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Analytical detection of paraoxon using acetylcholinesterase as an enzyme on polyaniline/$FeCl_3$ composite film by potentiostatic method

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Abstract: Polyaniline (PANI) film was synthesized by electrochemical method using sulfuric acid (H_2So_4) as a doping agent and ferric chloride ($FeCl_3$) as oxidants. Synthesized film was immobilized by enzyme, like Acetylcholinestrase (AChE) for cross linking detection of paraoxon. Thin film was characterized by X-ray spectroscopy, UV-visible spectroscopy, Fourier transformation infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), Energy dispersive X-ray spectroscopy (E-DAX) and result was analyzed. FTIR spectra recognize the existence of polarons and bipolarons within polymer film. UV spectra shows the absorption peaks due to π-π* transition of polyaniline and band gap energy. The enzyme inhibited film is used for amperiometric measurement of paraoxon solution in concentration range between 0.1µM to 0.9 µM and detection limit was found to be 0.1µM under optimal experimental conditions (phosphate buffer solution, pH 7.0 and temperature 25 ^{0}C). Biosensor shows good storage stability and retained 50% of its initial activity within 36 days at 4 ^{0}C.

Keywords: Polyaniline, Electrochemical, Biosensor, Acetylcholinesterase, Pesticide.

I. INTRODUCTION

Development of biosensor for analytical detection of pesticides is an excellent choice in conducting polymer films, biosensor is like a transducer, and it is self-contained device that integrates immobilized biological elements like enzyme [1-5]. Now the development of enzyme-based electrochemical sensor appear as a good choice because of their simple preparation and measurement process, high conductivity, short response time, sensitivity and reproducibility. The inhibition activity of enzyme is monitored by measuring the oxidation current by applied certain potential. Also they allow the structural, electrical and optical properties to use them as an immobilization point in biosensors. Therefore, AChE enzyme is easy immobilized on thin film for determination of pesticides [6-8]. AChE enzyme is excellent inhibitor for polymer film to produce sensor which is sensitive microenvironment for bio-molecules and good adhesion to the anlyte [9-11].

In present work the electrochemical polymerization takes place due to redox reaction with proper concentration of aniline, H_2SO_4 and $FeCl_3$. Electrodes like indium tin oxide (ITO), platinum foil and silver/silver chloride (Ag/AgCl) are used for polymerization. PANI/$FeCl_3$ composite film is prepared for development of pesticide sensor after cleaned with phosphate buffer solution and then immobilized by an AChE enzyme [12-14].

II. EXPERIMENTAL PROCEDURE

A. Electrochemical Synthesis of PANI/$FeCl_3$

Electrolytic solution was made by 0.5M H_2SO_4, 0.5M $FeCl_3$ and 0.1N an aqueous solution of aniline (98%) monomer in double distilled water in electrochemical cell [15-16]. The pH of aqueous medium was maintained by using phosphate buffer solution. The electrochemical polymerization of aniline monomer and $FeCl_3$ was synthesized by galvanostatic method [17- 22].PANI film is composed with $FeCl_3$ on ITO substrate.

B. Synthesis of enzyme based biosensor AChE/PANI/$FeCl_3$/ITO

By dropping 0.8 µL of purified AChE solution onto the surface of the PANI/$FeCl_3$/ITO electrode (PBS) and incubating it at 20^{0}C for 24 hrs. Then rinse with distilled water, dried and stored at 4^{0}C prior to use. Then this electrode was prepared for electrochemical measurement, it constituted a real contribution from the composite surface to the efficiency of the biosensor without the cross-linking agents to make bonding to the active sites of enzymes [23-24].

C. Electrochemical Measurement

The AChE/PANI/$FeCl_3$/ITO biosensor was immersed in a cell containing 10 ml phosphate buffer solution pH 7.0. The response of the sensor was measured when the current reached a steady state. The pesticide detection was carried out with a two-step procedure. The initial response of the biosensor when immersed in a cell containing 10 ml PBS current was recorded as I_0, then the biosensor was incubated for 20 min in 0.5µM of standard solution of pesticides. After the incubation the biosensor was washed with buffer solution and distilled water. Then biosensor was immersed to the cell containing phosphate buffer solution pH 7.0 and the peak current was measured as I_1. The inhibition rate of pesticides was calculated as follows,

$$\Delta I(\%) = \frac{(I0-I1\ )}{I0} X100$$

Where, ΔI was inhabited rate, I_0 was the response current value 0.8µL AChE enzyme on PANI/$FeCl_3$/ITO film without paraoxon inhibited and I_1 was the response current of 0.8µL AChE on PANI/$FeCl_3$/ITO film with paraoxon solution of different concentration for 20 min.[25].

