Conducting Polymer-Based Electrochemical Biosensor for the Detection of Acetylthiocoline and Pesticides via Acetylcholinesterase

Abdurrahman Akdag[1*], Mesut Işık[2], Hasan Göktaş[3]

[1] Department of Medical Laboratory Techniques, Vocational School of Health Services, Harran University, Turkey, ORCID ID: 0000-0001-5292-8001

[2] Department of Pharmacy Services, Vocational School of Health Services, Harran University, Department of Bioengineering, Faculty of Engineering, Bilecik Seyh Edebali University, Turkey, ORCID ID: 0000-0002-4677-8104

[3] Department of Electrical and Electronic Engineering, Faculty of Engineering, Harran University, Turkey, ORCID ID:0000-0002-2195-9531

[*]Corresponding author
Adress: Harran University Yenisehir Campus, Haliliye, Sanliurfa, 63300, Turkey
Tel: +904143183000
E-mail: ab.ak@hotmail.com

Abstract

A voltammetric biosensor for acetylthiocoline (ATCh) and paraoxon detection was successfully developed. To achieve this goal, polypyrrole (PPy) was synthesized onto the platinum (Pt) electrode surface in 0.03 M oxalic acid solution containing 25 mM pyrrole. PPy coated Pt (Pt/PPy) electrode surface was covered with chitosan (Chi) (Pt/PPy/Chi). The Acetylcholinesterase (AChE) enzyme was immobilized on the Pt/PPy/Chi electrode surface to build a voltammetric biosensor (Pt/PPy/Chi/AChE). The storage stability of the biosensor was determined to be 72% even after 60 days. The operational stability was determined to be 94% after 20 consecutive measurements. The linear range of the biosensor was determined to

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be 30-50 μM for ATCh and 0.46-1.84 nM for paraoxon. Limit of detection (LOD) was determined to be 0.45 μM for ATCh and 0.17 nM for paraoxon

Keywords: Biosensors, enzyme, immobilization, pesticide, polypyrrole

1. Introduction

Biosensors are type of a device that can commonly be used to detect and quantify target molecules. Due to their advantages such as high sensitivity, fast response time, low cost and selectvitiy, the usage of biosensors are getting very popular in clinical, environment, food and pharmaceutical applicatons [1–4]. There have been many different developments conducted on enyzme based sensors. Among these sensors, cholinesterase-based biosensor has a special place as it has been used to detect bacterial toxins, acetylcholine, pesticides, nerve agents and choline [5]. Acetylcholine (ACh), which affects learning, memory and muscle tones, is a neurotransmitter that has an important function in the cholinergic system and is effective in nerve conduction in cholinergic synapses. They actively take role in the neuromuscular junctons, motor neurons, preganglionic, spinal cord and centeral nervous system**.** Various neurodegenerative diseases such as Alzheimer's, Parkinson's and schizophrenia may occur due not only excessive accumulation in nerve tissue when acetylcholine is not metabolized, but also the decrease in the amount of acetylcholine in the perisinaptic region due to being exposed to execessive hydrolized process [6]. In the context of these facts, the determination of ACh, as in vitro/vivo, is crucial for anayltical methods [7].

The acetylcholinesterase (AChE) enzyme, which plays a role in the cholinergic system, regulates the level of neurotransmitter ACh. Organophosphates are widely used synthetic pesticides [8]. It is well known that small amount of organophosphate (OP) such as paraoxon inhibits the catalytic activity of AChE [9]. Investigation of organophosphates pesticides has been conducted via different tecniques such as mass spectrometry (MS), gas chromatography (GC), high-performance liquid chromatography, capillary electrophoresis, and GC–MS. However; these techniques have drawbacks including but not limited to being time consuming and offering expensive instrumentation. In addition, they require sample pre-processing, trained personnel and can not be operated outside laboratories. These challenges created a high demand for portable devices, that can enable simple, reliable, selective, rapid,

low cost and highly sensitive OP detection[10]. Enzym-based electrochemical biosensors have such requested properties. The working principle of the enzyme-based electrochemical biosensor is simply detecting and interpreting the change in the electrical signal. This signal is either the result of production or the reduction process of an electro-active species through enzymatic reaction. These types of biosensors not only eliminate complicated instrumentation need but also offer relatively low-cost solution [11]. That's why they have been adapted to countless of point-of-care applications with a wide range of analytes [11] and draw high demands with new application fields [12].

Chitosan, which is a biodegredable, is widely used in immobilization of enzymes. in the enzyme immobilization field, chitosan is one of the most popular polymer due to biocompatible, inexpensive and non-toxic properties [13,14].

Conductive polymers started to become more popular among the frequently studied materials especially after the discovery of their conductivity properties. Batteries, artificial muscles, membranes and corrosion technology can be given as an example for the application area of the conductive polymers [15]. Conductive polymers can be synthesized via chemical [16,17] or electrochemical methods [18–20]. Their easy preparation, high conductivity and stability enabled them to be used in enzyme immobilization [21]. Polyaniline [22,23], PPy [24,25] and their derivatives are conductive polymers that are widely used in enzyme immobilization. PPy can be used in electrochemical sensor materials, because it is easy to syhthesize and having good physical, chemical and electronic properties.

