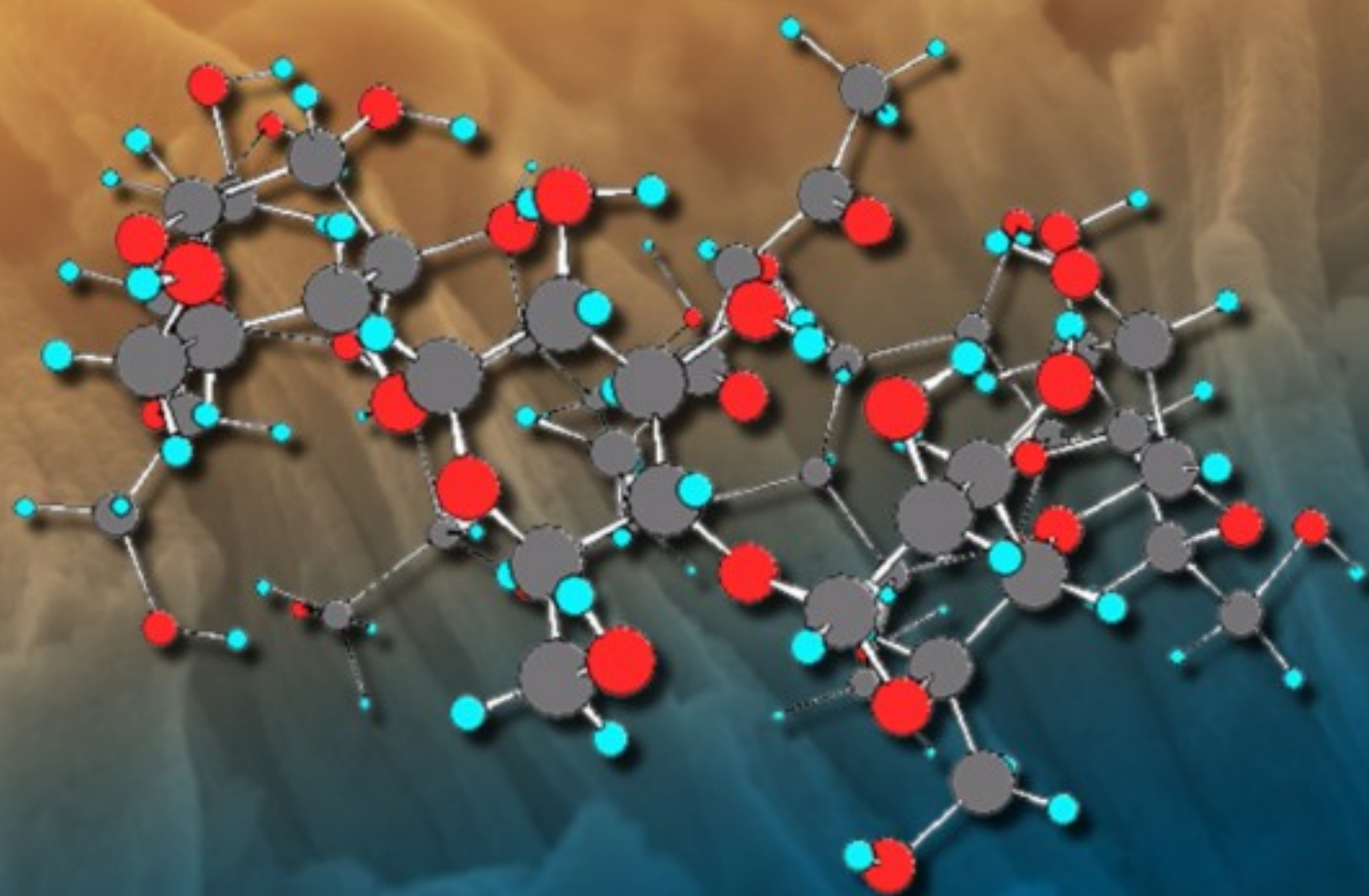


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Contents

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Research Articles

Diffusion in Carbon Nanotubes: Details, Characteristics, Comparisons at Nanolevel <i>Paolo Di Sia</i>	1
Synthesis Characterization and Humidity Sensing Properties of Sol-gel Derived Novel Nanomaterials of $\text{LaSr}_x\text{Fe}_{1-x}\text{O}_{3-\delta}$ <i>Mary Teresita V., Jeseentharani V., Avila Josephine B., Jeyaraj B., Arul Antony S.</i>	8
Gas Sensing Characteristics of ZnO Nanowires Fabricated by Carbothermal Evaporation Method <i>Roghayeh Imani and Mohammad Orvatinia</i>	17
In-Situ Decoration of Electrostatically Functionalized Multiwalled Carbon Nanotubes with $\beta\text{-Ni(OH)}_2$ Nanoparticles for Low Temperature NO_2 Detection <i>Richa Saggarr, Vasuda Bhatia, Prashant Shukla, Nitin Bhardwaj, Vinod K Jain</i>	28
Synthesis and Characterization of ZnO Nanoparticles as Prepared by Gel-combustion and ZnO Nanomorphologies by Sol-gel <i>Mario F. Bianchetti, Marjeta Maček-Krzmanec, Ines Bracko, Sreco D. Skopin and Noemi E. Walsøe de Reca</i>	36
Multiwalled Carbon Nanotubes Reinforced Cement Composite Based Room Temperature Sensor for Smoke Detection <i>Prashant Shukla, Vasuda Bhatia, Vikesh Gaur, Nitin Bhardwaj, Vinod Kumar Jain</i>	48
A Facile and Green Synthesis of Small Silver Nanoparticles in β-cyclodextrins Performing as Chemical Microreactors and Capping Agents <i>Giorgio Ventimiglia and Alessandro Motta</i>	59
Electrostatically Functionalized Multi-Walled Carbon Nanotubes Based Flexible and Non-Enzymatic Biosensor for Glucose Detection <i>Bhawana Singh, Vasuda Bhatia, V. K. Jain.</i>	69
Amperometric Acetylcholinesterase Biosensor Based on Poly (Diallyldimethylammonium Chloride)/Gold Nanoparticles/Multi-walled Carbon Nanotubes-chitosans Composite Film-modified Electrode <i>Xia Sun, Zhili Gong, Yaoyao Cao, Xiangyou Wang</i>	78
Structural, Morphological and Optical Properties of Spray Deposited Nano-crystalline CdO Thin Films <i>Maqbul A. Barote, Elahipasha U. Masumdar.</i>	90
A Novel Amperometric Immunosensor Based on $\{\text{MWCNTs-COOH-CHIT}\}_2/\text{GNPs}$ for Detection of Chlorpyrifos <i>Xia Sun, Lu Qiao, Xiangyou Wang</i>	96

