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Amperometric biosensing of organophosphate and organocarbamate pesticides utilizing polypyrrole entrapped acetylcholinesterase electrode

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ABSTRACT

The work presented here describes a novel, easy and low-cost method of fabrication of a highly sensitive acetylcholinesterase biosensor and its application to detect organophosphate and organocarbamate pesticides. Acetylcholinesterase was electro-immobilized into a thick conducting layer of polypyrrole. Porcine skin gelatin and gluteraldehyde mixture was used for stabilizing the system. Acetylthiocholine chloride was used as the substrate. Polypyrrole catalyzed the electrochemical oxidation of thiocholine and promoted the electron transfer, thus lowering the oxidation potential and increasing the detection sensitivity. Electro oxidation of thiocholine in polypyrrole matrix occurred at 0.1 V under low potential scan rate. The thiocholine sensitivity of the electrode was found to be 143 mA/M. The sensor was applied to detect the sample organophosphate pesticide ethylparaoxon and organocarbamate pesticide carbofuran. The detection limit for paraoxon was found to be 1.1 ppb and that for carbofuran is 0.12 ppb. The sensor showed good intra and inter state precision with relative standard deviation (RSD) 0.742% and 6.56% respectively. Both dry and wet storage stability were studied. The sensor stored at 0 °C in dry condition had a good storage stability retaining 70% of its original activity for 4 months. During wet storage, the activity decrease followed the same trend, however, the operational stability at the end of the storage period was found to be less compared to the dry storage case. The developed biosensor is as a promising new tool for analysis of cholinesterase inhibitors.

1. Introduction

Organophosphates (OPs) and organocarbamates (OCs) have been widely used as pesticides in modern agriculture due to their low persistence and high insecticidal activity (Amine et al., 2006; Carlsson et al., 2000; Rekha et al., 2000). Although they degrade faster than the organochloride, they have greater acute toxicity, posing risk to people who may be exposed to large amounts (Jeyaratnam, 1990). That is why rapid determination and reliable quantification of OPs and OCs have become of great importance. Numerous analysis methods including gas chromatography (Asensio-Ramos et al., 2010), liquid chromatography (Cappiello et al., 2002), ultraviolet spectroscopy (Ganzera et al., 2006), gas-mass spectroscopy (Wong et al., 2007), fluorimetry (Dams et al., 2002) and surface plasmon resonance (SPR) (Chand and Gupta, 2007) have been developed which can estimate the OPs and OCs in contaminated samples with high sensitivity, reliability and precision. Despite their advantage, these methods require expensive instrumentation, highly trained personnel, and long work up time, making them unsuitable for rapid analysis of field samples. Hence, simple, sensitive, selective, and field deployable tools are still highly desired for monitoring of OP and OC contamination. Enzyme based electrochemical biosensors have emerged in the past few years as the most promising alternative to detect pesticides (de Albuquerque, Ferreira, 2007; Marinov et al., 2010; Pohanka et al., 2011; Pohanka et al., 2012; Roepcke, et al., 2010; Skladal, 1996; Xavier et al., 2000). Among them, amperometric acetylcholinesterase (AChE) biosensors based on the inhibition of AChE have shown satisfactory results for pesticides analysis (Crew et al., 2011; Du et al., 2010a,2010b; Raghu et al., 2012; Sun and Wang, 2010; Tuoro et al., 2011) in which the enzyme activity is employed as an indicator of quantitative measurement of insecticides.

Effective immobilization of enzyme to solid electrode surface still remains as a great challenge for the fabrication of biosensor. A key consideration for immobilizing enzyme is how to retain its bioactivity. The usual immobilization methods include direct physical adsorption on a solid support (Sotiropoulou et al., (2005)), cross linking (Kandimalla and Ju, 2006), encapsulation into a hydro gel (Anitha et al., 2004; Yadavalli et al., 2004), covalent binding