In this study, it was aimed to detect of acetylthiocholine and pesticides. For this purpose, the conducting polymer/enzyme-based electrochemical biosensor was designed with AChE immobilization on Pt/PPy/Chi. The linear range, operational and storage stability of this designed biosensor (Pt/PPy/Chi/AChE) were investigated by electrochemical method.

2. Material and Methods

2.1 Materials

All the chemicals were obtained from commercial suppliers (Sigma-Aldrich, Merck).

2.2. Preparation of chitosan solution

Chitosan (0.3 g) was slowly added to 10 mL of 1% acetic acid solution at room temperature and then dissolved by stirring in a magnetic stirrer (150 rpm) for 3 h. To prepare the coagulation liquid, 2 g of sodium hydroxide (1 N NaOH) was added to 50 ml of distilled water containing 26% ethanol [26,27].

2.3. Synthesis of Polypyrrole Film

Polypyrrole film was synthesized onto Platinum (Pt) electrode with cyclic voltammetry technique in 0.30 M oxalic acid solution containing 25 mM pyrrole monomer. The cyclic voltammetry technique was applied at a potential range between 0.00 and 1.00 V by 50 mVs^{-1} scan rates.

2.4. Preparation of Enzyme Electrode

Enzyme based electrode was prepared in following steps. Firstly, polypyrrole (PPy) homopolymer film was synthesized onto Platinum (Pt) electrode (Pt/PPy). Secondly, chitosan solution was dropped onto Pt/PPy electrode (Pt/PPy/Chi). Pt/PPy/Chi electrode immersed in coagulation solution for 20 minutes. The Pt/PPy/Chi electrode was washed until its pH was neutral. Thirdly, Pt/PPy/Chi electrode was incubated in 50 mM phosphate buffer (pH 7.0) containing 5 % gluteraldehyte solution for crosslinking along 2 h, and then, the excess of glutaraldehyde was removed with distilled water. Lastly, AChE enzyme ($5x10^{-3}$ EU) was immobilized onto Pt/PPy/Chi electrode by immersing in AChE solution for 24 h at 4 °C, and then, the excess of AChE was removed with distilled water. Electrodes were kept in Tris/HCl buffer (20 mM, pH 7.5) at 4 $^{\circ}C$, when they were not used.

2.5. Electrochemical Measurements

All the electrochemical experiments were performed in a single compartment cell with three electrode configuration. The reference electrode was an Ag/AgCl (3M KCl) electrode, the counter electrode was a platinum sheet. The working electrode was a platinum sheet with a surface area of 0.18 cm^2. A CHI 660E model digitally controlled electrochemical analyzer was used in the electrochemical experiments. The biosensor response was monitored by the

Differential Pulse Voltammetry (DPV) technique, where increment potential was 4mV, pulse ampltidue was 50 mV, pulse widht was 10 ms and pulse period was 40 ms. DPV measurements were performed at room temperature in Tris/HCl buffer (20 mM, pH 7.5) solution containing 5,50-dithiobis- (2-nitrobenzoic acid) (DTNB) for ATCh. The current responses and linear ranges of AChE immobilized on Pt/PPy/Chi (Pt/PPy/Chi/AChE) was investigated using a serie in different concentrations of acetylthiocholine iodide (ATChI) and pesticide.

2.6. Storage and operational stability

The operational and storage stability of Pt/PPy/Chi/AChE electrode were presented in a normalized form, where the highest value of each set being assigned to the value of 100% activity. Storage and operational stability were determined in Tris/HCl buffer (20 mM, pH 7.5) solution containing 50 mM ATCh by DPV.

2.7. SEM-EDX analysis

Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) of Pt/PPy/Chi electrode surface before and after AChE immobilization were used to understand the differences in morphology and elemental composition. The device was operated at a typical acceleration voltage of 10.000 kV.

3. Results and Discussion

The use of enzyme immobilization in different biotechnological applications has many advantages, such as the stabilization of the catalytic activities of the enzyme, its stability, reusability, the ability to obtain products with pure ease, high stability against environmental effects, continuity of production and cost reduction [28,29]. AChE based biosensors are used in both determination of pesticides, such as OP and carbamate in tap and river waters and soil extracts in food and environmental fields [30].

Electrochemical sensors convert chemical reactions of target species on electrodes into electrical signals where measure the changes in current. Electrochemical techniques

enables high selectivtiy with low detection limit in a relatively small volume of sample via interpreting the change in the electrical signal level [31]. These features have increased the interest in electrochemical biosensors and have led to intensification of studies in this field.

3.1. Synthesis of polypyrrole

Figure 1 shows the first cyclic voltammograms obtained in the solution of 0.3 M oxalic acid and 0.3 M oxalic acid with 25 mM pyrrole by using platinum electrode. Measurements were obtained between 0 and 1 V at a scan rate of 50 mVs^{-1}. The current values remained constant up to about 0.7 V (Figure 1) during anodic scanning in 0.3 M oxalic acid solution. The current increase above 0.7 V represents the oxygen gas. It was observed that there was no change in the current values up to approximately 0.6 V in 0.3 M oxalic acid + 25 mM pyrrole solution. However, above 0.6 V, it was observed that the rate of increase in the current was higher compared to the environment without monomer. This corresponds to the oxidation of the pyrrole monomer.

