-PANI-PPy modified Graphite electrode biosensor

The graphite electrode was first rinsed with distilled water, ultrasonicated for 1 hr in deionized water followed by 1 hr in acetone. Then the electrode was dried for 2 hr at 70 °C. For electrochemical deposition, polypyrrole (PPy) film was electrochemically synthesized on graphite electrode from an aqueous solution containing 0.1 M pyrrole and 0.1 M potassium chloride. For this 0.1M pyrrole (monomer) was dissolved in 0.1M KCl solution³⁴ and ultrasonicated this mixture about 1 hr for uniform solution at room temperature. Electrochemical deposition was performed on graphite electrode by cyclic voltammetry technique from potential window -0.6 V to 0.9 V for 10 cycles at scan rate 50 mV/s as shown in **Figure 1 (a)**. It can be easily seen that polypyrrole film formation with successive cycles on bare graphite electrode. It can be noted that during first cycle there was no redox waves was observed and from next cycle gradual increase in current intensity with broad redox waves was observed. A broad oxidation wave was observed at the peak potential of $+0.15$ V and reverse cathodic wave was seen at a peak potential of -0.05 V as reported earlier, the oxidation wave is seen to shift slowly towards higher voltages with repeated scans,

whereas the reduction wave is observed to shift to even lower negative voltages.³⁵

The resulting polypyrrole modified film on graphite electrode was rinsed carefully with distilled water to remove loosely bound species and dried at room temperature for 1 hr.

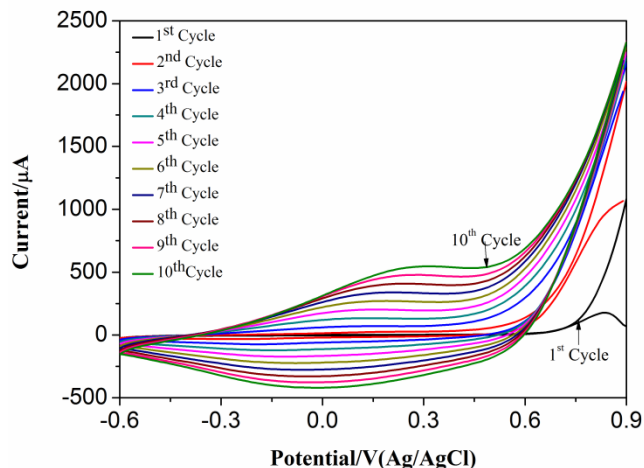


Figure 1(a): Electrodeposition of polypyrrole (PPy) on graphite electrode

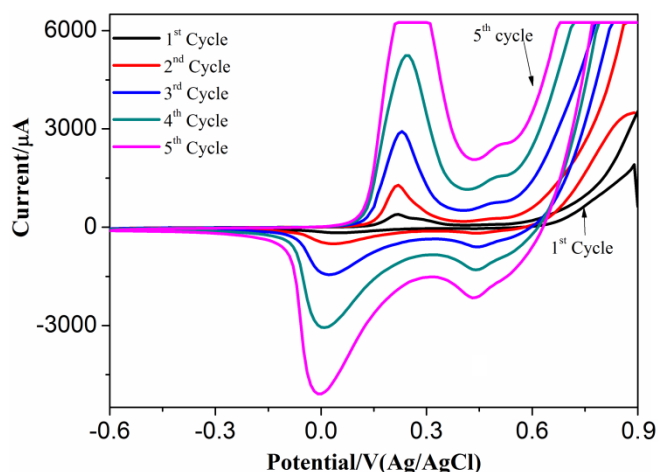


Figure 1(b): Electrodeposition of Polyaniline/CNT on PPy coated graphite electrode

Polyaniline (PANI) with carbon nanotubes (CNTs) was subsequently grown over polypyrrole modified graphite electrode surface by the process of polymerization of aniline in the presence of CNTs. In a typical process, the functionalized CNTs were added into 1M H₂SO₄ solution for ultrasonication about 3 hr to get CNTs disperse in the solution. Then, 0.5M aniline was dissolved in this solution under continuous stirring for 20 min at room temperature and electrochemically deposited on the polypyrrole modified graphite electrode by CV technique from -0.6 to 0.9V for five cycles at the scan rate of 50 mV/s as shown in **Figure 1(b)**. The electropolymerisation process of PANI/CNT on modified PPy based graphite electrodes exhibits well-defined redox peaks corresponding to a series of redox transitions. In the present case, two oxidation and reduction waves can be identified. Both oxidation and reduction currents progressively increase with the number of cycles, thus evidencing the growing of a conductive

film on the electrode surface. The resulting polymer nanocomposite modified electrode was rinsed with water and dried at room temperature. An enzyme solution was prepared by dissolving 2 mg of acetylcholinesterase in 1000 µl Tris HCl buffer (20 mM, pH 7.2). 5 µl enzyme solution was drop casted on prepared modified electrode and incubated the electrode at 30 °C for 30 min to obtain AChE-CNT-PANI-PPy electrode. The prepared enzyme immobilized electrode was then stored at 4 °C (when not in use).