Y³⁺ Added Nanocrystallite Mg-Cd Ferrite LPG, Cl₂ and C₂H₅OH Sensors <i>Ashok B. Gadkari, Tukaram J. Shinde, Pramod N. Vasambekar.....</i>	110
Immunosensor Based on Gold Nanoparticles-multi-walled Carbon Nanotubes-chitosans Composite and Prussian Blue for Detection of Chlorpyrifos <i>Xia Sun, Falan Li, Xiangyou Wang.....</i>	121
Nanostructured CdFe₂O₄ Thick Film Resistors as Ethanol Gas Sensors <i>S. V. Bangale, R. D. Prakshale, S. R. Bamane.....</i>	133
A Novel Combustion Route for the Preparation of Nanocrystalline LaAlO₃ Oxide Based Electronic Nose Sensitive to NH₃ at Room Temperature <i>K. A. Khamkar, S. V. Bangale, V. V. Dhapte, D. R. Patil, S. R. Bamane.....</i>	145
Gold Nanoparticle Amplification Combined with Quartz Crystal Microbalance DNA Based Biosensor for Detection of <i>Mycobacterium Tuberculosis</i> <i>Thongchai Kaewphinit, Somchai Santiwatanakul and Kosum Chansiri.....</i>	156
Structural, Morphological and Optical Properties of Spray Deposited Nanocrystalline ZnO Thin Films: Effect of Nozzle to Substrate Distance <i>Elahipasha U. Masumdar, Maqbul A. Barote.</i>	164
Zinc and Pyrrole-added Akaganeite (β-FeOOH) Films by Ultrasonic Spray Pyrolysis Assessed as Propane Sensors <i>Carlos Torres Frausto, Alejandro Avila-Garcia.....</i>	170
Potentiometric Determination of Low Content of Water in Different Organic Solvents Using NASICON Based Probe <i>Parul Yadav and M. C. Bhatnagar.....</i>	182
Development of Electrochemical Sensors for the Detection of Mercury by CNT/Li⁺, C₆₀/Li⁺ and Activated Carbon Modified Glassy Carbon Electrode in Blood Medium <i>Muhammed M. Radhi, Dawood S. Dawood, Nawfal K. Al-Damlooji and Tan W. Tee</i>	191

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Amperometric Acetylcholinesterase Biosensor Based on Poly (Diallyldimethylammonium Chloride)/Gold Nanoparticles/Multi-walled Carbon Nanotubes-chitosans Composite Film-modified Electrode

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Abstract: A novel amperometric biosensor was developed for the detection of organophosphorus pesticides (OPs). Acetylcholinesterase (AChE) was immobilized onto poly(diallyldimethylammonium chloride) (PDDA)/gold nanoparticles (AuNPs)/multi-walled carbon nanotubes-chitosans (MWCNTs-CHIT) composite film modified glass carbon electrode (GCE). The biosensor had good sensitivity owing to the excellent properties of MWCNTs and AuNPs, which promoted electron transfer and improved the electrochemical response. PDDA with positive charge was bound electrostatically to AChE with negative charge by layer-by-layer (LbL) self-assembly technique, which can increase AChE loading amount. The morphologies and the sensitive steps of surface modification were characterized by scanning electron microscopy (SEM), cyclic voltammetric (CV) and electrochemical impedance spectroscopy (EIS), respectively. Based on the inhibition of OPs on the AChE activity, under the optimized conditions, the biosensor exhibited a linear response to chlorpyrifos in the concentration range from 0.8 to 20 $\mu\text{g/L}$ and from 20 to 300 $\mu\text{g/L}$, with a detection limit of 0.56 $\mu\text{g/L}$. The biosensor exhibited good reproducibility and acceptable stability. Moreover, PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE biosensor can also be used for direct analysis of practical samples, which may open a new door for pesticide analysis in vegetables and fruits. *Copyright © 2012 IFSA.*

Keywords: Acetylcholinesterase, Multiwall carbon nanotubes, Gold nanoparticles, Poly (diallyldimethylammonium chloride), Biosensor.