Abbreviations: Geltn, gelatin; Glut, gluteraldehyde

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(Du et al., 2007; Lin et al., 2004), entrapment in different substrate materials (Gong and Lin, 2003; Jeanty et al., 2001), self-assembly into multilayer film (Liu and Lin, 2006), immobilization on controlled-pore glass (Lee et al., 2002), and immobilization on magnetic particles (Kindervater et al., 1990). The conducting polymers have attracted much attention due to their interesting electrical and electrochemical properties (Cosnier, 1999; Mu et al., 1997) and also due to their potential application in miniaturized electronic devices. Polypyrrole (PPy), a key member of the organic conducting polymers, has been widely used as the enzyme-hosting matrix in electrochemical biosensors due to its advantages of permitting a facile electronic charge flow through the polymer matrix, easy preparation, high conductivity and good stability (Ramanavičius et al., 2006). In these PPy based biosensors the enzyme was either electro entrapped inside the PPy matrix or attached to the surface of PPy film in pure or composite form. These include electro entrapment of glucose oxidase (GOX) (Fortier et al., 1990; Njagi and Andreescu, 2007; Umana and Waller, 1986) for glucose determination, electro entrapment of tyrosenase for determination of phenolic compounds (Narlı et al., 2006), surface attachment of acetylcholinesterase (AChE) on PPy and polyaniline (PANI) composite polymer film doped with multi-walled carbon nanotube (Du et al., 2010a,2010b) and on Au nanoparticles–polypyrrole nano wire composite film (Gong et al., 2009) for determination of OP and OC pesticides. Though the surface immobilized PPy-AChE-nanomaterial sensors can give high sensitivity, their durability is poor due to bio-fouling and washing out of the enzyme. Moreover the fabrication processes are very much cumbersome and expensive due to involvement of multiple chemical steps and costly chemicals such as the nanoparticles. Electro entrapment procedures are easy, single step procedure and due to the encapsulation of the enzyme by the macromolecular matrix, the enzyme is better protected from washing out during analytic application of the sensor and hence expected to be more durable compared to the surface immobilized AChE sensors. To our knowledge so far no work has been reported that has documented the use of electro entrapped AChE for pesticide sensing.

Aim of the present work was to test the feasibility of using PPy electro entrapped AChE as a biosensor for OP and OC pesticides without using nanoparticles, and hence to optimize the fabrication process and operational conditions for high sensitivity, reusability and long time stability. An optimum fabrication process has been developed in which AChE electro entrapped inside PPy using a low (0.02 M) concentration of KCl. The enzyme was stabilized inside the conducting polymer matrix by cross linking with gluteraldehyde and porcine skin gelatin. The performance of the sensor was tested with sample organophosphate pesticide ethyl paraoxon and organocarbamate carbofuran. This approach may help in imparting selectivity to the enzyme biosensors through control of the film porosity. Also, since polypyrrole film is not easily degraded by some of the organic solvents, therefore, such an approach may pave the way for real sample analysis in organic phase.

2. Experimental

2.1. Materials

Acetylcholinesterase, Type VI-S (500 U/mg) EEL, acetylthiocholine chloride (ATChCl) (99%), Bovine Serum Albumin (BSA) (96%), were procured from Sigma Chemicals Co., USA. Pyrrole (98%), gelatin powder (Type A, from porcine skin), 5,5′-dithiobis (2-nitro benzoic acid) (DTNB) (99%), carbofuran (98%), paraoxon-ethyl (90%), gluteraldehyde (50 wt% in H_2O) were all procured from Sigma-Aldrich, USA. KH_2PO_4 and K_2HPO_4 were of analytical reagent grade and procured from E. Merck, Germany. Doubled distilled water was used throughout the experiments.

2.2. Instrumentation

PAR 273-A potentiostate/galvanostate was used both for film deposition and electrochemical measurements. Platinum working electrode used was from CH Instrument, USA. UV–vis spectrophotometer used was UV-2550, Shimadzu, Japan.

2.3. Preparation of the sensor

The sensor probe was prepared by immobilizing AChE at the tip of a platinum (Pt) electrode (2 mm) with PPy as the support matrix. The Pt electrode was cleaned by polishing with fine alumina powder followed by sonicating for 15 min. For electro deposition 2 mL of 0.5 M pyrrole solution in phosphate buffer (pH 7.2) containing 0.02 M KCl and 5 μL (100 U mL^{-1}) of the enzyme were mixed together in a three electrodes cell setup comprised of platinum working electrode, platinum coil auxiliary electrode and Ag/AgCl saturated with 3 M NaCl as the reference electrode. Electrolysis was carried out at 0.8 V for 30 min. Before starting the electrolysis, the solution mixture was sonicated for 5 min. A thick film of PPy-AChE was developed at the tip. The film was then washed with phosphate buffer saline (PBS) (pH 7.2) and dried at room temperature for 30 min. Then, holding the electrode vertical, with its film containing end upwards, 5 μL of 5% gelatin solution (prepared by warming gelatin water mixture up to 60 °C followed by cooling down to room temperature) was added to it and then kept at room temperature for 1 h. Then gluteraldehyde (35% in water) was added in two steps. At first, 5 μL gluteraldehyde was added to the electrode tip with a micro syringe, keeping the electrode vertical. The electrode was then kept at room temperature in the same vertical position until the film appeared dry (for approximately 1 h). Then the electrode tip was again treated with gluteraldehyde by immersing it in the stock solution (35%) for 1 min. The electrode was then kept at room temperature for 4 h followed by overnight storage at 0 °C. Finally it was transferred to a −20 °C freezer where it was stored for 5 days before use.