RESULTS AND DISCUSSION

Scanning Electron Microscopy (SEM)

The surface morphology of the graphite electrode, CNT-PANI-PPy modified graphite electrode before and after AChE immobilization were investigated by scanning electron microscopy. SEM image of CNT-PANI-PPy modified graphite electrode after AChE immobilization was done after incubation at 30 °C. **Figure 2(a-c)** shows typical scanning electron microscopy images of bare graphite electrode, CNT-PANI-PPy modified electrode and AChE-CNT-PANI-PPy electrode. Bare graphite electrode has an irregular graphite layer and a rough surface.

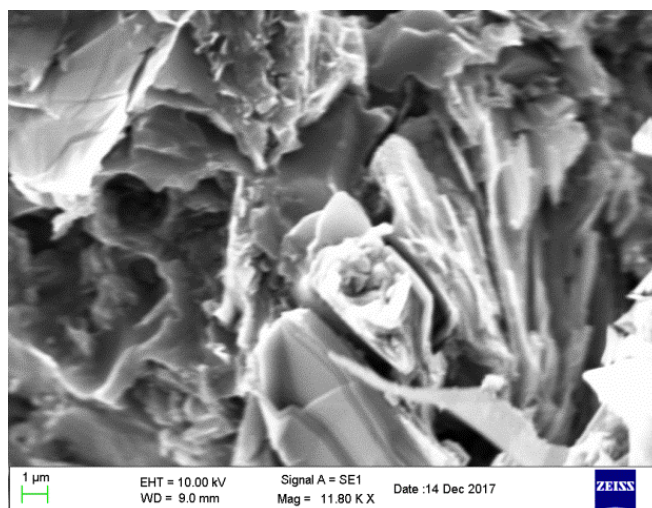


Figure 2(a): SEM image of graphite electrode

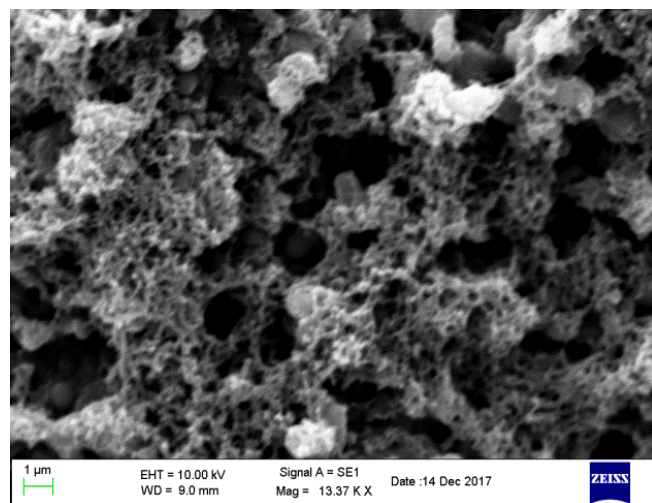


Figure 2(b): SEM image of CNT-PANI-PPy coated electrode

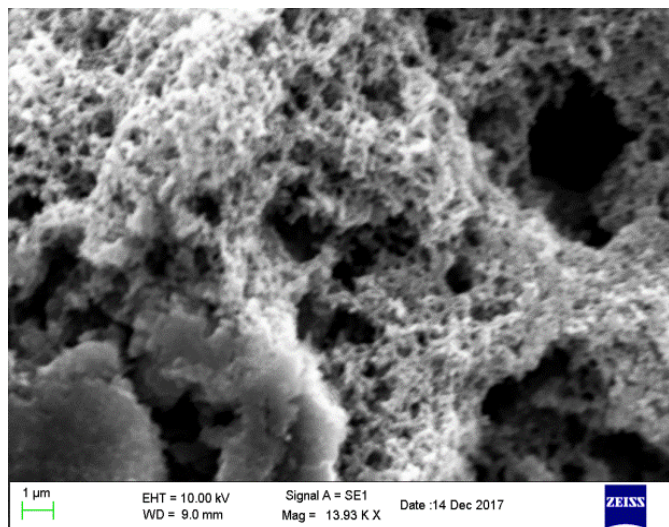


Figure 2(c): SEM Image of AChE- CNT-PANI-PPy coated electrode

Image corresponding to polymer nanocomposite film shows uniform morphology with well-packed structure. In addition, we can easily observe that the porous nature of polymer nanocomposites film, which provides suitable biocompatible environment for the enzyme entrapment. Globular shapes of the enzyme AChE over polymer nanocomposites film can also be clearly seen.

Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy is a powerful and sensitive tool to characterise mechanical analysis of interface properties and for evaluating impedance changes of the modified electrodes during the fabrication processes of biosensor. In a Nyquist plot, semicircle portion correspond to the charge transfer resistance R_{ct} at the higher frequency range and linear part of the plot corresponds to the diffusion-limited process at low frequency range.³⁶ **Figure 3** illustrated typical Nyquist plots obtained from bare graphite electrode (a), CNT-PANI-PPy modified graphite electrode (b) and AChE-CNT-PANI-PPy modified graphite electrodes (c) using 0.1M KCl with equimolar of the combination ferricyanide $[\text{Fe}(\text{CN})_6]^{3-}$ and ferrocyanide $[\text{Fe}(\text{CN})_6]^{4-}$ available as $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as redox probe with a frequency range 0.01Hz to

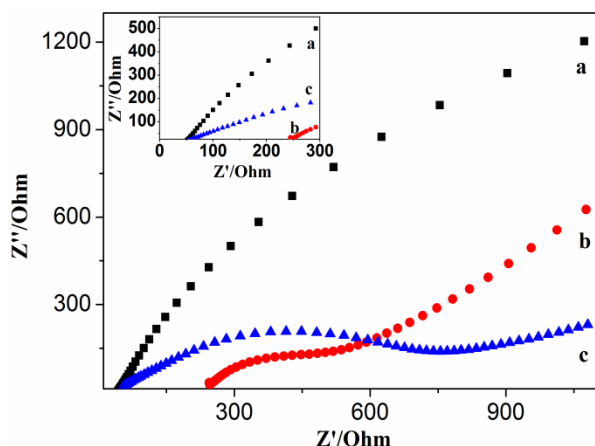


Figure 3: Electrochemical impedance spectra of bare graphite electrode (a), CNT-PANI-PPy electrode (b), AChE-CNT-PANI-PPy electrode (c) in 0.1M KCl with equimolar $[\text{Fe}(\text{CN})_6]^{3-/4-}$

1MHz. Bare graphite electrode presented a very high charge transfer resistance with an almost straight tail line. After modification of bare graphite electrode with polymer nanocomposites resulting layer shows semicircle with largely decreasing value. Decrease in charge transfer resistance indicates that electron transfer on polymer nanocomposites was very fast and it also promotes electron transfer due to their excellent unique electronic properties. After the immobilization of AChE enzyme on the electrode surface resistance was found to increase due to immobilization of insulating protein layer. This was a direct evidence of successful immobilization of enzyme on the electrode surface.

Electrochemical behavior of AChE-CNT-PANI-PPy modified graphite electrode

To evaluate the catalytic activity of acetylcholinesterase enzyme at the CNT-PANI-PPy modified graphite electrode, the modified electrode was characterize using a cyclic voltammogram in presence of enzyme substrate ATCl in the potential range from -0.1 to 0.1V. **Figure 4** shows CV of the AChE-CNT-PANI-PPy electrode in a solution containing 0.1 mol phosphate buffer solution (PBS), pH 7.2 without ATCl (curve a) or with 1mM ATCl (curve b) at a scan rate of 50 mV/s. No peak was observed for the AChE-CNT-PANI-PPy electrode in PBS (curve a). When 1 mM ATCl was added into PBS, the CV of AChE- CNT-PANI-PPy electrode shows an irreversible oxidation peak at 705 mV (curve b) which was attributed to the oxidation of the thiocholine, the hydrolysis product of ATCl catalyzed by the immobilized AChE.^{37,38}

Detection of pesticide (acephate) by AChE-CNT-PANI-PPy electrode

The pesticide determination was carried out by chronoamperometry which involves two steps. First step is measuring the response of electrode in 0.1 M phosphate buffer, pH=7.2 and 1 mm substrate. This value corresponds to I_i , the current before inhibition of pesticide. In the next step electrode was washed with the same buffer and incubated in pesticide sample solution at a known concentration, this value corresponds to I_f , the current after inhibition of pesticides. The percentage of enzyme inhibition and residual enzyme activity percent was determined according to the equations 1 and 2 respectively.³⁹

$$\% \text{ Inhibition (I \%)} = \frac{[I_i - I_f]}{I_i} \times 100 \quad (1)$$

$$\% \text{ Residual enzyme activity (REA \%)} = \frac{[I_f]}{I_i} \times 100 \quad (2)$$