Film growth curves obtained in 0.3 M oxalic acid + 25 mM pyrrole solution are given in Figure 2. Five cycles were taken for a film development. The first segment in these curves has the highest current values, and the current values of each segment are lower in comparison to the previous segment. This indicates that in each segment taken, the electrode surface is slightly more coated with polypyrrole film in comparison to the previous segment.

3.2. Characterization of Pt/PPy/Chi/AChE

Cyclic voltammetry curves obtained from the Tris/HCl buffer (20 mM, pH 7.5) solution of Pt/PPy and Pt/PPy/Chi/AChE electrodes are given in Figure 3. In these curves, it is observed that the current values of the Pt/PPy/Chi/AChE electrode decreases considerably compared to the Pt/PPy electrode. This is due to the fact that Chitosan/Enzyme was immobilized on the Pt/PPy/Chi/AChE electrode surface.

Scanning electron microscopy (SEM) image and corresponding energy dispersive X-ray (EDX) diagram of Pt/PPy/Chi/AChE electrode are given in Figure 4. As can be seen in Figure 4a and b, the images Pt and Pt/PPy/Chi/AChE electrodes are different from each other. The uncoated Pt electrode surface appears to have a smooth structure, whereas the Pt/PPy/Chi/AChE electrode surface has a wavy structure. This is due to the presence of PPy/Chi/AChE on the Pt electrode surface. The appearance of the sulfur element (0.06 wt%)

in the EDX diagram in figure 4c shows that AChE is immobilized to the Pt/PPy/Chi/AChE electrode surface. Because methionine, containing sulfur, is an amino acid and is found in the structure of enzymes.

3.3. Substrate detection

DPV curves at different substrate concentrations of the Pt/PPy/Chi/AChE electrode are given Figure 5a. The current values in the solutions containing the substrate appear to be higher than the medium without the substrate. The current values increase as the substrate concentration increases in a media containing substrate. These findings are also compatible with the literature. For example, Somerset et al. [32] immobilized polyaniline-AChE onto the gold electrode surface. They stated that amperometric biosensor current signals increased with the increase in the concentration of the acetylthiochloride. Parsajo and Kauffmann [33] found that the current values of AChE immobilized electrodes increase in direct proportion to the ACh concentration. The linear range of the Pt/PPy/Chi/AChE was determined to be 30-50 μM for ATCh. The linear regression equation is y (μA) = 0.305x (μM)-0.1067 with a correlation coefficient of 0.9921. Limit of detection (LOD) was calculated to be 0.45 μM (LOD based on $3S_b/m$ where S_b and m are the standart deviation of the blank and the slope of the calibration graph, respectively). This LOD value is low compared to some other electrochemical biosensors [33][34][35][36].

3.4. Pesticide detection

Figure 6 shows the DPV curves of Pt/PPy/Chi/AChE electrode in solutions containing paraoxon at different concentrations. It can be seen that the current values in solutions containing paraoxon are lower in comparison to the one in a solution without paraoxonethyl. This is due to the irreversible binding of paraoxon to the active site of the AChE enzyme. These findings are also compatible with studies identified pesticides. For instance, Zheng et al. [37] performed the amperometric detection of paraoxon with the nano silver electrode that they immobilized with AChE/Chi. They observed that current values decreased with the increase in paraoxon concentration. Rodriguez et al. [38] modified the screen printed electrode

with AChE/Chi/Fe_3O_4 to detect malation. They stated that the concentration of malation is inversely proportional to the current values. The linear range of the Pt/PPy/Chi/AChE electrode was determined to be 0.46-1.84 nM for paraoxon, where the linear regression equation is y (μA) = 2.4326x (nM) + 0.480 with a correlation coefficient of 0.9939. Limit of detection (LOD) was calculated to be 0.17 nM ($3S_b/m$), and this LOD value is low compared to some other electrochemical biosensors [39][40][41][42]

3.5. Stability

Immobilization is an important factor that increases the stability of enzymes. In a study conducted by Işık, the storage and operational stability of AChE, immobilized to the chitosan surface, was reported to be quite high compared to the free enzyme [43]. The immobilized AChE activity was found to be 75% more stable than the free enzyme in a study conducted by Gabrovska et al [44], where AchE was immobilized on acrylonitrile copolymer membranes. In another study on immobilization of AChE on porous silicon, immobilized AChE maintained its activity 50% longer than the free enzyme [45].

In our study, the operational stability of the Pt/PPy/Chi/AChE electrode was examined in buffer solution containing 50 mM ATCh. 20 Measurements were taken for this purpose. To determine relative activity Pt/PPy/Chi/AChE electrode, the currents obtained from all separate measurements were calculated as the percentage of the initial current. The results obtained in this way is plotted in Figure 7. As can be seen in Figure 6, the current values keep on decreasing til 17th measurement and show a very little change after the 17th measurement. 94.13% of the initial current was obtained at the end of the 20th measurement. This value shows that operational stability is very good. Aynacı et al. [5] took 18 measurements from the ATCh solution to test the operational stability of the AChE-choline oxidase enzyme electrode that was immobilized to the polypyrrole-polyvinylsulfonate film. They found that the activity at the end of the 18th measurement was 83.1% of the initial activity. Moradzadegan et al. [46] stated that AChE, which they immobilized to poly (vinylalkol) nanofibers, had 70% of the initial activity after 10 measurements.

