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Acetylcholine esterase enzyme doped multiwalled carbon nanotubes for the detection of organophosphorus pesticide using cyclic voltammetry

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ABSTRACT

Use of immobilized acetylcholine esterase (AChE) for detecting organophosphorus pesticides in water sources and body fluids can bring down the detection costs dramatically. In the present study, AChE was directly doped on multiwalled carbon nanotube (MWCNT) surface modified with carboxylic groups through amide bond and used for organophosphorus pesticide detection. Amide bond formation between MWCNTs and the enzyme molecules avoid use of any intermediate membranes, cross-linkers or binding materials. This strategy overcomes the hindrance to electron transfer posed by membranes or cross-linkers and increases the sensitivity of detection. MWCNTs carrying carboxyl groups were deposited on glassy carbon electrode and were subsequently immobilized with AChE. The activity of AChE was monitored by cyclic voltammetry after immobilization. Scanning electron microscopy and atomic force microscopy were used to characterize the electrode surface. FT-IR spectra were taken to characterize enzyme-MWCNT complex. Under optimized parameters, the electrode showed linear range between 10 and 50 nM, which is promising for detection of trace amounts of the pesticide. The lower and higher detection limits of the sensor are 0.1 nM and 500 nM respectively. The stability and reusability of the electrode were determined. Finally, successful detection of organophosphorus compounds in real samples established it as reliable for sensor applications.

1. Introduction

Organophosphorus compounds are the most common environmental pollutants, contaminating water bodies, food and soil and cause many diseases to humans by inhibiting proteins of various metabolic pathways and processes. Use of pesticides in agriculture and production of agents of chemical warfare by industries are the major source of these toxic compounds. The toxicity of organophosphorus compounds depends on the type and structure of the pesticides [1–3]. Amongst various pesticides used worldwide, 30% are organophosphorus pesticides [4]. Organophosphorus compounds irreversibly bind the enzyme AChE and block its activity leading to accumulation of the neurotransmitter acetylcholine. Increased level of acetylcholine in nerve synapses causes toxicity to respiratory and nervous systems. Severe exposure to organophosphorus compounds can even result in mortality [5]. Therefore, sensitive and fast detection of these pollutants is necessary.

Customary methods used for organophosphorus pesticide detection are thin layer chromatography (TLC), gas chromatography (GC), HPLC (High performance liquid chromatography), LC/GC–MS and some spectroscopic techniques [6]. However, all these techniques have their limitations that do not permit on-site testing. They are expensive requiring trained technicians and specialized equipments. Besides, they are usually time consuming and tedious. On the other hand, electrochemical detection of pesticides need no labelling or technicians and being cost-effective, fast, non-laborious, has the potential to overcome the above pitfalls successfully [6]. Immobilization of the enzyme is the crucial step to make enzyme based sensors, as the enzyme should retain its native structure, should be in close proximity of the transducer and should not experience hindrance due to membrane. Different chemistries and membranes have been used by various workers for immobilization of enzymes in electrochemical sensors. Tuoro et al. in 2011 physically entrapped the AChE in carbon paste but the amount of the enzyme needed was as high as 2500 U and the sensitivity was not effective [7]. Immobilization of AChE onto Chitosan-silver-nanosilver ink was done by Q. Zheng and his co-workers in 2016. They have used chitosan as a binding membrane but it may hinder free electron transfer generated during catalytic process and hence may result in decreased sensitivity and detection limits [8]. In the present study, an electrode was developed without using any binding membrane or crosslinker. Instead GCE was coated with modified MWCNTs carrying carboxylic groups on their surface and subsequently AChE was directly loaded on the MWCNT surface. Carboxyl groups on modified MWCNT forge amide bonds with the enzyme molecules. The sensor performance is based on enzymatic activity of AChE coated on the electrode. The quantity of pesticide in the sample is measured with the help of the electrode, as

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the difference in enzymatic activity before and after its exposure to the pesticide.

Being highly sensitive, fast and reusable electrochemical biosensors are applicable for on-site detection of organophosphorus. Biosensors use two types of enzymes namely, Acetylcholinesterase (AChE) and organophosphorus hydrolase (OPH) in combination with different types of transducers such as metal nanoparticles and carbon based nanoparticles. Here carbon nanotubes have been chosen to fabricate the electrode as they offer higher mechanical strength, chemical inertness, higher surface area and high conductance to the biosensor [9,10]. Organophosphorus hydrolase (OPH) works on hydrolysis of organophosphorus but its use in biosensor makeup may not be advantageous as commercial preparations of the enzyme are not readily available and purification of the enzyme is difficult involving high costs. On the other side, AChE is readily available and is cost effective compared to organophosphorus hydrolase (OPH). Acetylcholinesterase (AChE) mounted sensor works on inhibition method. The enzyme catalyzed reaction leads to produce thiocholine from acetylthiocholine Iodide and this response is measured by the electrode due to oxidation reaction of thiocholine. Organophosphorus pesticide inhibits the activity of AChE which can be measured by oxidation reaction of thiocholine [8].

2. Materials and methods

2.1. Materials

Multiwalled carbon nanotubes (size <100 nm and 5–8 μm), acetylthiocholine iodide (ATChI) (A5751), acetylcholine esterase (AChE) from *Electrophorus electricus* (C3389) and paraoxon ethyl (36186) were purchased from Sigma-Aldrich (India). Bovine serum albumin (BSA) (65186), Sodium phosphate dibasic dodecahydrate, sodium dihydrogen phosphate dehydrate, potassium chloride and potassium ferricyanide was purchased from Sisco Research Laboratories (India). Stock solution of AChE was prepared in 1% BSA (w/v) solution in Phosphate buffer (pH 7.2) and stored at 4 °C. All other reagents were used without any further purification and were of analytical grade. Double distilled water was used in all the experiments, unless otherwise mentioned. Potatoes used as samples in the study were bought from a local vegetable market (Vadodara, India). Untreated tap water collected from the laboratory was also used as sample in this study.