2.4. Measurement procedure

Quantitative estimation of sensor response was done mostly through the chronoamperometric (CA) method, but cyclic voltammetry was also applied in few cases. The cell setup comprised of three electrodes, the sensor probe as working, Pt coil as auxiliary and Ag/AgCl saturated with 3 M NaCl as reference electrodes in PBS (pH 7.2) electrolyte. Initial voltage $E_0 = 0.0$ V was applied for 60 s, final potential $E = 0.70$ V for 400 s and ATChCl was added at 300 s, i.e., after sufficient current stabilization. After taking out from −20 °C freezer, prior to its application, the sensor was soaked in PBS for 30 min and then potentiostated at 0.8 V for 15 min. The experiments were performed at 32 °C with constant magnetic stirring. Triplicate measurements were made in all experiments, with four cycles of potential sweep for the CVs, unless stated otherwise.

Time dependence of the inhibitory action of the pesticides was studied by evaluating the percent residual activity (A_r%) with time and the concentration dependence of the same was studied by evaluating the relative inhibition percentage (I%) with concentrations, using respectively Eqs.(1) and (2).

$$A_r\% = \frac{I_2}{I_1} \times 100\% \quad (1)$$

$$I\% = \frac{I_1 - I_2}{I_1} \times 100\% \quad (2)$$

Here I_1 is the initial CA response of the biosensor to 2 mM ATChCl before incubation, and I_2 is the same after incubation in inhibitor solution.

3. Results and discussion

3.1. SEM analysis

The morphology of PPy film, PPy-AChE, and PPy-AChE-Geltn-Glut were characterized by SEM as shown in Fig. 1. Electrodeposited PPy film showed a granular morphology (A). Electro deposition from a sonicated mixture of the pyrrole and enzyme resulted into a film with compact growth pattern and uniformly distributed enzyme units (B). In Fig. 1C, the gelatin-gluteraldehyde layer is seen covering the scattered enzyme units.

3.2. Enzyme leaching test

Ellman's spectrophotometric experiment (Ellman et al., 1961) was performed to check if any trace amount of enzyme can leach out from the immobilization matrix during electrochemical treatment of the sensor. This was done by performing more than 40 blank CV and CA runs with the sensor, taking PBS as the electrolyte followed by subjecting the same electrolyte to Ellman's spectrophotometric test at 412 nm. Assay was prepared by adding ATChCl and DTNB to the PBS solution in the UV cuvette but not the enzyme. No increase in absorption with time was observed, indicating that, the enzyme units cannot leach out from the sensor during the electrochemical treatment.

3.3. Optimization of the fabrication process

3.3.1. Saturated substrate concentration

The saturated substrate concentration was determined through the Michaelis–Menten plot (Fig. S1) and found to be 2 mmol L^{-1}. The apparent Michaelis–Menten constant, K_m^{app} was evaluated through the CA method found to be 1.09 mmol L^{-1}, which was calculated from the lower linear part of the Michaelis–Menten plot (inset, Fig. S1), following the Lineweaver–Burk equation (Eq.(3)).

$$\frac{1}{i} = \frac{1}{i_{max}} + \frac{K_m^{app}}{i_{max}[ATCh]} \quad (3)$$

Here i_{max} corresponds to the saturation current of thiocholine oxidation.

This value of K_m^{app} is in the range of the values reported by other authors: for AChE adsorbed on polyethyleneimine modified electrode (1.5 mmolL^{-1}) obtained by Vakurov et al., (2004) and for AChE adsorbed on screen-printed carbon electrode covered with Prussian Blue and Nafion (0.84 ± 0.44 mmolL^{-1}) obtained by Suprun et al., (2005). The thiocholine sensitivity of the sensor was calculated from the linear part of the Michaelis–Menten plot and found to be 143 mA/M. K_m^{app} of the free enzyme was also determined under the same experimental condition and found to be 1.39 mmolL^{-1} and the thiocholine sensitivity was 4 mA/M. The lowering of K_m^{app} value of the immobilized enzyme was probably due to creation of diffusion channels in the enzyme loaded film which helped fast oxidation of the analyte. The Michaelis–Menten plot for the kinetics of the free enzyme is shown in Fig. S2.