While free enzymes cannot maintain their long-term activities under certain conditions, immobilized enzymes can maintain their activities for a longer time. Increased storage stability of enzymes shows that these activities are preserved for a longer period of time. In our study, immobilized AChE enzymes on Pt/PPy/Chi electrode surface were stored at 4 ° C for 60 days. As seen in Figure 8, the immobilized AChE maintained approximately

72% of the initial activity after 60 days at 4 ^{o}C. This value shows that storage stability is very good. ACh biosensor prepared by Aynacı et al. [5] retained 31% of its initial activity after 35 day. Morinov et al. [47] found that the sensor maintained 75% of initial activity after 20 days of storage and 25% after 30 day, where they immobilized AChE on Poly(acrylonitrile-methylmethacrylate-sodium vinylsulfonate) membranes. In this study, the results show that the immobilized enzyme has significant advantages for many reasons such as longer shelf life, proper use, improved stability and prevention of contamination with enzyme products

3.6. Kinetic Parameters

In our study, the kinetic parameters of the immobilized AChE were determined using ATCh as substrate at different concentrations. The Km and Imax value for immobilized AChE were calculated from Michaelis-Menten (Figure 9). Km(app) and Imax were found to be 2,204 μM and 87,84 μA, respectively. These kinetic parameters show the change in the affinity of the substrate to the active site of the enzyme. The Km value that shows the affinity of the biosensor was found to be 2,204 μM in comparison to 1.74mM [48] and 0.4mM [49]. The high affinity of the AChE enzyme immobilized onto Pt/PPy/Chi used in this study can be understood from the Km value, that is lower than Km values in the literature.

4. Conclusions

AChE was successfully immobilized onto the platinum electrode surface coated with PPy and Chi. The operational and storage stability of Pt/PPy/Chi/AChE was found to be quite good. After 20 consecutive measurements, the operational stability was determined as 94.13%. In addition, the storage stability was found as 70% even after 60 days. The linear range was found to be between 0.46-1.84 nM for paraoxon and 30-50 μM for ATCh according to the results. LOD was determined to be 0.45 μM for ATCh and 0.17 nM for paraoxon. It was found that the biosensor with these properties is quite suitable to detect ATCh and paraoxon.

Conflict of interest statement

All authors declare that they have no conflicts of interest.

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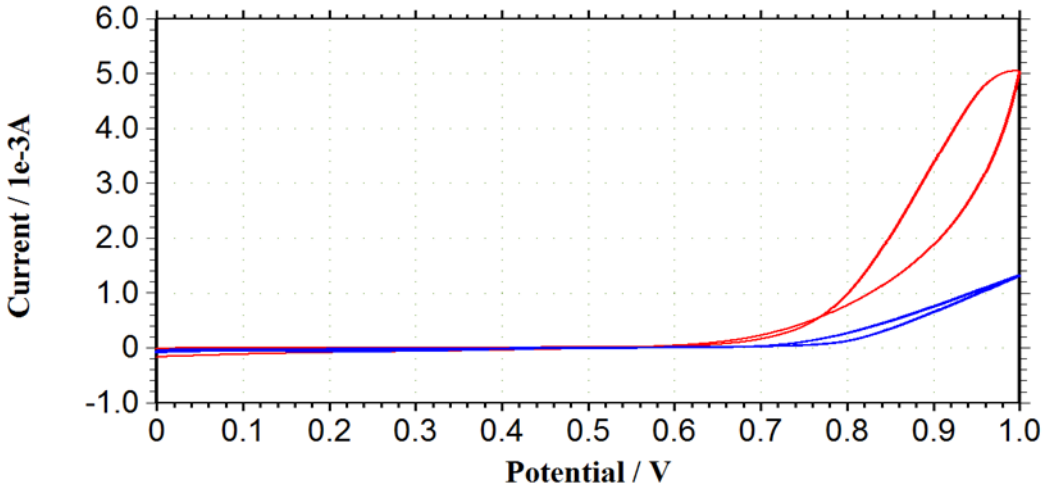
Figures



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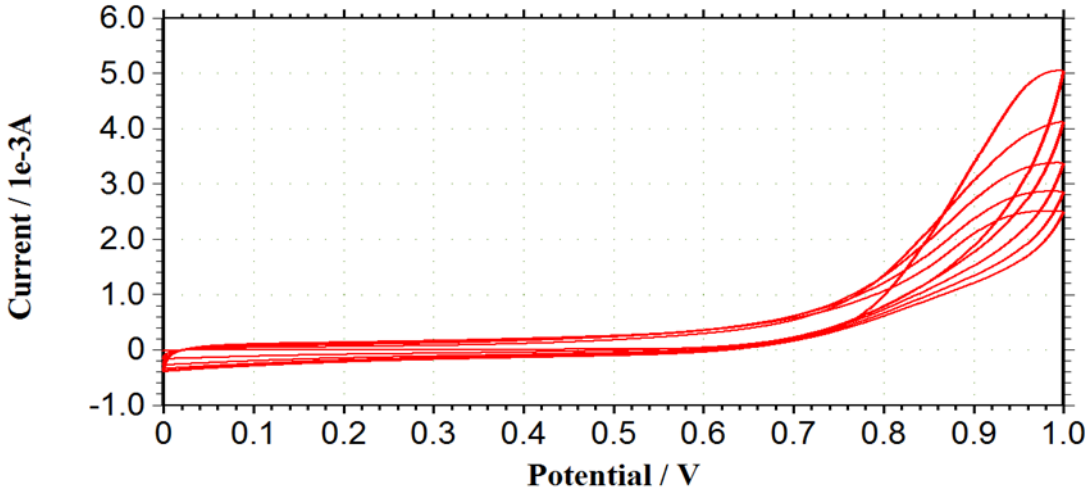