1. Introduction

Organophosphates pesticides (OPs) are widely used as insecticides in agriculture due to their high effectiveness for insects control [1-3]. However, long-term accumulations in environment, OPs could cause respiratory paralysis and death due to their high toxicity to acetylcholinesterase (AChE), which is crucial for the function of the central nervous system in humans [4-8]. So it is of great significance to develop fast, reliable and inexpensive testing devices for pesticides detection. Among various methods to detect pesticides, AChE biosensors have attracted great attention due to their ability to determine and quantify trace level of OP compounds in the environment [9]. A significant challenge to develop any enzyme-based biosensors is the effective immobilization of enzyme on the solid electrode surface [10, 11]. Therefore, it is necessary in the biosensor field to explore new materials and effective immobilization methods that can maintain inherent biological activity of biomolecules and enhance the performance of the biosensors [12].

In this regard the use of multiwall carbon nanotubes (MWCNTs) for the fabrication of electrochemical biosensors has attracted considerable attention in the last few years. MWCNTs have advantages of their special structures, a large edge/basal plane ratio, good mechanical strength, enhanced electronic properties, rapid electrode kinetics, strong adsorptive ability, and excellent biocompatibility [13-16]. These features have made MWCNTs highly suitable in the biosensor field [17]. However, untreated MWCNTs are extremely hydrophobic and tend to assemble into bundles, making them tricky to process [12, 18]. So we need to find effectual dispersants for CNTs to solve this problem. Chitosan (CHIT) is a biological cationic macromolecule with primary amines. It has been widely used for dispersion because of its good biocompatibility and film-forming ability [19-23]. Furthermore, CHIT can provide a favorable microenvironment, load a large amount of enzyme and prolong the enzyme storage life [24]. Here, we used CHIT to disperse MWCNTs for modifying glassy carbon electrode. Recent years, gold nanoparticles (AuNPs) have drawn people's wide concern in analytical electrochemistry due to their high conductivities and high surface to volume ratios. Willner et al., have highly researched the electrochemical adhibitions of AuNPs and their ability to promote the electron transfer [25-28]. AuNPs are ideal platforms for biosensors, especially for those with electrochemical detection. Moreover, AuNPs could combine with -NH₂ group of CHIT, and then AChE was bound through Au-N [29]. As a water soluble and linear positively charged polyelectrolyte, poly (diallyldimethylammonium chloride) (PDDA) has been widely used for the immobilization of biomolecules [30-32]. AChE possessed negative charge at pH 7.0 due to the isoelectric point (pI) of AChE lying around pH 5.5 [33, 34]. Thus, AChE was used for adsorbing PDDA with positive charge layer by layer to increase AChE loading amount.

CHIT could make MWCNTs uniformly disperse onto the surface of GCE, which promoted electron transfer. AuNPs could combine with -NH₂ group of CHIT, and then AChE was bound through Au-N, which improved AChE immobilization effects. Finally, AChE was absorbed electrostatically with PDDA layer by layer, which increased AChE loading amount. Based on the above consideration, herein, we developed an electrochemical AChE biosensor, in which AChE was immobilized onto PDDA/AuNPs/MWCNTs/GCE. To the best of our knowledge, this kind of AChE biosensor has been still very less reported. The preparation and assembly of multilayer films and the performance of AChE biosensors were investigated in detail.

2. Materials and Methods

2.1. Apparatus

A three-electrode system was employed with the modified GCE (d = 3 mm) as the working electrode, a platinum electrode as the auxiliary electrode and saturated calomel electrode (SCE) as the reference

electrode. Electrochemical measurements were performed with CHI660D electro-chemical workstation (Shang-Hai Chenhua Co., China).

2.2. Reagents

Acetylcholinesterase (Type C3389, 500 U/mg from electric eel), acetylthiocholine chloride (ATCl) and Poly (diallyldimethylammonium chloride) (PDDA) (35%, w/w in water) were purchased from Sigma (St. Louis, USA). MWCNTs were obtained from Nanotech Port Co. (Shenzhen, China). HAuCl_4 was from Shanghai Sinopharm Chemical Reagent Co. Ltd. (China). 0.1 M pH 7.0 phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 . Chlorpyrifos was standard product, CHIT (95% deacetylation), ethanol and other reagents were of analytical grade.

2.3. Preparation of Sensor

MWCNTs-CHIT composite was prepared according to the literature with minor modifications [35]. Briefly, 0.5 wt% of CHIT solution was prepared prior by dissolving 0.5 g of CHIT powder in 100 ml of 1.0 % (v/v) acetic acid solution. Then 1 mg of MWCNTs was put into 1 mL of the above CHIT solution, ultrasonicated at room temperature until homogenous black suspension obtained, and stored at 4°C when not in use.

The gold colloidal nanoparticles were prepared as described in the literature with minor modifications [36]. All glasswares used in the following procedures were cleaned in a bath of freshly prepared aquaregia (3:1 HCl-HNO_3), thoroughly rinsed with distilled water, and dried prior to use. 100 mL of 0.01 % HAuCl_4 solution was taken in a flask, brought to boiling under vigorous stirring, and then 2.5 mL of ice cold 1 % sodium citrate solution was quickly added with the reduction of gold by citrate to give a color change from blue to wine red. The solution was kept boiling for 15 min with vigorous stirring, then cooled down to room temperature, and stored at 4°C in a dark bottle.