D. AChE reactivation

For the purpose of multiple usage and cost-saving, PANI/$FeCl_3$/ITO electrode was recovered by pyridine 2-aldoxime methochloride (2-PAM), the biosensor was exposed to pesticides, it was washed with PBS (pH7) and distilled water and reactivated with 4.0 mM 2-PAM for 10 minutes, then transferred to the electrochemical cell of 10 ml PBS (pH7) under slow magnetic stirring. Paraoxon was added and the peak current was recorded as I_2. The reactivation efficiency (R %) is calculated as the following equation [26-27].

$$R\ \% = (I_2/I_0) \times 100\%$$

Where I_2 was the response current value on PANI/$FeCl_3$/ITO with AChE after 4.0 mM/L 2-PAM reactivation for 10 minutes.

III . RESULTS AND DISCUSSION

A. UV–Visible spectroscopy

The UV-Visible absorption spectra of PANI/$FeCl_3$/ITO film was recorded at room temperature by Analytic Jena specord210 plus spectrophotometer with wavelength range 200 -800 nm as shown in fig.1 the spectra shows three peaks at around 264nm, 331nm and 491nm. The peak at around 264 nm shows $\pi \rightarrow \pi^*$ transitions which is available in compounds with unsaturated centers and aromatics compounds.

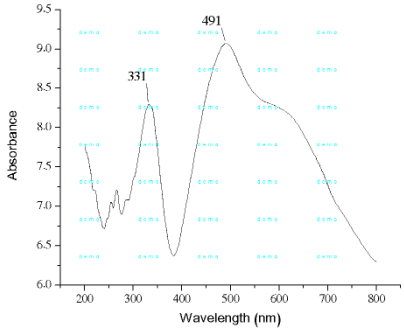

Fig .1 UV–Vis studies of PANI/$FeCl_3$/ITO Electrode

Second peak 331nm absorption is responsible for bi-polaron state and peak 491 nm shows charge transfer bands indicates conductivity of thin film having band gap energy is 2.56 eV. More doped aniline has resulted in stronger absorption at 491 nm. The absorption spectra observed for synthesized composite electrode gives good agreement with the earlier reported work [28-29]. This shows very good resemblance with the polymerization potential.

B. FTIR analysis

The FTIR spectrum of synthesized PANI/$FeCl_3$/ITO composite film with immobilization of AChE enzyme is shown in fig.2 the spectra show various peaks, the peak at 3325.33 cm^{-1} corresponds to N-H stretching. The peaks at 1230.38 and 1338.19 cm^{-1} which is assigned to evidence of the presence of anion in the polymer film i.e. S=O stretching in sulfonate. The vibration bands are observed at 1709.33 cm^{-1} (C=O) these peaks correspond to the characteristic for aniline it shows very good agreement with earlier reported work [30]. Thus the FTIR spectral results confirm the presence of polyaniline.

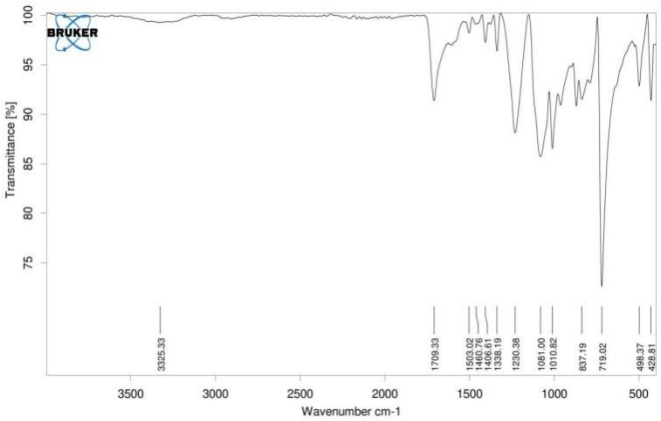

Fig. 2 FTIR studies PANI/$FeCl_3$/ITO based Electrode

C. X-ray diffraction

X-ray spectroscopy of PANI/$FeCl_3$/ITO film is shown in fig.3 The XRD pattern of PANI/$FeCl_3$ film clearly indicates that the intensity of observed peaks is better developed on the composites prepared using di and tri basic acid solutions compared with the monobasic acid. The profile of the characteristic peak of PANI/$FeCl_3$/ITO at $2\Theta = 25.11^o$ the obtained patter shows a mostly amorphous material with few crystalline phases. Thus the fraction of crystalline phase found to be increased with voltages.