2.2. Apparatus

Scanning electron microscopic images of the electrode surfaces after different stages of coating were taken with SEM (JSM-7600F; Jeol, USA) and Atomic force microscopy images were taken with AFM (Flex AFM, Nanosurf). All electrochemical measurements were carried out by CHI660C electrochemical workstation (CH Instruments, Austin, TX, USA). A three electrode system with AChE/MWCNTs/GCE electrode as working electrode, Ag/AgCl as reference electrode and a platinum wire as counter electrode (CH Instruments, Austin, TX, USA) was used. FT-IR spectra of MWCNT and MWCNT-AChE were recorded with FTIR spectrophotometer 8400S (Shimadzu, Singapore). Ultrasonic cleaner (Digital sonifier 450, Branson 50/60 HZ, USA) was used for dispersing MWCNTs. Chopped vegetable sample was centrifuged with centrifuge (Sigma, 3–18 K, Germany).

2.3. Fabrication of AChE/MWCNTs/GCE electrode

Previously a sensor was designed for the detection of acrylamide using carboxylic group modified single walled carbon nanotubes [11]. In the present study carboxyl group modified MWCNTs were used with GCE. Glassy carbon electrode was polished with 0.3–0.05 μm alumina slurry and rinsed several times with double distilled water to get mirror like surface. After that the electrode was dried at room temperature. Different stock solutions of carboxylic group functionalized MWCNTs were prepared in DMF (Dimethyl formamide). Various concentrations of MWCNTs (3–8 mg/mL) prepared in DMF were ultrasonicated for 4 h for dispersion. 5 uL aliquot of MWCNTs solution from each stock was layered onto the surface of glassy carbon electrodes separately and were allowed to dry at room temperature for 1 h. This resulted in thin films of MWCNTs having carboxyl functional group on the electrode surface. Different stock solutions of acetylcholine esterase enzyme were prepared in PBS (pH 7.2). Different aliquots of enzymes (0.025–0.9 Units) were drop casted onto the electrode and were allowed to dry at room temperature. Thus, the MWCNTs bind with the enzyme via amide bond formation (Fig. 1). The fabricated electrode was then gently washed in PBS to remove the loosely immobilized enzyme acetylcholine esterase. The electrode was stored at 4 °C in air when not in use.

2.4. Electrochemical measurements

Electrochemical measurements were carried out with potentiostat CHI660C (USA) with a three electrode configuration system. Potentials developed between the electrodes were measured versus the reference Ag/AgCl electrode (Sinsil Internationals, USA). A thin platinum wire was used as the counter electrode (Sinsil Internationals, USA). Cyclic voltammetry (CV) technique was performed and potential was cycled between +0.7 to −0.2 V with scan rate of 0.1 V/s and sample interval of 0.001 V for the electrode AChE/MWCNTs/GCE. Optimal concentrations of MWCNTs and AChE to be coated on GCE were determined by recording the peak current in electrolytic solution. Concentrations of the substrate and inhibitor, inhibition time and detection limit of the sensor were also optimized with the same parameters. The electrolyte composition used was as follows: 10 mM KCl + 0.1 mM $K_3[Fe(CN)_6]$ + 10 mM Phosphate buffer solution (PBS pH 7.2).

After optimization, the AChE/MWCNTs/GCE biosensor was used for organophosphorus pesticide detection using cyclic voltammetry (CV). AChE enzyme loaded on the electrode surface bind the pesticide covalently. This resulted in reduced enzymatic activity of the electrode and is proportional to the concertation of the pesticide. This inhibited enzyme's performance was tested by CV response in PBS (pH 7.2) solution containing 12.0 mM ATChI and current was compared with initial current before inhibition. Enzymatic reaction forms thiocholine intermediate which further gets oxidized anodically generating the dimer Dithio-bis-choline and

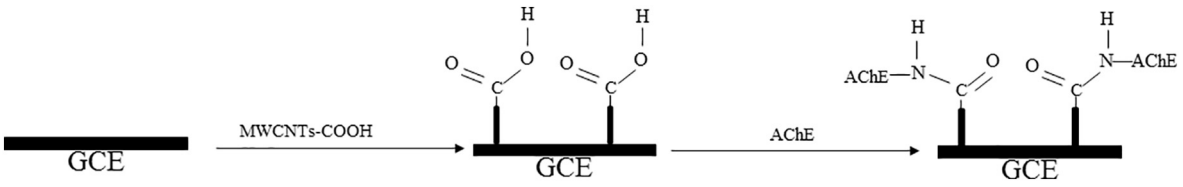

Fig. 1. Assembling AChE on glassy carbon electrode: ultrasonicated MWCNTs were adsorbed and layered on polished surface of GCE following doping of AChE through amide bond formation.

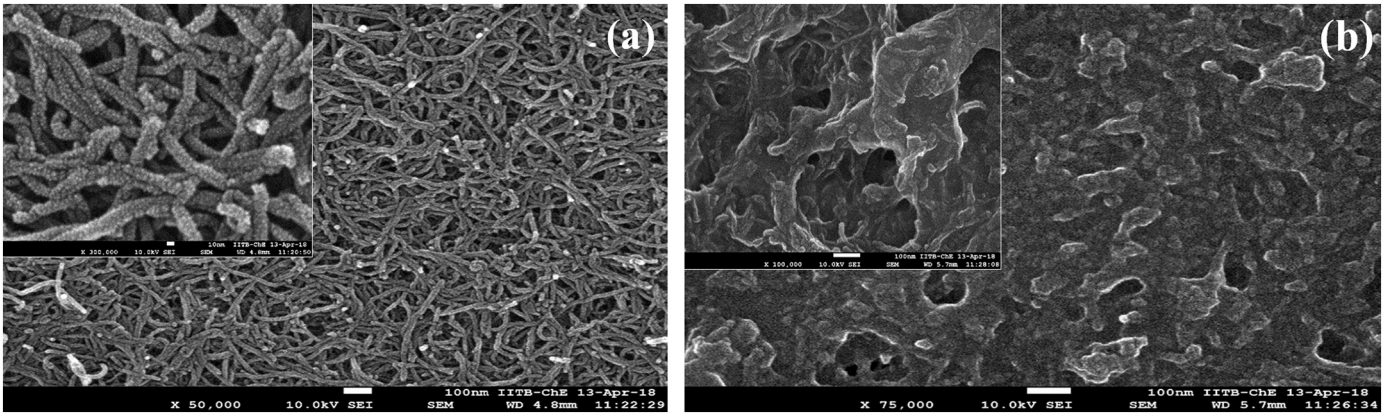

Fig. 2. SEM Images of modified electrode surfaces: (a) MWCNTs adsorbed (MWCNTs/GCE); (b) AChE doped on MWCNTs (AChE/MWCNTs/GCE).