Prior to modification, a GCE was polished progressively with 0.3 μm and 0.05 μm alumina powder, then sonicated in distilled water, absolute ethanol, 6.0 M HNO_3 and distilled water 5 min respectively. Before modification, the electrode was applied a potential scanned from -0.2 to 1.2 V in 0.5 M H_2SO_4 until a reproducible cyclic voltammogram (CV) was obtained. Finally, the electrode was thoroughly rinsed with distilled water, and dried at room temperature. 7 μL of the above prepared MWCNTs-CHIT nanocomposite was first dropped on the surface of the clean GCE (denoted as MWCNTs-CHIT/GCE), and allowed to dry at room temperature. Then 7 μL of AuNPs was coated on the MWCNTs-CHIT/GCE (denoted as AuNPs/MWCNTs-CHIT/GCE), and dried it in air under natural condition. Mean-while, AuNPs were immobilized on the electrode through chemisorption and used for immobilizing AChE via the Au-N bond [29]. And then 10 μL AChE solution (100 mU) was dropped onto the modified GCE, incubated at 20 °C for 30 min. Further, 7 μL PDDA (1 %, w/w in water) was spread onto the modified electrode surface and let stand for 30 min. Finally, the alternate deposition of PDDA (1 %, w/w in water) and AChE was repeated three times for optimizing AChE loading, which was regarded as PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE. The prepared biosensor was stored at 4 °C when not in use. The stepwise fabrication process of the biosensor was shown in Fig. 1.

2.4. Electrochemical Detection of Pesticide

For the measurement of chlorpyrifos, the prepared PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE was first immersed in pH 7.0 PBS (0.1 M) containing different concentrations of standard chlorpyrifos solution for 14 min, then immediately transferred to the electrochemical cell of 3 mL pH 7.0 PBS (0.1 M)

containing 4.0 mM ATCl to study the electrochemical response. The inhibition was calculated as follows:

$$\text{Inhibition (\%)} = (i_{P, \text{control}} - i_{P, \text{exp}}) / i_{P, \text{control}} \times 100 \%, \quad (1)$$

where $i_{P, \text{control}}$ is the peak current of ATCl on PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE without chlorpyrifos inhibition, $i_{P, \text{exp}}$ is the peak current after the incubation with inhibitor.

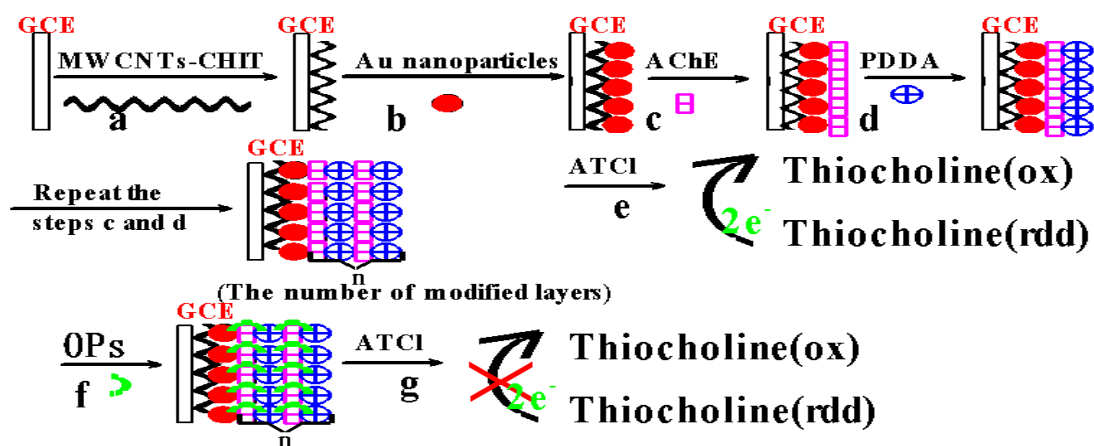


Fig. 1. The fabricating procedures of the biosensor.

2.5. Preparation and Determination of Real Samples

The pakchoi, lettuce, garland chrysanthemum and Chinese chives bought from a supermarket were rinsed three times with double-distilled water and then chopped. Then, different concentrations of chlorpyrifos solution were sprayed on their surfaces. After 24 h of storage at 4 °C, each sample was mixed with the 10 mL mixed solution of acetone/0.1 M pH 7.5 PBS (1/9, v/v), which was obtained through 15 min of ultrasonic treatment. After the suspensions were centrifuged (10 min, 10000 rpm), the acquired supernatants were detected by CV directly without extraction or preconcentration. The content of chlorpyrifos in the samples can be achieved from the calibration curve.

3. Results

3.1. SEM Characterizations of MWCNTs-CHIT and AuNPs

The morphologies of different composite modified electrodes were characterized by the scanning electron microscope (SEM), as shown in Fig. 2. It can be clearly seen that the MWCNTs-CHIT presented a large number of filamentous structure and formed a uniform film. After decorated with AuNPs, the surface morphology of MWCNTs-CHIT was largely changed. The AuNPs were well-distributed along the surface of the MWCNTs-CHIT, which indicated that the AuNPs were well adsorbed on the surface of the MWCNTs-CHIT.

3.2. Electrochemical Impedance Analysis

Fig. 3 shows some Nyquist diagrams obtained from bare GCE (curve a), MWCNTs-CHIT/GCE (curve b), AuNPs/MWCNTs-CHIT/GCE (curve c), PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE

(curve d) using 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ with 0.1 M KCl as the redox probe. As shown in Fig. 3, the bare GCE (curve a) had a very big R_{ct} . When MWCNTs-CHIT nanocomposites were deposited on the GCE (curve b), the R_{ct} was decreased obviously. This phenomenon suggested that the MWCNTs-CHIT nanocomposites greatly improved the conductivity of the modified electrode due to their excellent and unique electronic properties. After AuNPs were coated on the surface of MWCNTs-CHIT/GCE, the R_{ct} further decreased to a very small value (curve c), implying that AuNPs are good electron conducting materials. However, when AChE was immobilized on the electrode, the R_{ct} increased sharply (curve d), confirming the immobilization of enzyme. The reason maybe that with the increase of the thickness of the interface, the conductive support was insulated and the electron transfer between anionic $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and GCE was reduced [13, 37, 38].