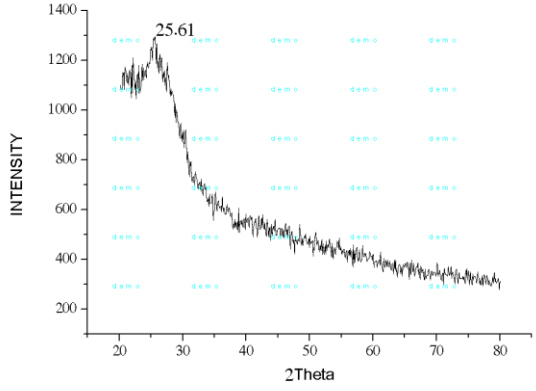

Fig. 3 XRD of PANI/$FeCl_3$/ITO

D. Scanning Electron Microscopy and Energy dispersive X-ray spectroscopy

SEM image of PANI/$FeCl_3$/ITO of synthesized thin films with and without immobilization of enzyme are shown in fig.4 (a, b, c and d) having magnifications 30KX and 50KX. Image a, b shows porous morphology of composite film. Such nature is easily supportive for immobilization of enzymes for the biosensor application. Image c and d show morphology changed with enzyme immobilized onto the modified electrode whose surface can be easily observed further, homogeneous coating of the enzyme proved that the proposed electrode before immobilization serves as an excellent host-guest platform for biomolecules immobilization. Fig. 4.e and f shows E-DAX spectrum and graphical representation of electrode. It indicates composition of various chemical compounds used for synthesized film. E-DAX image also finds parentages of chemical elements present in a sample.

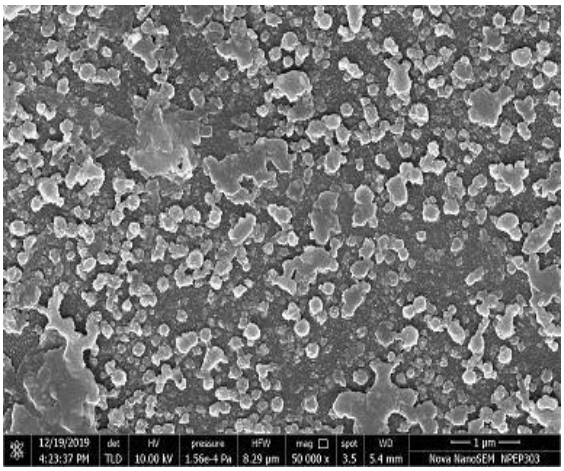

Fig.4 (a) SEM of PANI/$FeCl_3$/ITO film without enzyme (Mag. 30 KX)

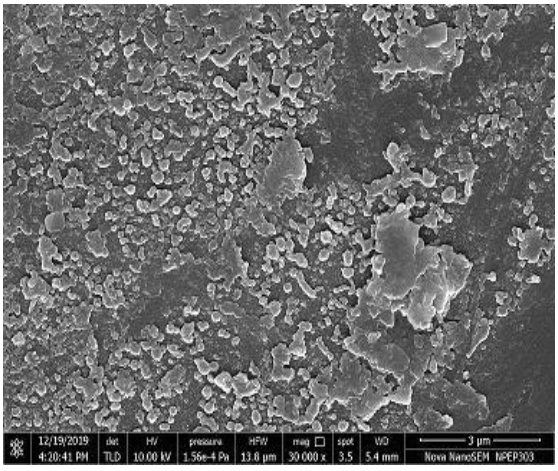

Fig. 4. (b) SEM of PANI/$FeCl_3$/ITO film without enzyme (Mag. 50 KX)