3.3.2. Effect of enzyme loading

To see the effect of enzyme loading, the enzyme was immobilized in PPy taking different quantities ranging from 1 to 50 μL (100 U mL^{-1}) and their response to a fixed amount (2 mM) of ATChCl were examined through cyclic voltammetry (Fig. S3). From Fig. S3 it is obvious that the peak current decreases with increasing enzyme amount. The difference was not very significant between 1 and 5 μL (0.1–0.5 U) but significant decrease was seen from 5 μL onwards. With a loading of 50 μL, the peak almost disappeared. The decrease is probably due to increasing diffusion limitation as reported in literature for PPy entrapped enzymes (Ramanavicius et al., 2005). The peak potentials for curve “b” through curve “e” were found to be close to 0.80 V (RSD ranging from 0.21% to 0.26%) while in curve “a” it was 0.85 V with RSD 0.31%. Shift in peak potential in case of curve “a” is probably due to reduction in the number of diffusion channels in the film.

3.3.3. Effect of pH

The pH dependence of the enzyme electrode over the pH range 6.4–7.8 was studied through cyclic voltammetry. Fig. S4 shows the cyclic voltammetric response of the sensor towards 2 mM ATChCl at different solution pH. The peak potentials and the base line corrected peak currents (with RSDs) found to be 0.80 V (0.31%), 121.8 μA (0.73%), (pH 7.8); 0.85 V (0.20%), 195.4 μA (0.56%), (pH 7.4); 0.80 V (0.24%), 427.6 μA (0.37%), (pH 7.2); 0.80 V (0.21%), 329.9 μA (0.44%), (pH 7.0); 0.84 V (0.12%), 152.1 μA (0.66%), (pH 6.8) and 0.83 V (0.17%), 140.8 μA (0.53%), (pH 6.4). Variation of peak currents with the pH of the electrolyte is shown in Fig. S5. It was found that the peak currents were higher near the biological pH (pH 7). The maximum value was obtained at pH 7.2. Thus it is clear that PPy does not alter the optimum pH for the catalytic behaviour of cholinesterase.

3.3.4. Effect of KCl concentration

Effects of KCl quantity during electro deposition on signal intensity and sensor operational stability were studied in the range 0.02–0.1 M KCl using cyclic voltammetry. At higher KCl concentration, film formation found to be easier but the film thus formed showed higher background current and lower signal reproducibility during analytic application. The increase of the background current at higher chloride doping is probably due to the potential response behavior of the film as reported in the literature

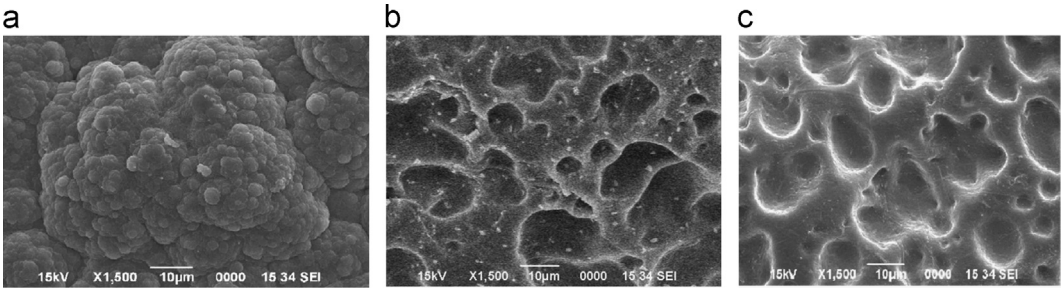

Fig. 1. SEM images of (A) PPy film, (B) AChE doped PPy film, and (C) PPy-AChE-Geltn[1]-Glut[2] film.

(Dong et al., 1988; Cadogan et al., 1992; Shimidzu et al., 1987). The instability in the amperometric signal with number of measurements at higher chloride doping is probably due to leakage of chloride ions, which in turn affects the background current, as is obvious from Fig. S6. To minimize the artificial increase in the sensor signal and also to have better stability, a lower amount of KCl (0.02 M) was used during electro deposition of the film.