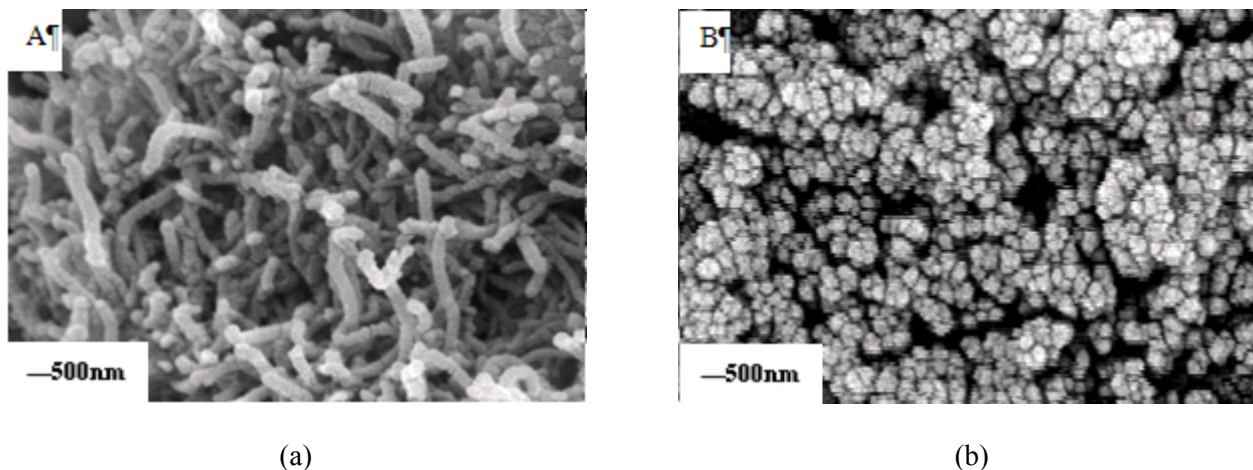


Fig. 2. SEM images of (A) MWCNTs-CHIT/GCE and (B) AuNPs/MWCNTs-CHIT/GCE.

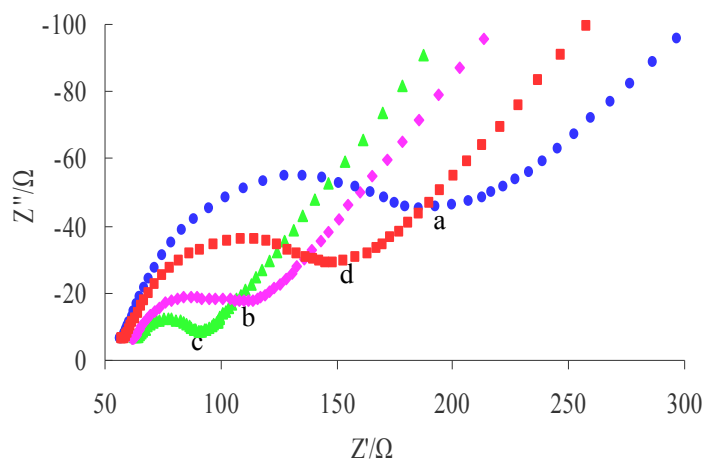


Fig. 3. EIS of (a) bare GCE; (b) MWCNTs-CHIT/GCE; (c) AuNPs/MWCNTs-CHIT/GCE; (d) PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE recorded in pH 7.0 PBS containing 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl.

3.3. Cyclic Voltammetry Analysis

Fig. 4 illustrated the cyclic voltammetry (CV) of 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ with 0.1 M KCl at different modified electrodes. As shown in Fig. 4, the immobilization of MWCNTs-CHIT nanocomposites on the bare GCE (curve b) led to a significant increase in peak current of the redox probe, compared with

the bare GCE (curve a). After AuNPs were coated on the surface of MWCNTs-CHIT/GCE (curve c), the response of the system was higher than before. When AChE was immobilized on the surface of AuNPs/MWCNTs-CHIT/GCE (curve d), an obvious decrease in the amperometric response was observed, which indicated that AChE was successfully immobilized. All these indicated that the CVs of different electrodes were consistent with those of EIS results.

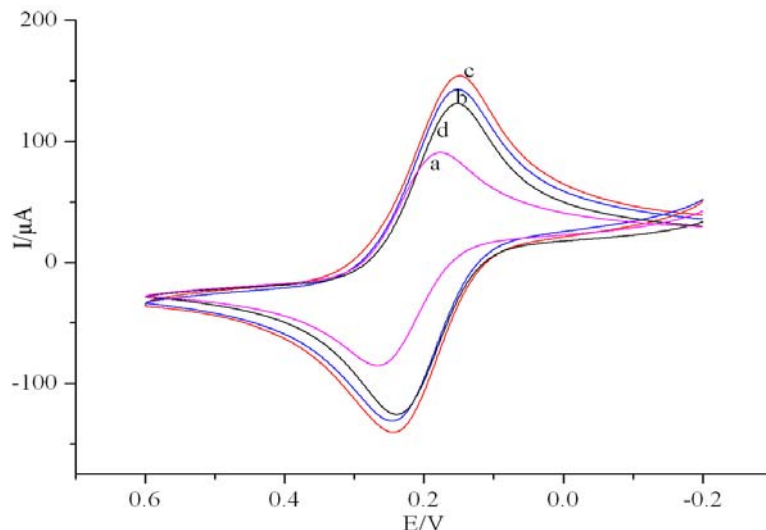


Fig. 4. CV of (a) bare GCE; (b) MWCNTs-CHIT/GCE; (c) AuNPs/MWCNTs-CHIT/GCE; (d) PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE recorded in pH 7.0 PBS containing 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl.