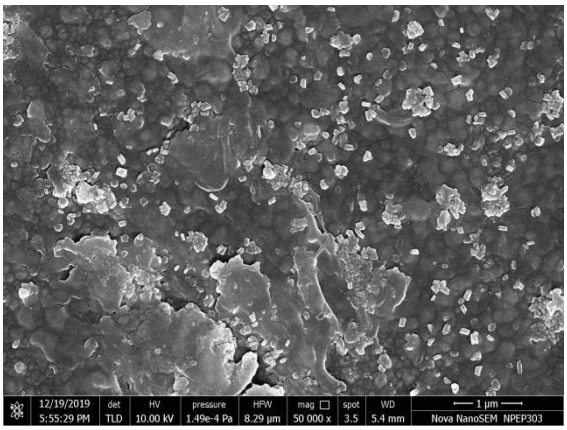

Fig.4 (c) SEM of PANI/$FeCl_3$/ITO film with enzyme (Mag. 30 KX)

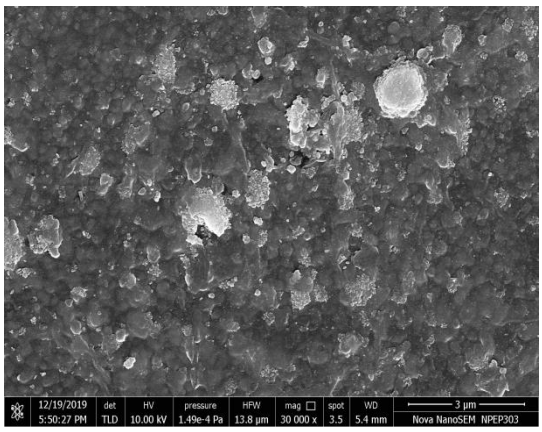

Fig. 4. (d) SEM of PANI/$FeCl_3$/ITO film with enzyme (Mag. 50 KX)

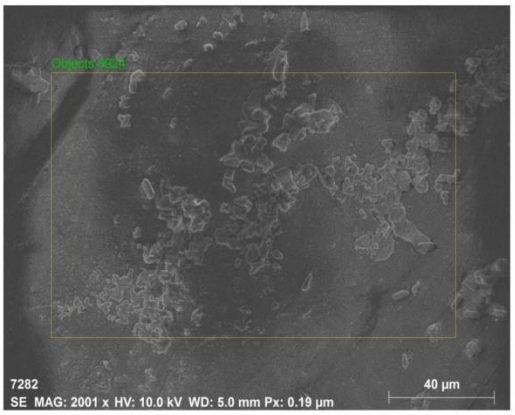

Fig.4 (e) E-DAX of PANI/$FeCl_3$/ITO film

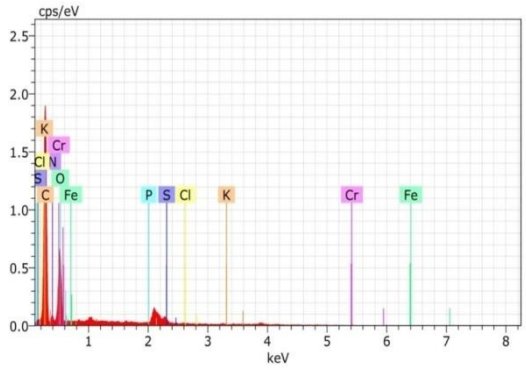

Fig.4 (f) E-DAX shows composition of PANI/$FeCl_3$/ITO film

E. Optimization parameters of experimental

E1. Effect of potential

The response current increases rapidly with increase in potential fig.5 which indicates that the response of the AChE electrode was controlled by the electrochemical method. It is well known that the rate of redox reaction is related to the concentration of electro active group, the pH value of solution and applied potential [31-32]. The response of the electrode at higher potential is decreased but at 0.9 V, the current was almost steady, which could be explained by the rate-limiting process of enzyme kinetics, diffusion-control of H_2O_2 and substrate [33-34]. So we had set the potential at 0.9 V for the further studies of AChE/PANI/$FeCl_3$/ITO electrode.