3.4. Cyclic voltammetric behavior towards thiocholine oxidation

Fig. 2 shows the typical cyclic voltammograms (CVs) of the sensor and the immobilization matrix towards thiocholine oxidation at scan rate 20 mV/s. When CV was performed with the sensor in ATChCl, three peaks were seen (Curve 'b'). That these peaks were due to enzyme-substrate interaction only, is clear from a comparison of curve 'b' with curve 'c' (Pt-PPy-AChE-Geltn-Glut in PBS) and curve 'd' (Pt-PPy-Geltn-Glut in ATChCl). That none of these peaks is due to interaction of ATChCl with bare platinum electrode or PPy modified platinum electrode is obvious from curve 'f' and curve 'g' which are respectively the CVs of bare platinum electrode and polypyrrole coated platinum electrode in 2 mM ATChCl. That electro oxidation of gelatin and /or gluteraldehyde is not behind these peaks, is obvious from curve 'e', where the same three peaks appeared in absence of them. Inset in Fig. 2 is curve 'e' with those three peaks. One intense anodic peak (A) at 0.80 V (RSD 0.26% and peak current 605 μA with RSD 0.12%), one intense cathodic peak (B) at −0.65 V (RSD 0.3% and peak current 532.7 μA with RSD 0.44%) and a low intensity anodic peak (C) at −0.30 V to 0 V region. Intensity of both the peaks A and B found to vary with ATChCl concentration, although their trends of variation were not same. Variation of A with ATChCl followed Michaelis–Menten plot characteristic. So it is attributed to be due to oxidation of TCh produced during enzymatic hydrolysis of ATChCl. Variation of peak B was nonlinear initially but showed Michaelis–Menten plot characteristic when few initial values were discarded during continuous CV run in same ATChCl solution using the enzyme electrode. It indicates the possibility of occurring of two reduction processes at −0.65 V (RSD 0.3%), one of which might be the reduction of RSSR formed during anodic oxidation of ATChCl. Peak C tends to disappear at low scan rate, i.e., when relatively more enzyme substrate contact time is allowed. So it is probably due to some slow reaction between PPy surface and unhydrolyzed acetylthiocholine (ATCh).

The effects of scan rate on the cyclic voltammetric behavior of the sensor are shown in Fig. 3. The peak current found to increase and peak maxima shifted towards more positive potential with increasing scan rate. Inset I (Fig. 3) is curve 'a' which is the *CV* at 1 mV/s. Here two anodic peaks D (0.20 V, RSD 0.35%, 71.2 μA, RSD 0.32%) and E (from 0.65 V onwards, with RSD 1.5%), and one cathodic peak F (−0.60 V, RSD 0.20%, 124.1 μA, RSD 0.80%) are seen. With increasing scan rate peak D shifted to the higher potential side and got merged with peak E at a scan rate of about 8 mV/s and at a potential of 0.60 V (RSD 0.17%, peak current 234.9 μA with RSD 0.47%, Fig. 3, Curve 'c'). With further increase in scan rate the peak maxima of both anodic and cathodic peaks got shifted further (0.80 V with RSD 0.26%, 429.6 μA with RSD 0.37%; −0.80 V with RSD 0.21%, 409.7 μA with RSD 0.51%, Fig. 3, curve 'e'). It indicates that both the peaks D and E, originated from the electro oxidation of the same species—thiocholine (further justification to this point comes from Fig. S7). Peak F is same as the cathodic peak of Fig. 2b, which appears due to reduction of the dithio species. It has been attributed that peak D appears due to reduction of thiocholine ions those diffuse into the PPy matrix, whereas peak E is due to reduction of thiocholine ions in the solution at the vicinity of the PPy surface. Peak currents measured from the base line of charging current was found to be linear with the square root of the scan rate which indicates a diffusion controlled electrochemical process (inset II, Fig. 3). As seen in the Fig. 3 (Curve 'a'), the thiocholine oxidation peak appeared at 0.20 V (with RSD 0.35%) when a completely fabricated electrode was used, the same was obtained at 0.10 V (RSD 0.69% and peak current 103.8 μA with RSD 0.19%) with a freshly prepared Pt-PPy-AChE electrode (Fig. S7). The reason probably is, when the PPy surface is coated with gelatin and gluterldehyde the diffusion of thiocholine ions to PPy matrix gets hindered and becomes slower, which cause the oxidation peak to shift towards the higher potential side.

Thus, it is inferred that thiocholine electro oxidation in PPy occurs at 0.1 V. This potential is much lower than the oxidation potential of 0.70 V of thiocholine on solid electrodes, as reported in literature (Liu et al., 2005). The enhancement of the amperometric signal and lowering of the oxidation potential are attributed to be due to its inherent conductivity and electro catalytic property of PPy. It is noteworthy to mention that, although the thiocholine oxidation in presence of PPy occurs at 0.1 V, a potential of 0.7 V was used during chronoamperometric analysis so that the sensitivity is maximum.