releases a pair of electrons (Eq. (1)) [2]. Thus, the enzymatic reaction and generation of electrons were monitored as a current response by CV method.

$$\begin{aligned}&\text{Acetylthiocholine} + H_2O \overset{\text{AChE}}{\rightarrow} \text{Thiocholine (red.)} + \text{Acetic acid}\\ &2\ \text{Thiocholine(red.)} \rightarrow \text{Dithio-bis-choline(ox.) (Dimeric)} + 2H^+ + 2e^-\end{aligned} \quad (1)$$

The electrode was then rinsed in double distilled water and was incubated in PBS (pH 7.2) solution having desired concentration of paraoxon (organophosphorus pesticide) for 10 min. After that, its performance was tested in 12.0 mM ATChI solution by CV measurement under the same experimental conditions. The rate of inhibition by paraoxon was calculated as follows:

$$\%Inhibition = (I_0 - I_1)/I_0 \times 100 \quad (2)$$

where, I_0 = peak current of ATChI on the AChE/MWCNTs/GCE before paraoxon inhibition. I_1 = peak current of ATChI on the AChE/MWCNTs/GCE after paraoxon inhibition.

The electrode AChE/MWCNTs/GCE was then regenerated by pralidoxime iodide after paraoxon inhibition. The electrode was immersed in 1.0 mM pralidoxime iodide for 15 min and then was run in 12 mM ATChI solution in PBS (pH 7.2) for CV analysis to measure electrochemical response.

2.5. Preparation of samples

Two real samples tap water and potatoes were chosen for pesticide determination. Potato (5 g) was finely chopped into 3 mm cubes and soaked in distilled water for 45 min. Following that, potato soaked water was decanted and used for analysis. Tap water from the laboratory was directly centrifuged at 5000*g* for 5 min. Thus, prepared samples were directly used for pesticide determination. The fabricated sensor was first incubated in the prepared samples for 10 min., then the current of the sensor was estimated in 12 mM ATChI prepared in $K_3Fe[CN]_6$ and phosphate buffer as a response of enzymatic activity. It did not show any decrease in current which showed no inhibition of the enzyme. Therefore, to test the sensor's efficiency, pesticide was added to both the samples and the sensor was incubated in the samples for 10 min. Following that, current of the sensor was estimated in 12 mM ATChI before and after the incubation. Decrease in current was observed due to inhibited enzyme which is proportional to pesticide concertation.

While dealing with real samples on-site it is not always possible to control the chemical constitution of the samples. Hence, it is better to check the suitability of electrode with untampered real samples.

3. Result and discussion

3.1. Characterization of MWCNTs/GCE and AChE/MWCNTs/GCE electrode

The morphological and surface characterization of the fabricated electrodes was carried out using scanning electron microscopy (SEM) and atomic force microscopy (AFM). It was not possible to insert electrode surface for SEM and AFM analysis since the electrode does not have any detachable parts. Instead, glass slide was used in place of glassy carbon electrode assuming that it would effectively mimic the surface of the electrode to provide support for MWCNTs layering. The aim of the analysis was to ensure enzyme loading on MWCNTs. Similar methodology was used to analyze the film of deposited nanoparticles in literature [30–32]. The glass surface was coated with the functionalized carbon nanotubes followed by layering of the enzyme. It was observed using SEM that a large number of uniformly layered filamentous structures of MWCNTs modified the surface (Fig. 2(a)). Subsequently, successful loading of AChE on MWCNT surface was observed (Fig. 2(b)). This establishes loading of the enzyme on the MWCNTs and can be attributed to the amide bond formation between carboxylic group of MWCNTs and amine group of the enzyme. Fig. 3(a) and (b) represent the AFM images of bare surface and MWCNTs/AChE modified surface of glass slide respectively. AFM image of AChE/MWCNTs modified surface shows higher surface roughness compared to the bare surface (Fig. 3(b)). MWCNTs/AChE has a *Z*-mean fit value of 374 nm, which is higher than 25.5 nm observed with bare slide surface. Further, the results obtained by carrying out electrochemical analysis with GCE/MWCNTs/AChE, GCE/MWCNTs and bare GCE showed that the layering of MWCNTs enhanced the surface area for enzyme loading, besides increasing its electric conductivity when coated on GCE, which is discussed in Section 3.2. However, only topological and morphological characterization are not sufficient to ensure enzyme loading on MWCNTs. To confirm the above observations, spectroscopic and electrochemical characterization of the fabricated electrode was done by FT-IR spectra and cyclic voltammetry method respectively [12,13].

FT-IR spectra of MWCNTs and the complex of AChE-MWCNTs were recorded and compared (Fig. 4). The spectrum was magnified between 1600 and 1750 cm^{-1} to highlight and mark the peaks. The peak at 1654 cm^{-1} corresponds to α- helical structures while the peak at 1685 cm^{-1} indicates presence of β pleated sheet. The peak at 1718 cm^{-1} shows stretching of C═O bond in amide-I region. The shifts observed in the peaks can be attributed to interactions of other functional groups with each other and less intensity of the observed peaks may be due to the low amount of enzyme used for preparing the complex. Thus, it confirms the binding of AChE to MWCNTs [14].