3.4. Cyclic Voltammetric behavior of ATCl

Fig. 5A illustrated the CVs of different fabricated electrodes in absence and presence of ATCl. As shown in Fig. 5A, no peak was observed at bare GCE (curve a) and PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE (curve b) in pH 7.0 PBS without ATCl. CV of PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE (curve d) showed an irreversible oxidation peak in the presence of 4.0 mM ATCl, whereas no detectable signal was observed for AuNPs/MWCNTs-CHIT/GCE (curve c). Conspicuously this peak arose from the oxidation of thiocholine, hydrolysis product of ATCl, catalyzed by the immobilized AChE. The peak current produced by the oxidation of thiocholine was used as an indicator for quantitative measurement of the enzyme activity, which could reflect the biological effect of chlorpyrifos involved in the inhibition action.

For comparison, as shown in Fig. 5B, the CV behaviors of ATCl at PDDA-AChE/GCE (curve a), PDDA-AChE/MWCNTs-CHIT/GCE (curve b), PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE (curve c) were further studied. PDDA-AChE/GCE (curve a) showed a small peak current, which may be due to the low enzyme loading, because of the weak combination between AChE and GCE. While the oxidation peak at PDDA-AChE/MWCNTs-CHIT/GCE (curve b) was larger than that at PDDA-AChE/GCE owing to the excellent properties of MWCNTs, which possessed inherent conductive properties and catalytic behavior. Whereas much larger peak current was obtained at PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE (curve c), this was due to the presence of AuNPs in nanocomposite film, which possessed the ability to promote the electron transfer. In addition, the response of PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE also indicated that the combination of MWCNTs and AuNPs further improved the electronic transport due to their synergistic effects.

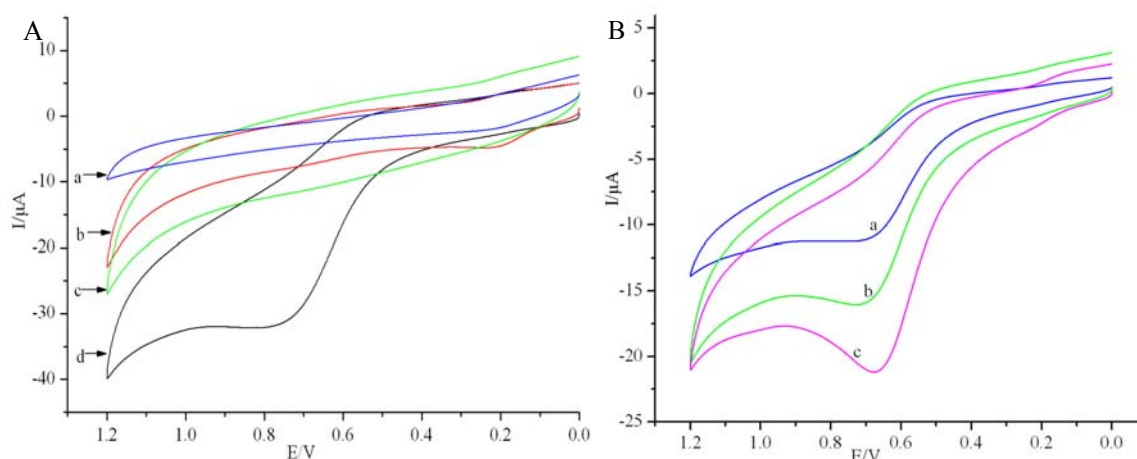


Fig. 5. (a) CV of (a) bare GCE; (b) PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE in the blank PBS (0.1 M, pH 7.0); and (c) AuNPs/MWCNTs-CHIT/GCE; (d) PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE in 0.1 M PBS (pH 7.0) containing 4.0 mM ATCl. (B) CV of (a) PDDA-AChE/GCE; (b) PDDA-AChE/MWCNTs-CHIT/GCE; (c) PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE in the presence of 4.0 mM ATCl in 0.1 M PBS (pH 7.0).

3.5. Optimization of Experimental Parameters

3.5.1. Influence of PDDA-AChE Layers

The effect of the layers of PDDA-AChE multilayer films on the peak current as an important parameter to the proposed biosensor was studied in 0.1 M PBS (pH 7.0) containing 4.0 mM ATCl. As shown in Fig. 6A, the peak current of the resulting biosensor increased from one to three layers of PDDA-AChE multilayer films, while the response decreased by further increasing the number of the PDDA-AChE layers. Before modified three layers, it can be explained that as a result of an insufficient loading of AChE, the current depends on the rate of the hydrolysis reaction of ATCl catalyzed by AChE [9]. However, when the number of PDDA-AChE layers exceeding three would result in a negative change of current response, which might be caused by the excessive compactness film obstructing electron transfer tunnels. Therefore, three layers were selected throughout this study.

3.5.2. Influence of Incubation Time on Inhibition

The incubation time was an important factor for capturing chlorpyrifos. As shown in Fig. 6B, the steady-state current increased sharply with an increase of incubation time up to 14 min in the 20 $\mu\text{g/L}$ chlorpyrifos solution, and then it trended to a stable value. However, the maximum value of inhibition of chlorpyrifos was not 100 %. It can be explained that the binding sites between pesticides and enzymes could reach an equilibration state [39]. As a result, the optimum incubation time was set at 14 min for the subsequent experiments.