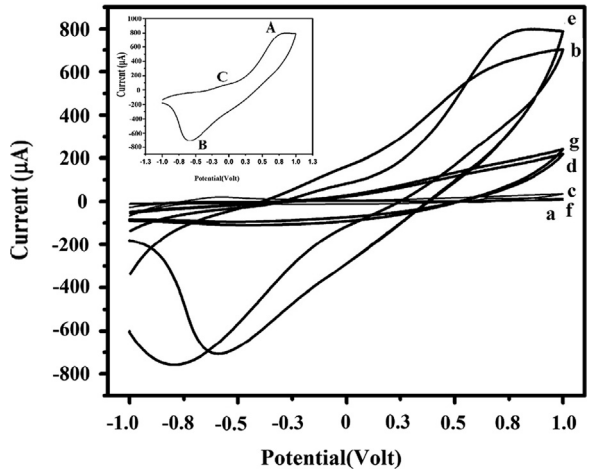

Fig. 2. Cyclic voltammograms at scan rate 20 mV/s of (a) Pt electrode in PBS, (b) sensor in 2 mM ATChCl, (c) sensor in PBS, (d) Pt-PPy-Geltn-Glut electrode in 2 mM ATChCl, and (e) Pt-PPy-AChE electrode in 2 mM ATChCl. (f) Pt electrode in ATChCl (g) Pt-PPy electrode in 2 mM ATChCl. *Inset*: Fig. 2e.

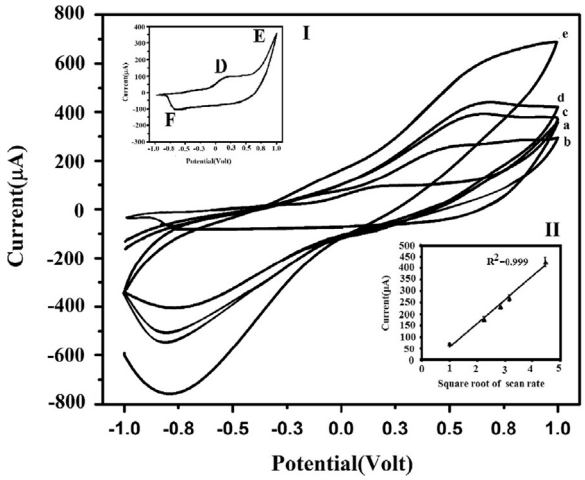

Fig. 3. Cyclic voltammograms of the sensor in 2 mM ATChCl at different scan rates. (a) 1 mV/s, (b) 5 mV/s, (c) 8 mV/s, (d) 10 mV/s, and (e) 20 mV/s. *Inset I:* curve a *Inset II*: Scan rate vs. peak currents.

3.5. Inhibition study

3.5.1. Effect on chronoamperometric signal

The effect of inhibition on the sensor response was studied by examining the biosensor response to 2 mM ATChCl before and after incubation in inhibitor solution (Fig. 4). Curve 'b' is the initial response of the sensor to 2 mM ATChCl and curve 'c' is the response after incubating the sensor in paraoxon solution for 30 min. The figure clearly indicates that, as a result of inhibition, amperometric response of the biosensor decreases. Response characteristic of the sensor towards sudden addition of inhibitor was studied for real time monitoring of inhibitor and shown in Fig. S8.

3.5.2. Incubation time

The variation of residual activities of the biosensor with incubation time was studied for four differently concentrated inhibitor solutions each of paraoxon and carbofuran. Observing that carbofuran has stronger inhibitory effect than paraoxon the concentration range 12.5, 60, 150 and 250 ppb for paraoxon and 5, 25, 60 and 100 ppb for carbofuran were selected for inhibition study. In each case the sensor was incubated for 1 h duration and its response to 2 mM ATChCl and hence the percent residual activities were calculated by using Eq.1 (Fig. S9). We observed that, as compared to the other immobilization matrices used by different workers, relatively more time is required for AChE inhibition in presence of the thick PPy matrix used here. The reason probably is that, the inhibitor molecules need to overcome the diffusion barrier at first to cause effective inhibition.

3.6. Enzyme reactivation studies

For reactivation of the inhibited enzyme, a 0.05 M NaF was used. The inhibited sensor was immersed in a solution of 0.05 M NaF for 15 min followed by washing and 15 min immersion in phosphate buffer (0.01 M) solution. When the inhibition is less than 15 %, 95–98% reactivation occurred. But when the percent inhibition is beyond 15%, reactivation decreased gradually.

3.7. Calibration plot

The calibration curves for paraoxon and carbofuran were obtained by plotting the percent relative inhibition (I%) (Eq.2) against concentrations, while allowing the incubation for 1 h.