Figure 2

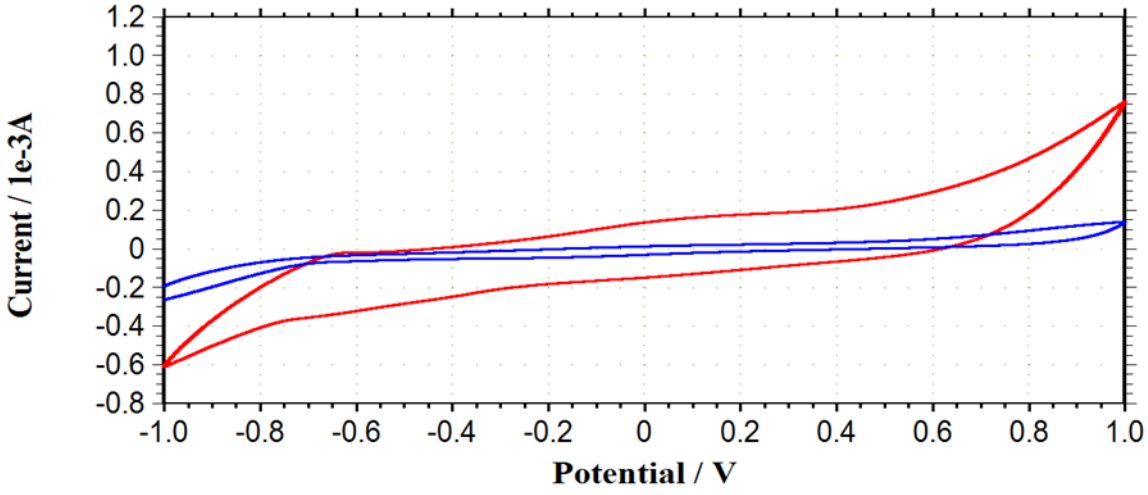


Figure 3