Electrochemical analysis of stepwise fabrication of the electrode was performed by cyclic voltammetry as shown in Fig. 5. The voltage was cycled between 0.7 to −0.2 V and the current passing through the bare GC

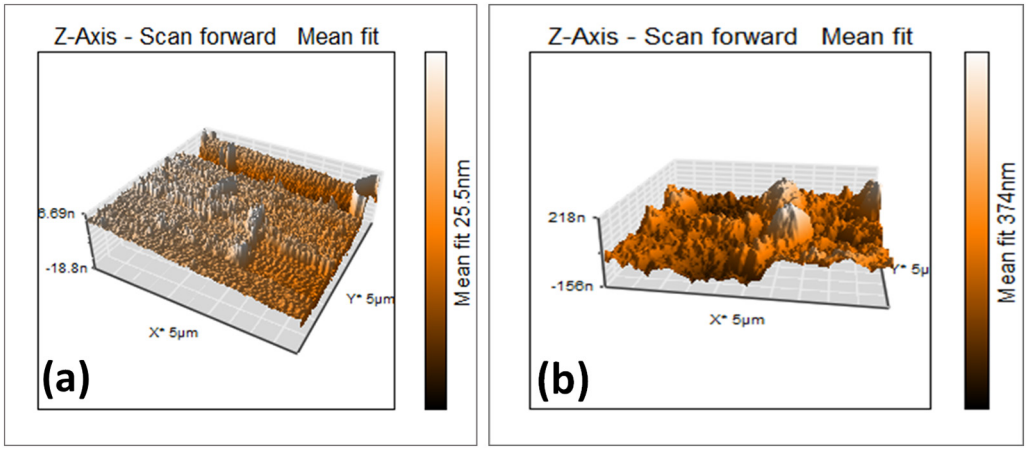

Fig. 3. AFM pictures of different electrode surfaces: (a) bare polished glassy carbon electrode; (b) MWCNTs modified and AChE doped glassy carbon electrode (AChE/MWCNTs/GCE).

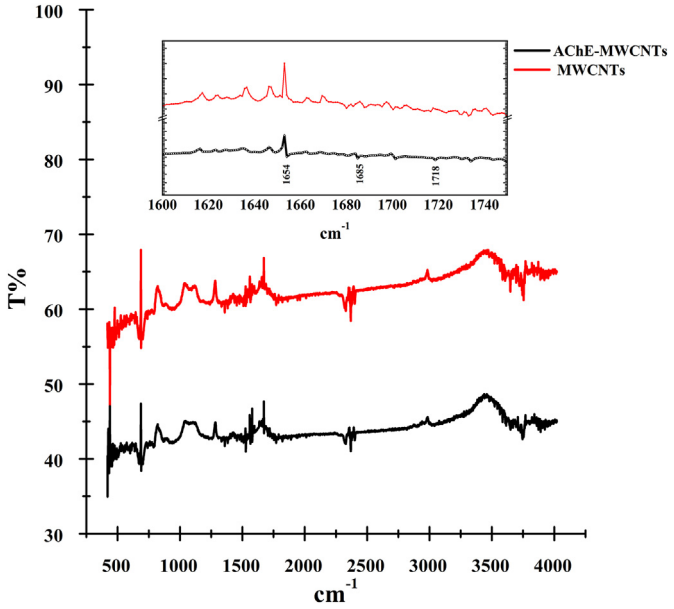

Fig. 4. FT-IR Spectrum of MWCNTs and AChE-MWCNTs complex: FT-IR spectra were recorded separately for MWCNTs and MWCNTs-AChE and were compared. The inset is showing magnification of FT-IR spectrum from 1600 to 1600–1750 cm^{-1} region.

electrode, MWCNTs/GC electrode and AChE/MWCNTs/GC electrode was measured. It was observed that current increased dramatically after coating GC electrode with MWCNTs, as a result of increased surface area and higher electric conductance property of carbon nanotubes. After the enzyme was coated on MWCNTs, GC electrode showed decrease in current. This decrease in current response may be a consequence of increased interface after enzyme coating causing deterrence to electron transfer. This also ensures that the enzyme itself is not getting oxidized or reduced during the voltage cycling [15–17].

Further, it is important to measure the response of the sensor towards thiocholine which is generated by the enzymatic reaction as shown in Eq. (1), because the pesticide is detected directly by measuring electrochemical behavior of thiocholine. Thiocholine has sulfydryl group which is responsible for its electrochemical activity and generates the dimer Di-thio-bis-choline and liberates electrons. But, thiocholine once generated is very unstable so it is very difficult to measure the response of the sensor directly against it. Further, it is not commercially available. Cysteine has thiol group and it is easier to obtain. Therefore, in the present study cysteine was used instead of thiocholine to measure its electrochemical behavior on AChE/MWCNTs/GCE [18]. Cyclic voltammetry was run in three cycles, in the absence and presence of 0.5 mM cysteine, to characterize the response of the fabricated electrode AChE/MWCNTs/GCE under the experimental conditions as mentioned

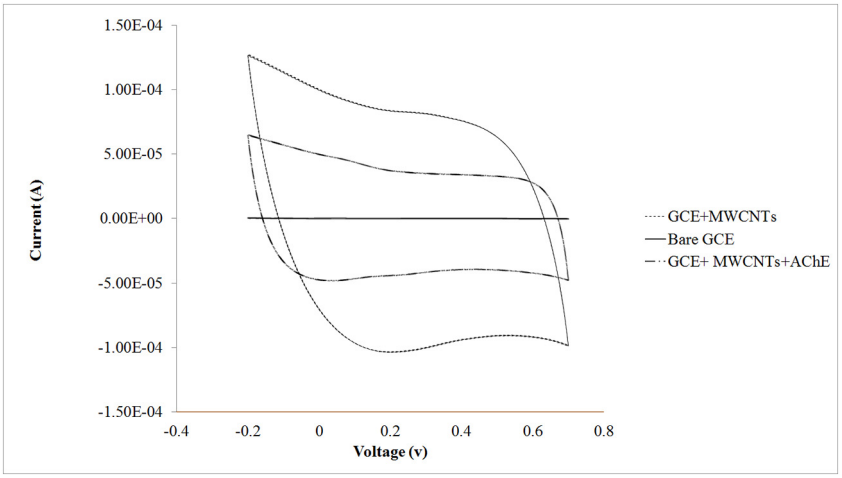

Fig. 5. CV of (a) bare GCE, (b) GCE + MWCNTs and (C) GCE + MWCNTs + AChE recorded in PB (pH 7.2) containing 0.1 mM K3[Fe(CN)6] and 10 mM KCl.