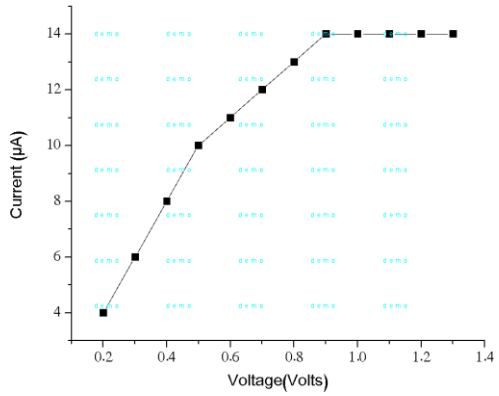

Fig.5 Current-potential curves for the AChE/PANI/$FeCl_3$/ITO electrode in 0.1M pH 7.0

E2. Current response

Current-time relationship of the AChE/PANI/$FeCl_3$/ITO electrode was set at 0.9 V is as shown in fig.6. It was found that, the current increases with increasing paraoxon concentration in the range 0.1μM to 0.9μM. In the present case, assuming that the enzyme is uniformly immobilized on the polymer film, the reaction takes place predominantly on the surface of the electrode in the lower concentration. However, the reaction on the surface of the electrode and the diffusion occurring simultaneously at higher concentrations delays the response time.

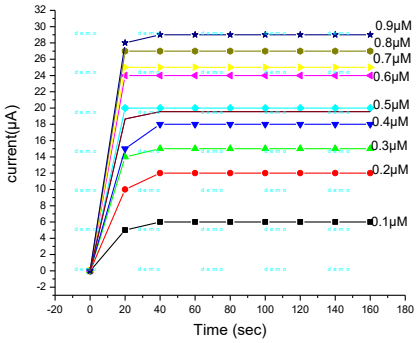

Fig. 6 Current–time curve of (AChE/PANI/$FeCl_3$/ITO) electrode

E3. Effect of pH

The pH study was carried out by taking the pH range from 1 to 9. The pH of the sample was adjusted using HCl and NaOH. It also prevents the loss of the enzyme activity under polymerization conditions [35].Therefore enzyme sensor response depends on the working pH of the sampling solution. The effect of pH on the behavior of the enzyme electrode was studied with 0.1 M phosphate buffer solution with 0.5 μM paraoxon. The current is measured at 0.9V by various pH values as is shown in fig. 7. The electrochemical response for doping electrolytic substance and dopants was quite better at pH ranging from 2 to 5 and the maximum current occurred at pH 7 [36].

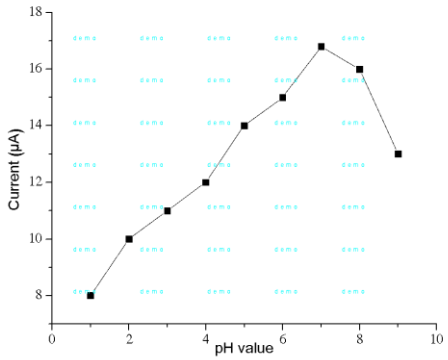

Fig.7 Effect of pH on the AChE/PANI/$FeCl_3$/ITO electrode response of paraoxon

E4. Michaelis–Menten constant (Km)

Michaelis–Menten shows the concentration of the substrate when reaction velocity is equal to one half of the maximum velocity. The relationship between 1/current against 1/paraoxon concentration in 0.1 M phosphate buffer is shown in Fig.8 the maximum current (I_{max}) = 29 μA. The value of Km is 0.9 it depends on immobilization of enzyme, at low Km reaction approaches more rapidly and we get faster response of the electrode to paraoxon.

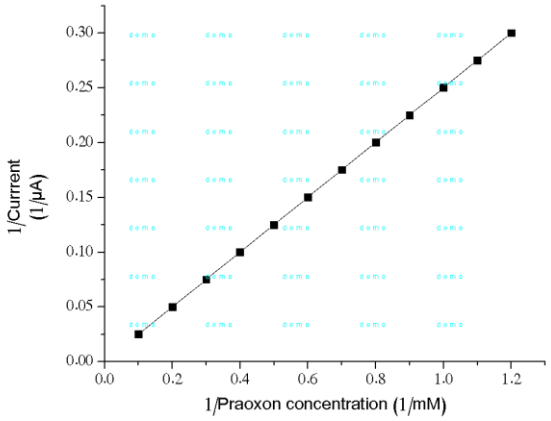

Fig. 8 Michaelis–Menten constant (Km) for the AChE/PANI/$FeCl_3$/ITO electrode in 0.1 M phosphate buffer, pH 7.0