The determination of acephate was performed by chronoamperometry after the electrode was immersed in different concentrations of acephate. 5 min of incubation time was selected for inhibition measurements of acephate in order to obtained lower detection limit and for rapid analysis. The current decreased with the increasing concentrations of pesticides, which was caused by the inhibition of AChE activity by pesticides. This was because acephate as one of the Organophosphates pesticides exhibited fairly high toxicity and involved in the irreversible action on AChE, thus reduced the enzymatic activity. Due to the notable change in current signal of AChE, a simple method for determination of acephate was established. Under optimum experimental conditions, the inhibition of acephate on prepared biosensor was

proportional to its concentration in its range, from 1 ppm to 4 ppm. As shown in **Figure 5**, the curve **a** represents chronoamperometry response of biosensor for 1 mM substrate thiocholine before inhibition. The curves **b** to **e** represents the response of the biosensor for increase in concentration of pesticide solution from 1 ppm to 4 ppm after inhibition. The linear regression equation is $I\% = 3.97 + 12.13 \log C$ with $R^2 = 0.9707$ from which the limit of detection (LOD) of acephate was calculated to be 0.007 ppm.⁴⁰⁻⁴²

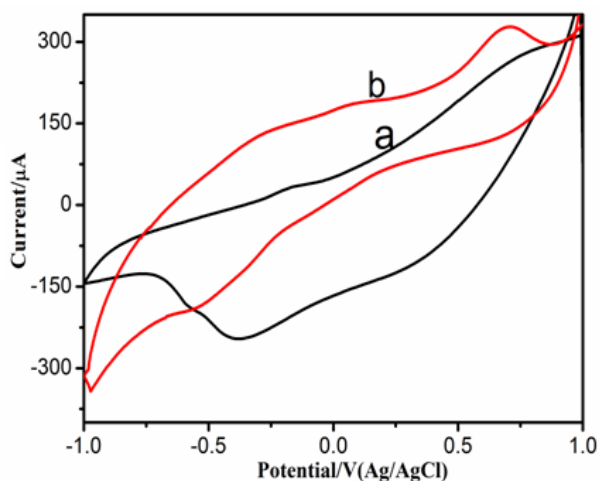


Figure 4: Cyclic voltammogram of CNT-PANI-PPy electrode (a) and AChE-CNT-PANI-PPy electrode (b) in pH 7.2 PBS containing 1mM ATCl at scan rate 50mV/s

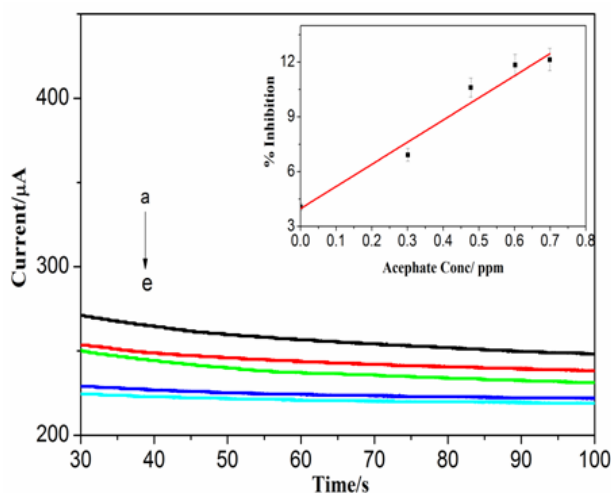


Figure 5: Chronoamperometry of AChE-CNT-PANI-PPy electrode and detection of pesticide (acephate)

CONCLUSIONS

AChE enzyme biosensor based on polymer nanocomposites modified graphite electrode for pesticide detection is successfully prepared. PAN/PPy polymer with CNT nanocomposite not only increased the surface area of modified electrode but also show excellent conductivity to immobilize the enzyme easily on the surface of polymer nanocomposites modified graphite electrode. As immobilization of AChE enzyme, the three dimensional polymer matrixes provided a favorable microenvironment to maintain the bioactivity of enzyme. AChE enzyme biosensor

showed good affinity to ATCl with very low limit of detection (LOD) of acephate. The developed biosensor could be used for the direct detection of various pesticides in real samples e.g. soils extract, vegetables, milk and water.

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Priyanka D. Virutkar is a research scholar registered for Ph.D. under the guidance of Dr. Subhash B. Kondawar, Department of Physics, Polymer nanotech laboratory, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, India. She has obtained her Master's degree in Physics from R. T. M. Nagpur University Nagpur. Her main research interest is Fabrication of enzymatic biosensor based on conducting polymer nanocomposites for pesticide detection.