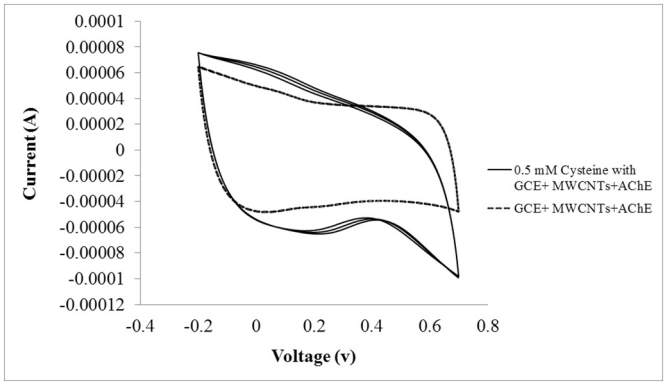

Fig. 6. Electrochemical behavior of cysteine on the AChE/MWCNTs/GCE recorded in PB (pH 7.2) containing 0.1 mM K3[Fe(CN)6] and 10 mM KCl.

earlier. The peak current increased after adding 0.5 mM cysteine in the electrolyte system as seen in the voltammograms in Fig. 6 [8,18].

3.2. Optimization of experimental parameters

3.2.1. Effect of MWCNTs concentration on GCE

The concentration of MWCNTs plays an important role in determining the conductance of the fabricated sensor. The current response of the electrode was measured by running cyclic voltammetry cycles in electrolyte system. It can be seen that the current increased significantly with the increased concentrations of MWCNTs. Increasing the concentrations beyond 0.03 mg of MWCNTs, did not show further enhancement in current, rather decreased the current. This may be owed to increased thickness of the film which hindered the electron transfer. Thus, 0.030 mg concentration of MWCNTs was used to fabricate the electrode (Figs. 7 and 8).

3.2.2. Effect of enzyme AChE loading

Effect of enzyme amount loaded on the MWCNTs/GC electrode was measured under the same parameters. With increase of the amount of enzyme on the electrode surface, peak current increased significantly up to 0.1 U of the enzyme. Any further increase in the enzyme amount decreased the current gradually. Therefore, we chose 0.1 U of AChE as optimized concentration for further experiment (Fig. 9).

3.2.3. Effect of ATChI amount

Influence of various concentrations of ATChI substrate on fabricated sensor was measured with the electrochemical parameters already standardized. When the concentration of ATChI was varied from 0.05 to 16 mM, current dramatically increased up to 12 mM. Subsequently, no significant or no large change was found in the current with increase in ATChI concentration (Fig. 10). For that reason, 12 mM of ATChI was used for further experiment [12].

3.2.4. Effect of inhibition time

Incubation time or response time of the sensor is time needed to inhibit the activity of the enzyme by covalent bond formation with the inhibitor paraoxon and is a key factor to assess the performance of the sensor. The inhibition curve showed a gradual increase till 6 min, as the incubation time was changed from 2 to 22 min. (Fig. 11). After 6 min the curve showed a sudden jump with increased inhibition at 10 min of incubation. However, when the electrode was immersed in pesticide solution for >10 min, it did not show further increase in inhibition. Hence, the inhibition curve tends to reach its saturation with

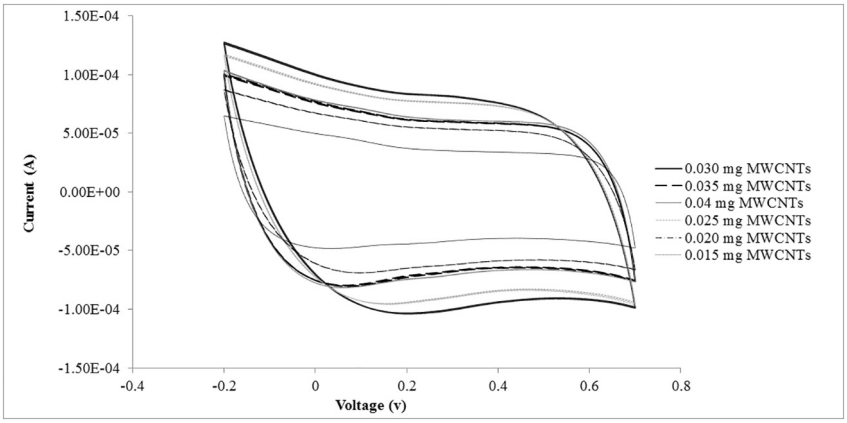

Fig. 7. Cyclic voltammogram of MWCNTs coated electrode with different concentrations of MWCNTs in PB (pH 7.2) containing 0.1 mM K3[Fe(CN)6] and 10 mM KCl.

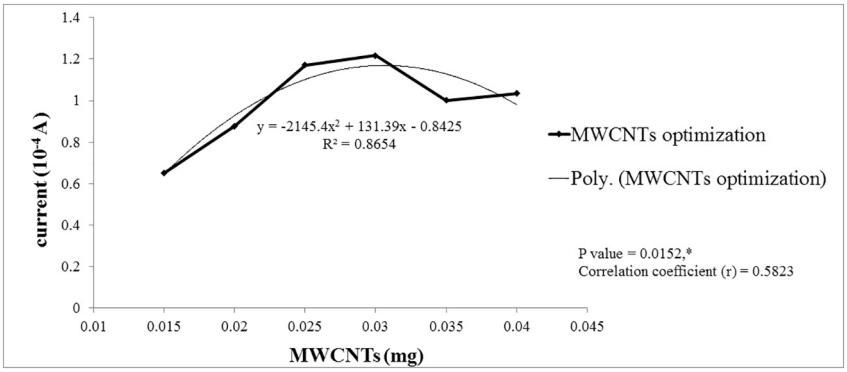

Fig. 8. Optimization of MWCNTs coating on GCE: effect of different concentrations of MWCNTs coatings in PB (pH 7.2) containing 0.1 mM K3[Fe(CN)6] and 10 mM KCl.