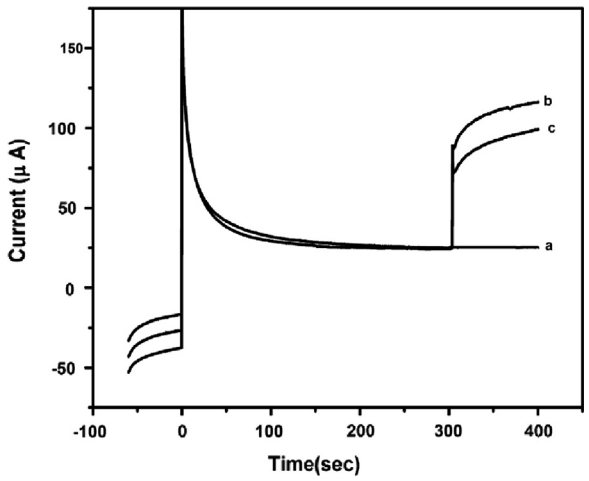

Fig. 4. Chronoamperometric response of the sensor towards (a) 200 μL KCl (0.05 M) in PBS, (b) 2 mM ATChCl, and (c) 2 mM ATChCl after incubation for 30 min in a 60 ppb paraoxon solution.

Paraoxon solutions were prepared in phosphate buffer while the carbofuran were in 5% acetonitrile. The calibration plots are shown in Fig. S10(A and B). In case of paraoxon the linear ranges were; from 0.1 ppb to 12.5 ppb ($y=1.081x+8.866$, $R^2=0.993$) and from 12.5 ppb to 150 ppb ($y=0.197x+20.33$, $R^2=0.998$). Maximum, but not complete, inhibition due to paraoxon occurred at 150 ppb, the amount of inhibition being 50%. In case of carbofuran the two linear ranges found were 0.025–2 ppb ($y=13.11x+8.432$, $R^2=0.998$) and 5–60 ppb ($y=0.256x+36.85$, $R^2=0.999$). Maximum inhibition occurred at 60 ppb with a value of 54%. Limit of detection (I_{10}%) were found to be 1.1 ppb and 0.12 ppb respectively for paraoxon and carbofuran. The difference of the linear range for the two pesticides could be attributed to the different degrees of pesticide inhibitions, which were caused by the different process of inhibition by the two pesticides. From the calibration curve it is obvious that the biosensor is more selective towards carbofuran than paraoxon. The ratios of the slopes of the two calibration curves in the two linear ranges are respectively 12.33 and 1.3 which indicates that carbofuran selectivity of the biosensor over paraoxon is more in the lower concentration range. Effect of 5% acetonitrile on the biosensor response and operational stability was examined and found that no significant changes in these two parameters occur as compared to the PBS solution. Similar results were also reported in the work of Valdés Ramírez (Valdés Ramírez's et al., 2008).

3.8. Reproducibility and stability

3.8.1. Precision measurement

Inter-assay precision or the fabrication reproducibility was confirmed by determining the response of six different fabrications to 2 mM ATChCl solution. The relative standard deviation (RSD) of the measurements was calculated. A value of 6.56% was obtained which indicates a good reproducibility of the fabrication process.

The intra state precision or the operational reproducibility was confirmed by evaluating the RSD of sensor response for six continuous CA run with a single fabrication, using 2 mM ATChCl. The RSD was found to be 0.742%, which indicates that the sensor response has acceptable precision for consecutive measurements of ATChCl.

3.8.2. Operational stability

The operational stability of the biosensor was confirmed by examining its repeated response to 2 mM ATChCl, both in absence and in presence of inhibitor. In absence of the inhibitor, the sensor signal found to be stable for 40 continuous measurements. After 40 measurements, the signals showed decrease and dropped to 50% of its original value at the 60th measurement. To study the operational stability in presence of inhibitor, a 60 ppb solution each of paraoxon and carbofuran were used. It was observed that in presence of inhibitor, the percent residual activity (%A_r) of the enzyme decreased gradually and consequently the sensor signals got reduced to a value close to 60%, after 10 number of measurements, in case of paraoxon. In case of carbofuran the sensor response dropped down to 60% after 7 number of measurements. Fig. S11 shows the response characteristics of the sensor in absense and presence of the inhibitors. Operational stability was found to vary with the aging period, i.e., storing of the sensor at −20 °C after fabrication (Bucur et al., 2006), and was maximum when subjected to a period of 5 days.

3.8.3. Storage stability

For evaluation of storage stability, a fresh fabrication was selected. After measuring the initial stable response, the sensor was stored at 0 °C and chronoamperometric responses were

Table 1
Comparison of the different parameters of the sensor with the surface immobilized PPy-AChE sensors.