3.5.3. Influence of Detection Solution pH

The sensitivity and stability of the biosensor was greatly influenced by the pH of the detection solution (0.1 M PBS containing 4.0 mM ATCl) from 6.0 to 8.0 range. The maximum peak current measured at the PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE was obtained at pH 7.0 (Fig. 6c). It is easy to lose the activity of AChE irreversibly at the lower or higher pH values. In order to get the highest sensitivity, pH 7.0 was used in the detection solution.

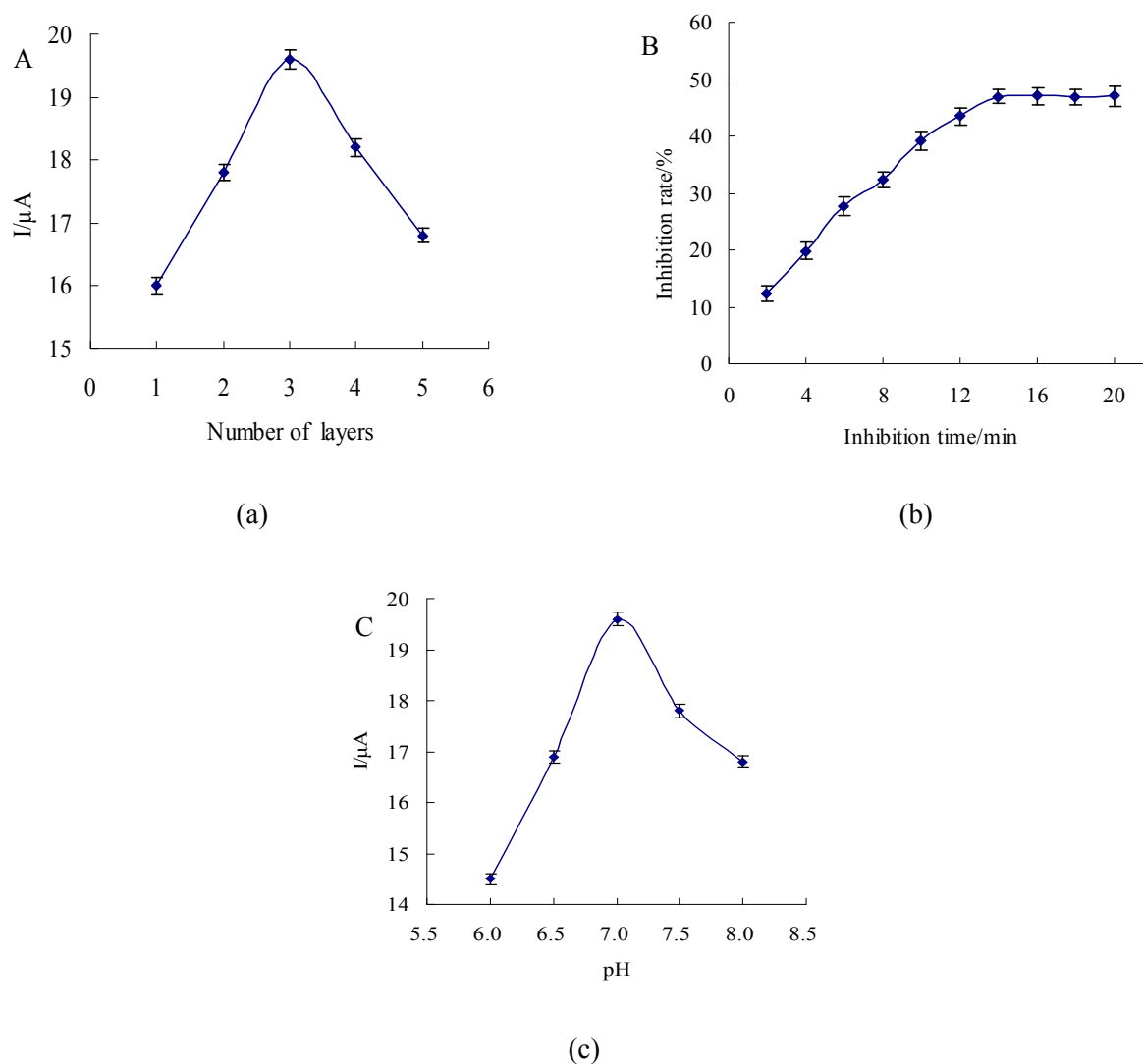


Fig. 6. (a) Effect of the number of PDDA-AChE layers on the amperometric response; (b) Relationship between inhibition rate and inhibition time; (c) Effect of the pH of the detection solution on the amperometric response.

3.6. Detection of Chlorpyrifos

Fig. 7A shows the CV of the proposed biosensor immersed in various concentrations of chlorpyrifos under the optimal conditions, the produced current decreased drastically with increasing chlorpyrifos concentrations. It may be due to the fact that chlorpyrifos as one of the OPs pesticides reduced the enzymatic activity to its substrate and exhibited irreversible inhibition action on AChE. The peak current changes observed in Fig. 7A reflected the activity of immobilized enzyme, which provided a simple method for determination of chlorpyrifos. Under the optimal experimental conditions, the inhibitions of chlorpyrifos on PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE were proportional to its concentrations in two ranges, from 0.8 to 20 $\mu\text{g/L}$ and from 20 to 300 $\mu\text{g/L}$ (Fig. 7B), the regression equation was $I\% = 1.6376c + 14.23$ and $I\% = 0.1394c + 46.213$, with the correlation coefficients of 0.9982 and 0.9949, respectively. The detection limit was calculated to be 0.56 $\mu\text{g/L}$ ($S/N = 3$).