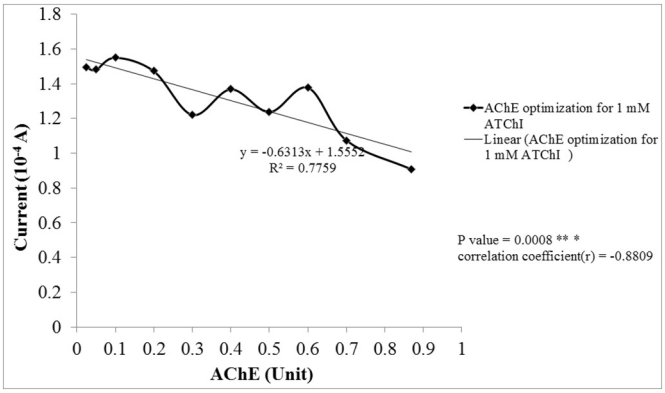

Fig. 9. Effect of AChE loading on modified MWCNTs/GCE recorded in PB (pH 7.2) containing 1 mM ATChI in 0.1 mM 820 K3[Fe(CN)6] and 10 mM KCl.

stable values after 10 min of incubation. The inhibition curve of paraoxon could not reach 100% of the inhibition because of lack of equilibrium between the binding sites of enzyme and pesticides [19]. Accordingly, 10 min was chosen as the optimum inhibition time for pesticide detection.

3.3. Detection of paraoxon

The electrode was incubated with different concentrations of paraoxon under optimal experimental conditions and change in peak potential and dramatic decrease in response current were observed with

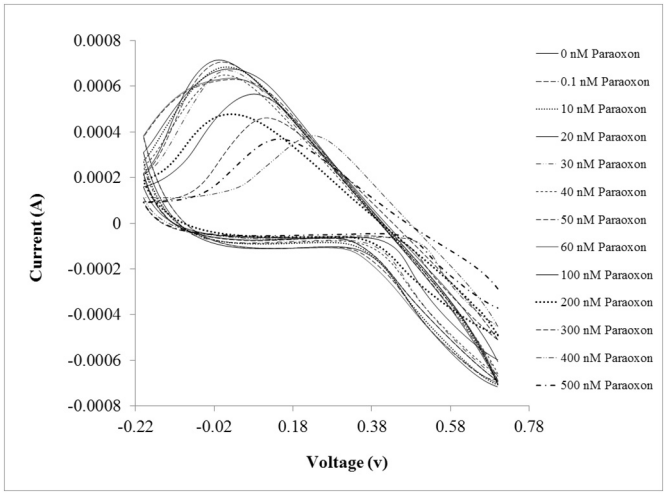

Fig. 12. Cyclic voltammetric detection of different paraoxon concentrations by fabricated electrode in presence of 12 mM ATChI with PB (pH 7.2), 0.1 mM K3[Fe(CN)6] and 10 mM KCl; Incubation time 10 min; concentration of paraoxon: 0.1 nM, 10 mM, 20 nM, 30 nM, 40 nM, 50 nM, 60 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM.

increased concentrations of paraoxon (Fig. 12). This may be attributed to increased inhibition of free catalytic sites of the enzyme available to the substrate. Under the optimal experimental conditions, inhibition of the enzyme by various paraoxon concentrations on fabricated electrode (AChE/MWCNTs/GCE) showed linear range from 10 to 50 nM with the regression coefficient $R^2 = 0.98$. The detection limit was

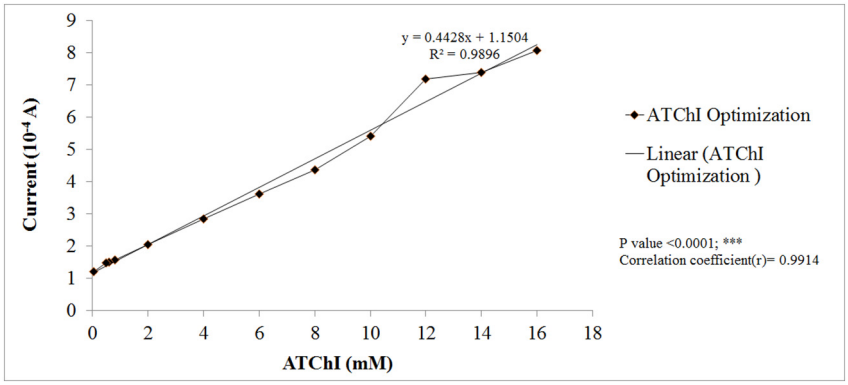

Fig. 10. Effect of various concentrations of ATChI on the sensor recorded in PB (pH 7.2) containing 0.1 mM K3[Fe(CN)6] and 10 mM KCl.

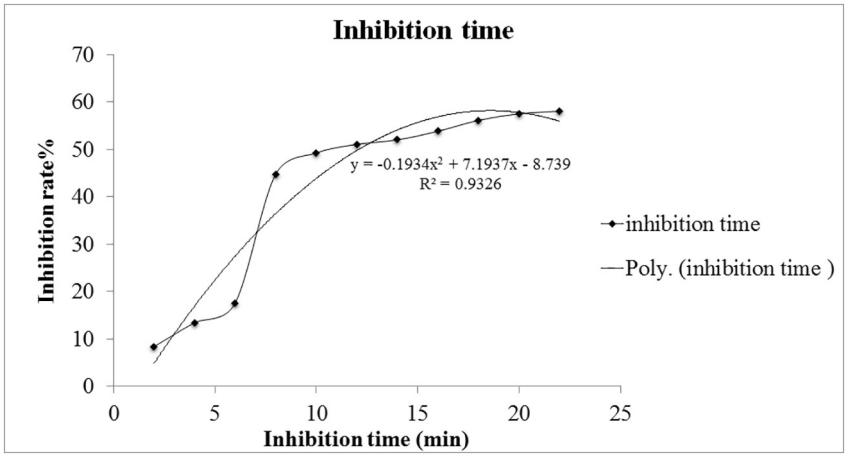

Fig. 11. Effect of Inhibition time: Response time of the sensor recorded in electrolytic system having 12 mM ATChI with PB (pH 7.2), 0.1 mM K3[Fe(CN)6] and 10 mM KCl.