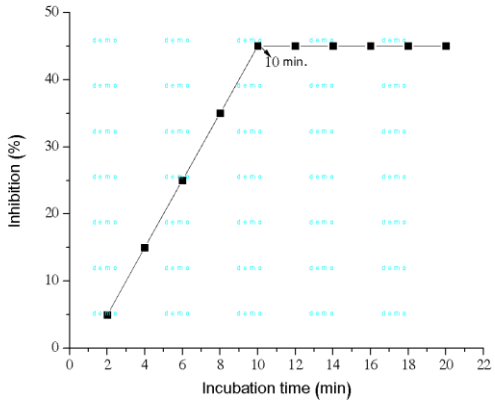

Fig.9 Effect of inhibition time on paraoxon (in 0.5 μmol L^{-1} concentration) at 0.9 V vs. Ag/AgCl

E5. Effect of incubation time

Paraoxon pesticides was tested for effect of AChE activity at incubation times (0–20 min) in a pesticide solution (0.5μM). It was found that enzyme inhibition increased with incubation period length until reaching a stable level fig.9 however the decrease in activity was less pronounced after 10 minutes for pesticides so incubation time was 10 min, which is better than earlier works [37].

E6. Reactivation of the AChE electrode with 2-pyridine aldoximemethiodide (2-PAM)

By irreversible inhibition of AChE pesticides electrode was reactivated by cleaned with phosphate buffer pH7.0 and immersed in reactivator 2-pyridine aldoximemethiodide (2-PAM, 4.0 mML^{-1} solutions). The activity of AChE was restored to 90% of its original value by 2-PAM treatment for 15 min.

Table.1. Comparison of performances of different biosensors for pesticide detection

Electrode	Analyte	Linear range	Detection limit	Inhibition time (min)	References
Au/PANI modified CPE	Atrazine	1-18μMol/L	0.3μMol/L	-	22
AChE/Fe_3O_4/GR/ SPE	Chlorpyrifos	0.05-100 μg/L	0.02 μg/L	15	24
AuNps/MPS/Au/AChE	Carbamate	0.003-3.0μM	1.0nM	10	34
AChE/SnO_2cMWCNTs/Cu	Methyl Parathion	1-160 μM	0.1μM	-	31
PANI/AuNPs/Pt/AChE	Paraoxon	3.6×10^{-7}-3.6×10^{-4} μM	0.026×10^{-5} μM	20	37
BChE immobilized on PBNPs/SPE	Paraoxon	7×10^{-9}M-4×10^{-8} M	4×10^{-9} M	10	36
AChE/HGO/GCE	Paraoxon	10-45 ng/ml	1.58 ng/ml	10	38
AChE/PANI/$FeCl_3$/ITO	Paraoxon	0.1μM-0.9μM	0.1μM	10	This work

F. Storage stability

It shows long term stability for the suitable application of a biosensor shown in fig.10. In order to calculate the storage stability, the sensor was tested for 6 weeks of storage in 0.1 M phosphate buffer pH 7.0, at 25°C. There was decrease in sensitivity of the sensor of about 10% from the initial value for every week, observing a very good preservation of the bioactivity than other work [38-39].

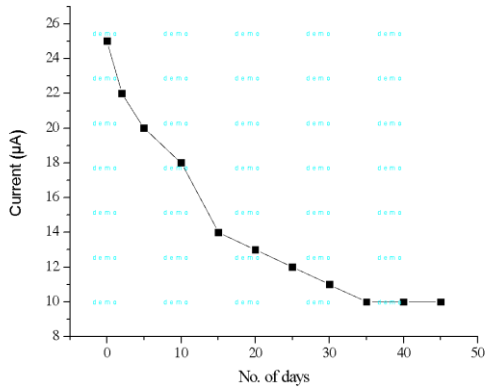

Fig.10.Stability of the AChE/PANI/$FeCl_3$/ITO electrode on storage in 0.1 M PBS, pH 7.0

CONCLUSION

The stability of electrode was enhanced by using ferric chloride as to immobilize the AChE. The AChE/PANI/$FeCl_3$/ITO electrode shows excellent conductivity, simple and uniform deposition on surface area of film and good stability it also shows high sensitivity, dynamic range of detection, short response time for paraoxon detection. This is inexpensive and simple method of fabrication of AChE/PANI/$FeCl_3$/ITO biosensor. The method not only can be used to immobilize enzymes to construct a range of biosensors but also may be extended to develop other biological molecules, such as antibody and DNA for biosensor.

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