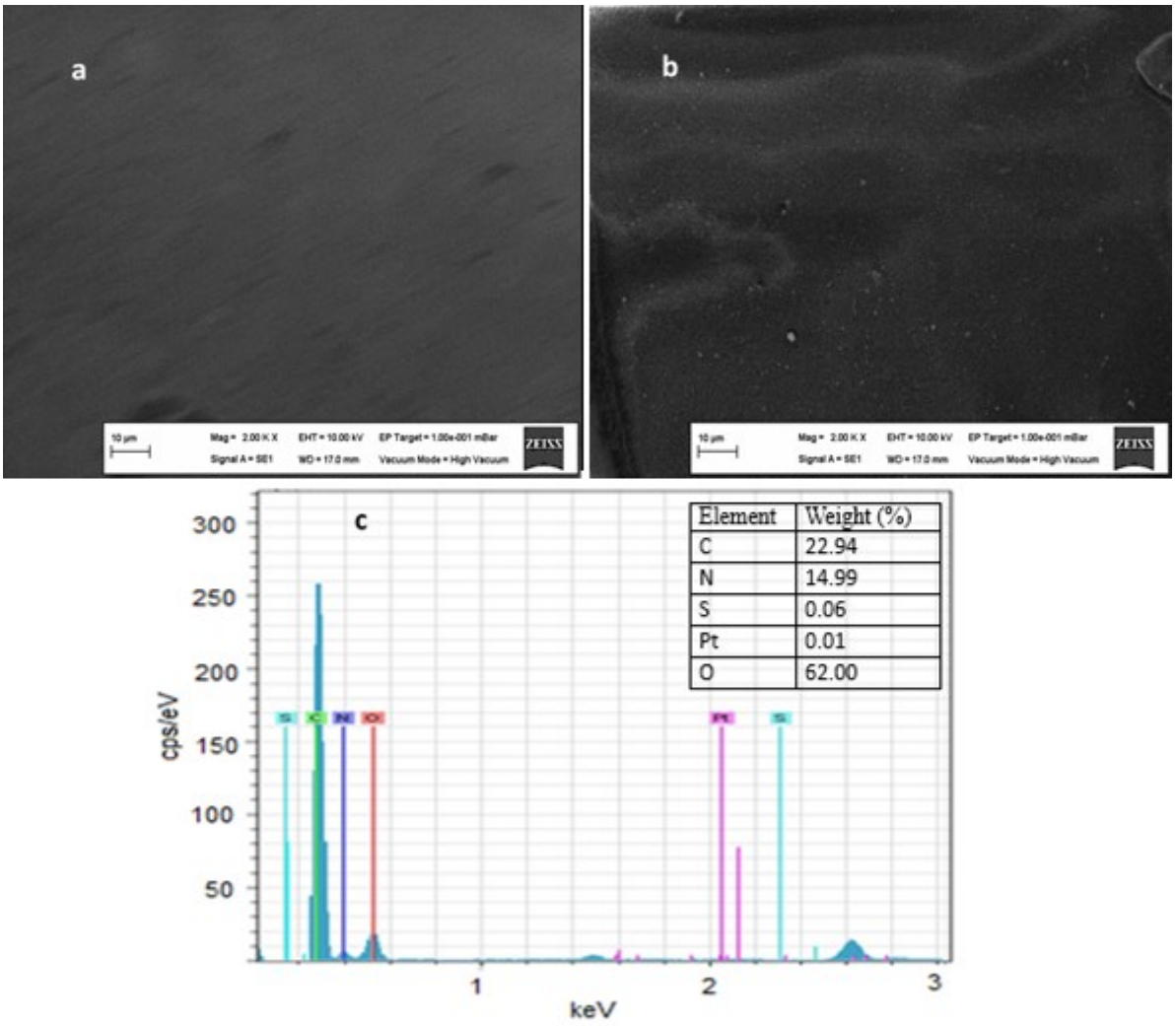
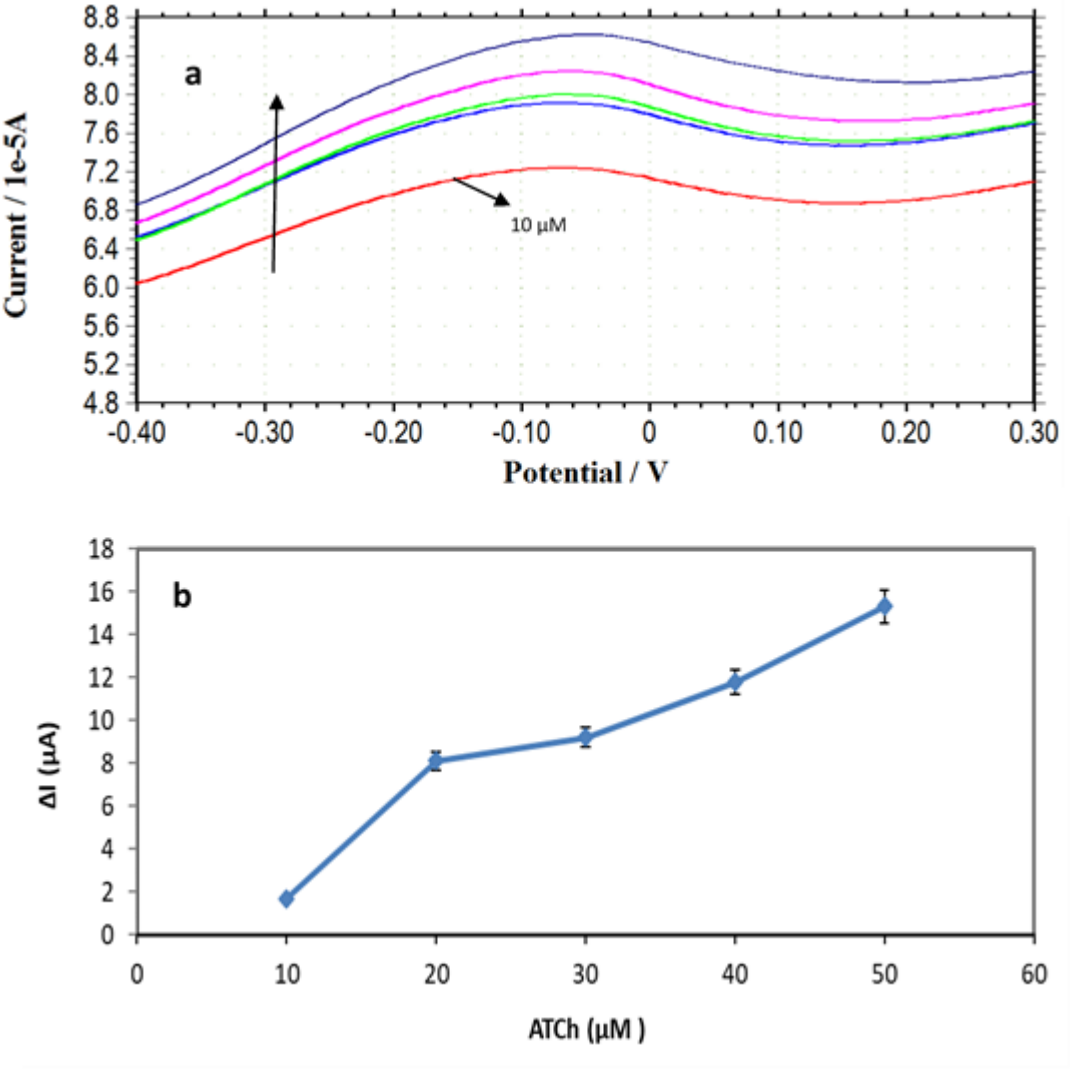