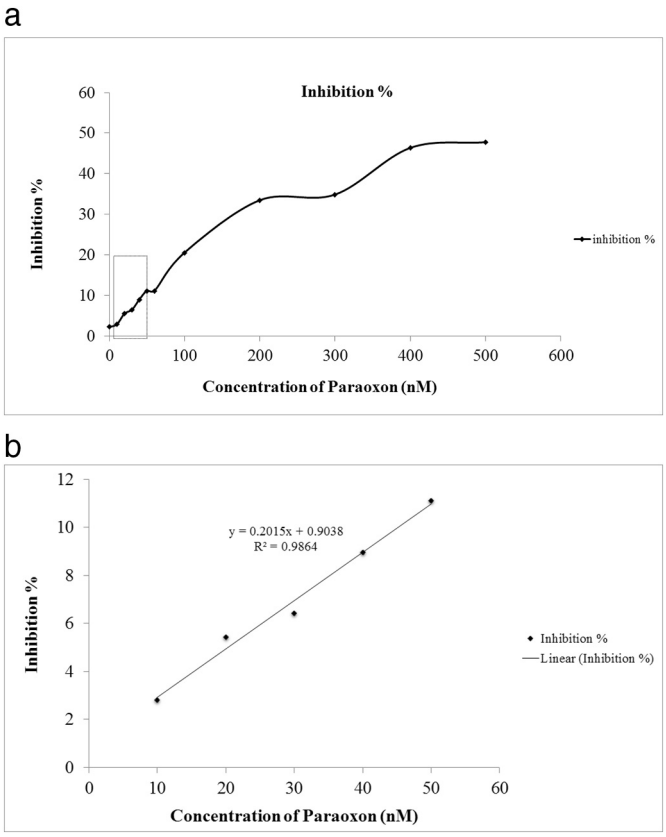

Fig. 13. Detection of paraoxon of the fabricated sensor: (a) Detection range 0.1–500 nM (b) Linear range 10–50 nM.

found to be 0.1 nM (Fig. 13(a) and (b)). Analytical characteristics such as sensitivity (linear range and detection limit) of a number of already reported biosensors were compared with the biosensor constructed (AChE/MWCNTs/GCE) and the results are summarized in Table 1.

The present sensor is based on inhibition of AChE by organophosphorus pesticides, where paraoxon has been picked up as model pesticide as it is known to be most toxic. Inhibition of the enzyme greatly depends on two things: affinity of a particular pesticide for AChE enzyme, general occurrence and concentration of the pesticide in the environment. Anti-acetylcholine activity of different pesticides greatly depends on their structures [24]. So, the same level of inhibition can be obtained with high concentration of low affinity pesticide and low concentration of high affinity pesticide [25]. To consider the cumulative effect of two or mixtures of pesticides and interference due to various organic and inorganic species is important while proposing a method of detection. In literature various studies have been carried out to examine the cumulative inhibition of the enzyme by different organophosphorus pesticides and interference of organic and inorganic compounds. Pogac'nik and Franko in 1999 examined Anti-AChE of each pesticide, mixture of two or more pesticides [26]. Amongst, various organophosphates (Chlorpyrifos, paraoxon, diazinon), paraoxon is found to be maximally toxic and showed the lowest limit of detection. While Chlorpyrifos being less toxic showed relative Anti-AChE activity of 0.01% compared to paraoxon. While looking at the cumulative effect, it was proved that Anti-AChE toxicity in the mixed solution of pesticides corresponded well with the sums of individual anti-AChE activity. Thus, an acetylcholinesterase-based sensor does not measure the total pesticide concentration, but gives the sum of anti-cholinesterase activity from each pesticide that can be expressed as paraoxon equivalent [26]. So, the present biosensor can be used to evaluate the total anti-AChE of the sample which corresponds to the concentration of the pesticide. Interference due to common electroactive species (ascorbic acid, uric acid), nitrophenyl derivatives (nitrobenzene, nitrophenol), inorganic ions (NO_3^-, PO_4^{3-}, SO_4^{2-}, NO_2^-, F^-, Cl^-, K^+, Ca_2^+, Mg_2^+) and sugars (Fructose, Sucrose) for AChE based electrochemical biosensor have been studied and found to show no significant inhibition or interference or proved to have negligible effect [27–29].

Inhibitory effects of a list of organic compounds and electrolytic species over the activity of AChE, screening of a series of real samples for organophosphorus compounds and comparing the results with the results obtained by other standard techniques such as HPLC and spectroscopic techniques can be considered as an extension of the present work and can be taken up as a new objective.

3.4. Replication, stability and reactivation

Fabrication of electrode was repeated 3 times simultaneously and their current response in 12 mM ATChI was checked. The SD value of the response current was found to be 0.071, which indicates that the replicability achieved is of acceptable value. The sensor electrode was stored in dry conditions at 4 °C and its enzyme activity was checked at four day intervals. Even after the long storage period of 32-days, it still retained its 54.28% current response compared to its initial current response. According to published reports, the inhibited AChE of the sensor can be reactivated by pralidoxime [20,21]. The sensor was reactivated in 1.0 mM pralidoxime iodide solution for 15 min, and then its activity was measured which showed 90.68% regeneration of AChE compared to its original value, suggesting that the reactivation achieved is of acceptable value.