Electrode type	Chemicals used for fabrication	Storage stability (days)	Reusability	Inhibition time (min)	Reactivation time	LOD ppb/pesticide	Ref.
AChE-Au-PPy/GCE	Pyrrole, $LiClO_4$, $HClO_4$, $HAuCl_4$, AChE	30 <	–	12	10	2 (methyl parathion)	Gong et al. (2009)
AChE/PAn-PPy-MWCNTs/GCE	Pyrrole, Aniline, HNO_3, H_2SO_4 MWCNTs, SDS, AChE	30	–	15	8	1 (malathion)	Du et al. (2010)
PPy-AChE-Geltn- Glut/Pt	Pyrrole, Gelatin Gluteraldehyde, AChE	120	8 assays above 70% residual activity	60	30	1.1 (paraoxon) 0.12 (carbofuran)	This issue

Table 2
Comparison of the analytic performance of the sensor with few of the contemporary AChE sensors when applied to the same analyte.

Analyte	Electrode	Limit of Detection (ppb)	Linear range range (ppb)	Ref.
Carbofuran	AChE/Nafion/BSA/CoPc-SPE	0.06	0.022–22	Laschi et al. (2007)
	AChE-TCNQ/SPE	1.1	–	Bucur et al. (2006)
	AuNP/AChE/Au	7.26	–	Shulga, Kirchhoff (2007)
	PPy-AChE-Geltn-Glut/Pt	0.12	0.025–2, 5–60	This issue
Paraoxon	AChE/Fe^{3+}-BSA-Nafion /SPE	10	14–173	Suprun et al. (2005)
	AChE/PAN-AuNPs/Pt	0.739	0.1–100	Marinov et al. (2010)
	AChE/Geltn-Cellulose/SPE	7975	–	Pohanka et al. (2012)
	AChE-Carbon Paste/Cu	0.86	0–33	Tuoro et al. (2011)
	PPy-AChE-Geltn-Glut/Pt	1.1	0.1–12.5, 12.5–150	This issue

measured at the intervals of 30, 60, 90 and 120 days. No loss in enzyme activity was observed till 60 days. Slight decrease in the response was observed at 90 days and at the end of 120 days 28% reduction in current signal was observed. To study the storage stability in wet condition, a freshly prepared sensor after initial treatment was immersed in phosphate buffer containing 0.05 M KCl and kept at 4 °C for 120 days. No significant loss in enzyme activity was found at the end of 120 days. We noticed that the wet stored sensor showed relatively poor signal reproducibility as compared to the dry stored one when subjected to continuous analysis at the end of the stored period (120 days). The dry stored sensor gave stable value up to 36–40 measurements, whereas the wet stored sensor gave the same up to only 20 measurements. The reason probably is that, prolonged exposure to water/phosphate buffer medium results in some changes in the microenvironment of the PPy matrix that makes the sensor signal unstable. We have also studied the wet storage stability in Bovain Serum Albumin (BSA) solution in phosphate buffer, but no significant difference in the result was observed as compared to the phosphate buffer/ KCl case.

3.9. Comparison with other AChE based biosensors

Performance of the fabricated biosensor has been compared with other contemporary AChE based biosensors and shown in Tables 1 and 2. From the tables it is obvious that the prepared biosensor has comparable analytical performance but some added advantage that includes ease of fabrication, reusability and stability. The fabrication process is very simple and cost effective due to involving only a limited number of steps, and limited number of chemicals. The operational and storage stability exhibited by the prepared sensor was not realized earlier with any other electro-entrapped biosensor. The enhanced stability is attributed to formation of cross linked network involving gluteraldehyde, polypyrrole, gelatin and the enzyme. Cross linking of PPy with monomeric or polymeric gluteraldehyde probably occurs through its N atom. Also, the porosity of the electro generated film and the vertical mode of addition of gluteraldehyde and gelatin during fabrication help penetration of these two components deep inside the film thus rendering better cross linking and stability. Gelatin serves the duel functions of cross linking with gluteraldehyde and providing of a biocompatible microenvironment to the enzyme inside the PPy matrix. The biosensor exhibited very enhanced signal output which was not seen earlier with any other AChE based biosensor.

4. Conclusions

We have developed a novel, simple and highly sensitive method of fabrication of acetylcholinesterase biosensor for OP and OC pesticides. The method involves electro entrapment of the enzyme AChE in polypyrrole in presence of very low amount of supporting electrolyte (KCl) followed by cross linking with gluteraldehyde and gelatin. Apart from the relatively longer analysis time, the other parameters of the sensor such as the sensitivity, stability, reproducibility, reusability and the ease of fabrication are very excellent and promising ones. Through this work we have demonstrated for the first time that polypyrrole amplifies the amperometric signal of thiocholine oxidation and lowers its oxidation potential from 0.7 V to 0.1 V. We have also demonstrated that gelatin and gluteraldehyde mixture can enhance the stability of the electro entrapped biosensors by providing a better cross linking than what was obtained earlier through mere use of gluteraldehyde.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.08.050.

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