Figure 4



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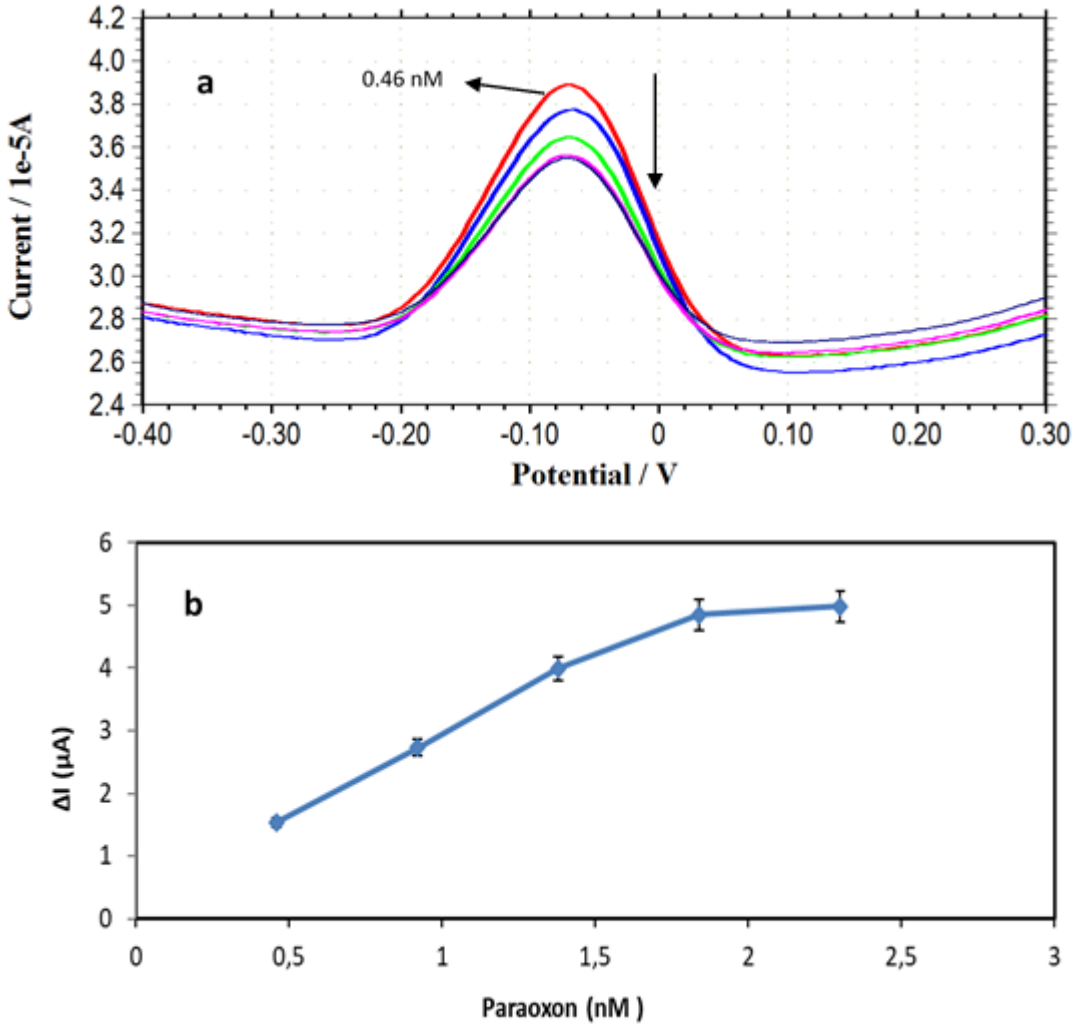


Figure 6

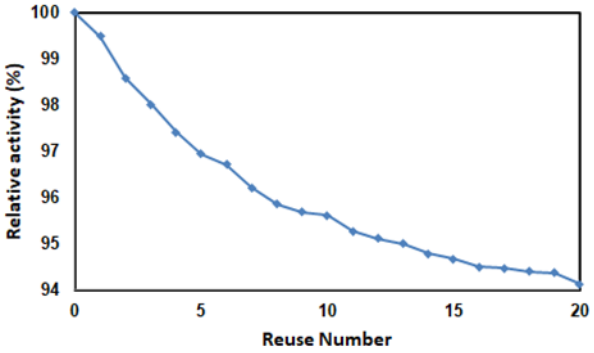


Figure 7

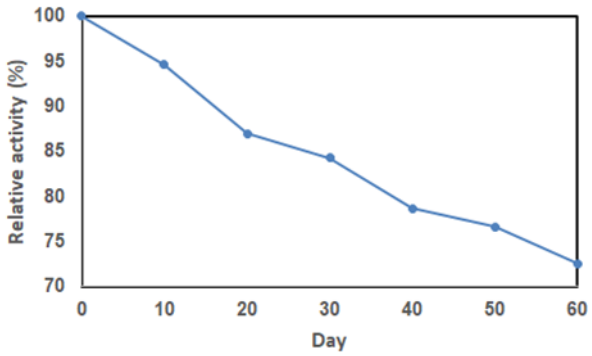


Figure 8

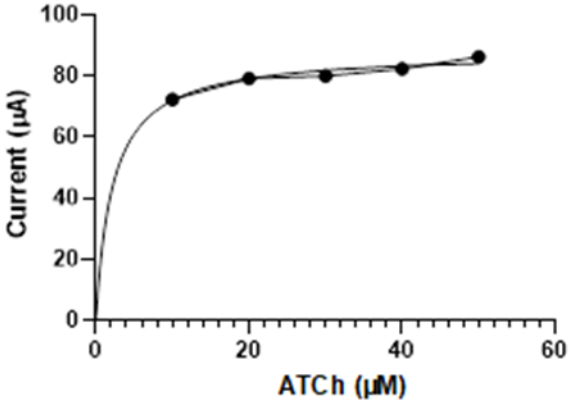


Figure 9

Figure Captions

Figure 1. The first cyclic voltammograms recorded for Pt electrode in 0.30 M oxalic acid and 0.30 M oxalic acid + 25 mM pyrrole