3.5. Real sample analysis

It is crucial to evaluate reliability of the sensor for its application in real sample analysis. Two real samples tap water and potatoes bought from local market were selected for pesticide detection. Potatoes were cut into 3 mm cubes and soaked in distilled water for 45 min. The potato soaked water was decanted and used for analysis. Untreated tap water from the laboratory was collected and centrifuged to at 5000 *g* for 5 min. The results of analysis of tap water and potatoes showed no inhibition. Hence, efficiency of detection of the sensor was tested by adding different pesticide concentrations to both the samples. Each of the sample was tested 3 times to check the reproducibility of the current in real samples and was found to be 6.924 ± 0.0041 (10^{-4} A) and 6.856 ± 0.0378 (10^{-4} A) for potato and tap water respectively (Fig. 14(a) and (b)). Table 2 shows the results calculated from inhibition ratio for the added concentrations of pesticides. Recovery for potato and tap water was calculated as 4.864 ± 0.2 nM (97.28%) and 10.356 ± 1.45 nM (103.56%) respectively out of 5 nM and 10 nM. This result suggests

Table 1
Comparison of analytical characteristics of the present sensor with reported sensors for detection of paraoxon

Electrode	Linear range (nM)	Detection limit (nM)	References
BChE/AgNw/PTTBO/Graphite	0.5-8 × 10^3, 10-120 × 10^3	212	(Turana et al. [23])
AChE/PDDA-MWCNTs-GR/GCE	2.74-171.6, 0.171-10.298 × 10^3	0.44	(Sun et al. [12])
AChE/carbon paste electrode	3.432-51.493 × 10^3	1.373 × 10^3	(Caetano and Machado [22])
AChE/MWCNTs/GCE	10-50	0.1	Present work

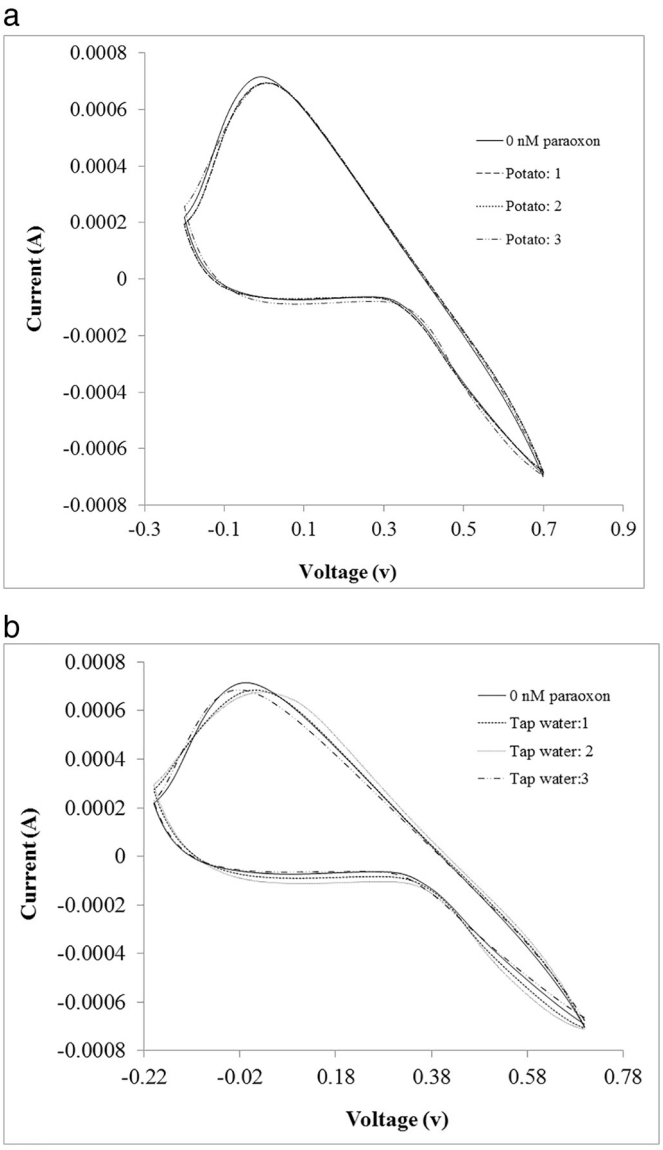

Fig. 14. Selective detection paraoxon from real samples: (a) 5 nM paraoxon added potato (b) 10 nM Paraoxon added tap water.

that the proposed fabricated sensor is reliable and can be used directly for analytical application.

Detection of real samples showed slightly altered inhibition of the enzyme compared to in buffer solutions for potato and tap water respectively. Thus, it did not show exact amount of the pesticide present in the sample (Fig. 14(a) and (b)) (Table 2). Similar effect was also reported with some sensors in literature. Exact reason behind this effect is still not identified. Probabilities of adsorption or entrapment of any organic matter onto the biolayer (MWCTs-AChE) which can block the supply of substrate and electrolyte and hinder the current is also negligible as the electrode was washed every time just after its incubation in pesticide added buffer-electrolyte system or real samples. It is also shown that pesticide did not get adsorbed on solid organic matter, which might be the result of lower pesticide concentrations detection

Table 2
Recovery studies of different paraoxon concetrations from real samples

Sample	Added (nM)	Found(nM)	Recovery
Potato (5 g)	5 nM	4.864 ± 0.2 nM	97.28%
Tap water	10 nM	10.356 ± 1.45 nM	103.56%

in case of potato [25,26]. It is said that some real samples or matrix somehow show limiting or protective effect to the enzyme. However, it is possible to make matrix-matched calibration curve which measure anti-AChE activity of real samples in buffer from the same origin without having any pesticide residue. These can give better estimation compared to estimation obtained from single point matrix-matched buffer solution. A standard addition method can also be opted for real sample analysis to avoid a matrix effect [26]. Direct analysis of real samples with the biosensor can also be a good option [25].

4. Conclusion

Successful fabrication of the MWCNTs and acetylcholine esterase based biosensor for rapid and onsite detection of pesticide paraoxon by doping the enzyme directly on to MWCNTs surface was done. Thus, the use of any binder or insulating membrane or ink unlike other fabrication chemistries, using harmful and noneco-friendly insulating inks, which may hinder electron transfer and affect the performance of the sensor was eliminated. In the present study, MWCNTs were chosen for loading enzyme due to their inherent properties such as higher surface area, higher electric conductance, higher mechanical strength and electrochemical properties which are best suited for nanosensor fabrication. Fabrication was carried out at room temperature, avoiding the use of any large instruments or harmful reagents. The sensor showed superior stability and linear range compared to other sensors in use. The limit of detection for paraoxon was 0.1 nM under optimized conditions. The sensor was used for real sample detection and was found to be useful for detection of pesticide residues in food and water. The sensor displayed good recovery. The results demonstrate that the sensor can be adopted for food and water safety.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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