Figure 2. The film growth curves recorded for Pt electrode in 0.30 M oxalic acid + 25 mM pyrrole

Figure 3. The cyclic voltammograms for Pt/PPy and Pt/PPy/Chi/AChE electrodes in Tris/HCl buffer (20 mM, pH 7.5) solution

Figure 4. SEM images of Pt (a) and Pt/PPy/Chi/AChE (b) and corresponding EDX diagram (c) of Pt/PPy/Chi/AChE

Figure 5. Differential pulse voltammograms (a) and calibration plot (b) of Pt/PPy/Chi/AChE electrode in inTris/HCl buffer (20 mM, pH 7.5) solution containing 10, 20, 30, 40 and 50 µM ATCh

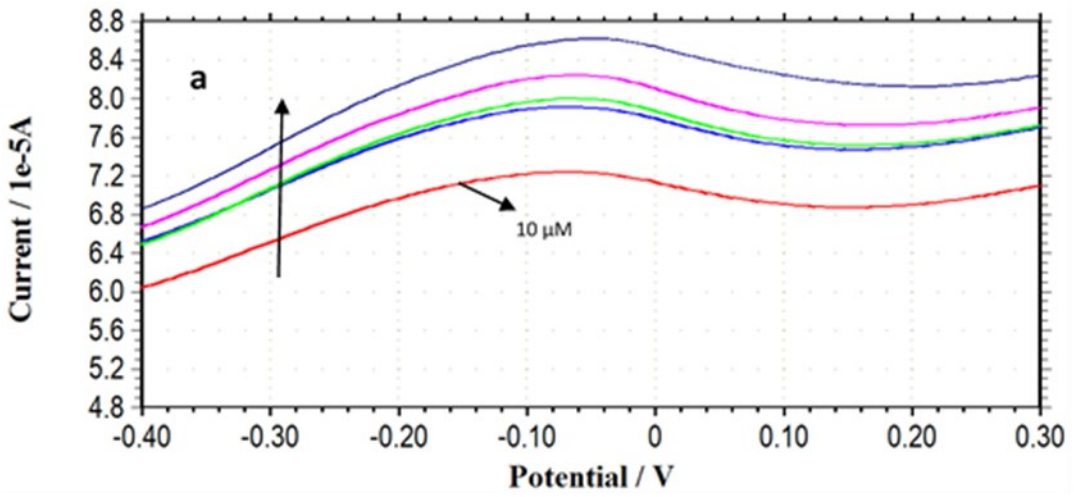
Figure 6. Differential pulse voltammograms (a) and calibration plot (b) of Pt/PPy/Chi/AChE electrode in inTris/HCl buffer (20 mM, pH 7.5) solution containing 0.46, 0.92, 1.38, 1.84 and 2.3 nM paraoxon

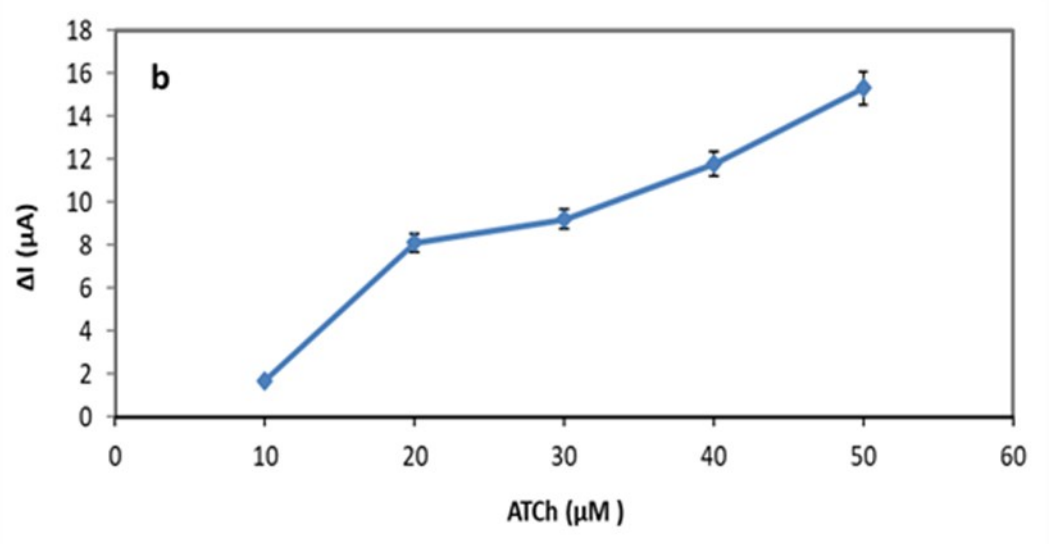
Figure 7. Operatonal stability of Pt/PPy/Chi/AChE electrode

Figure 8. Storage stability of immobilized AChE on Pt/PPy/Chi electrode

Figure 9. Michaelis-Menten graph of Pt/PPy/Chi/AChE electrode

Graphical Abstract





Highlights

- ✓ An electrochemical biosensor was proposed by using polypyrrole/acetylcholinesterase for acetylthiocoline and pesticides detection.
- ✓ The biosensor exibited good operational and storage stability.
- ✓ The biosensor has low detection limit.