A comparison of analytical characteristics of the present biosensor with other reported sensors for the detection of chlorpyrifos was summarized in Table 1.

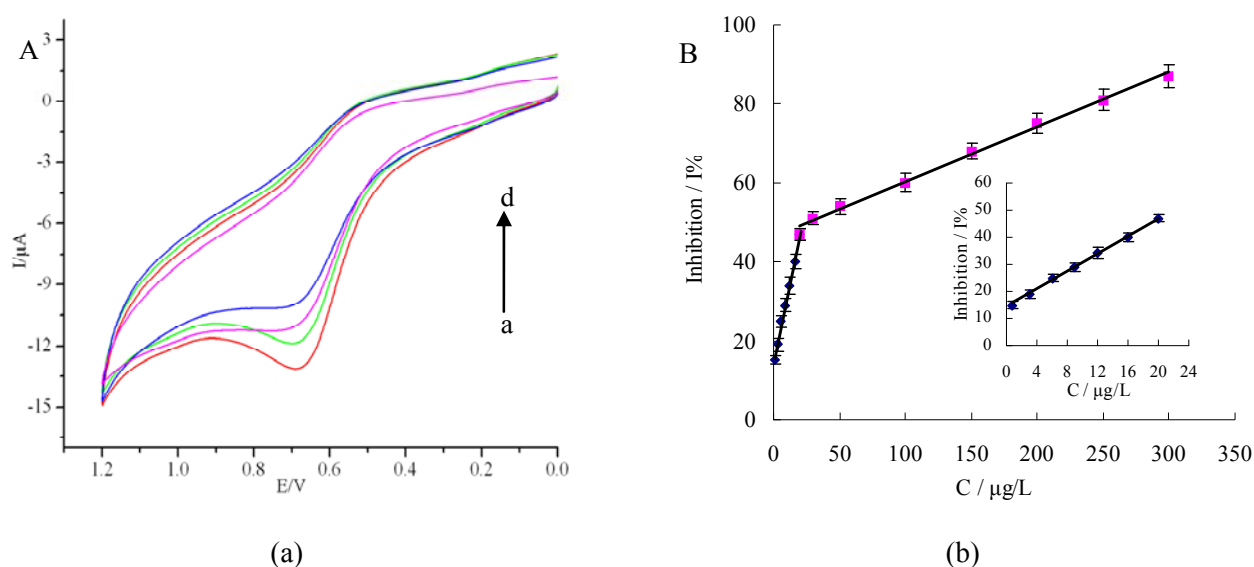


Fig. 7. (a) The CVs of PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE after inhibition by chlorpyrifos of different concentrations (from a to d): 0, 1.0, 5.0, 10 $\mu\text{g/L}$ under the optimal conditions; (b) Relationship between inhibition rates and chlorpyrifos concentrations. Inset: linear relationship between low inhibitions and low concentrations.

Table 1. Comparison of analytical characteristics with other reported biosensors for the detection of chlorpyrifos.

Electrode	Linear range ($\mu\text{g/L}$)	Detection limit ($\mu\text{g/L}$)	References
AChE-[BMIM][BF ₄]-MWCNT/CP	3.5-350	1.4	[40]
AChE-BTB/sol-gel film	50-2000	40	[41]
PATP membranes/AuNPs/GCE	180-3500	100	[42]
PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE	0.8-20, 20-300	0.56	This work

3.7. Evaluation of the Biosensor and Analysis of Real Samples

The inter-assay precision was estimated at 6 different electrodes for the determinations to 4.0 mM ATCl after being treated with 10 $\mu\text{g/L}$ chlorpyrifos solutions for 14 min. Similarly, the intra-assay precision of the biosensor was evaluated by analyzing chlorpyrifos levels for six replicate measurements at one enzyme electrode. The RSD values of inter-assay and intra-assay were found to be 5.4 % and 6.8 %, respectively, indicating acceptable precision and replicability. The enzyme electrode was stored at 4 °C in dry condition for 7 days when not in use, and no obvious decrease in the response of ATCl was observed. The sensor still retained 87 % of its initial current response after a 30-day storage period, indicating satisfactory stability of biosensor.

In order to evaluate the feasibility of the proposed biosensor, it was evaluated by processing real samples. We performed the recovery tests by adding different concentrations of chlorpyrifos into pakchoi, lettuce, garland chrysanthemum and Chinese chives samples, summarized as Table 2. The recoveries were from 87 % to 109 %, suggesting that the proposed biosensor can be feasible for direct analysis of relevant samples.

4. Conclusions

In this work, we have developed a sensitive and efficient biosensor, PDDA-AChE/AuNPs/MWCNTs-CHIT/GCE, for the detection of chlorpyrifos. The assembled MWCNTs-CHIT and AuNPs increased

the sensitivity of the sensor. In addition, AuNPs deposited onto the electrode created a more favorable surface for the attachment of the AChE. In order to optimize the loading of AChE and enhance the detection sensitivity, the alternate deposition of PDDA and AChE was repeated three times by LBL self-assembly technique. The proposed amperometric biosensor showed satisfactory results for OPs determination such as high sensitivity, good precision and long-term stability. The proposed system has a high potentiality in detection of other toxic compounds against to AChE.

Table 2. Recovery studies of chlorpyrifos in real samples.

Sample	Taken ($\mu\text{g/L}$)	Found ($\mu\text{g/L}$)	Recovery (%)
Pakchoi	10	10.2	102
	30	27.3	91
Lettuce	10	8.7	87
	30	31.2	104
Garland chrysanthemum	10	10.5	105
	30	27.9	93
Chinese chives	10	9.6	96
	30	32.7	109

Acknowledgements

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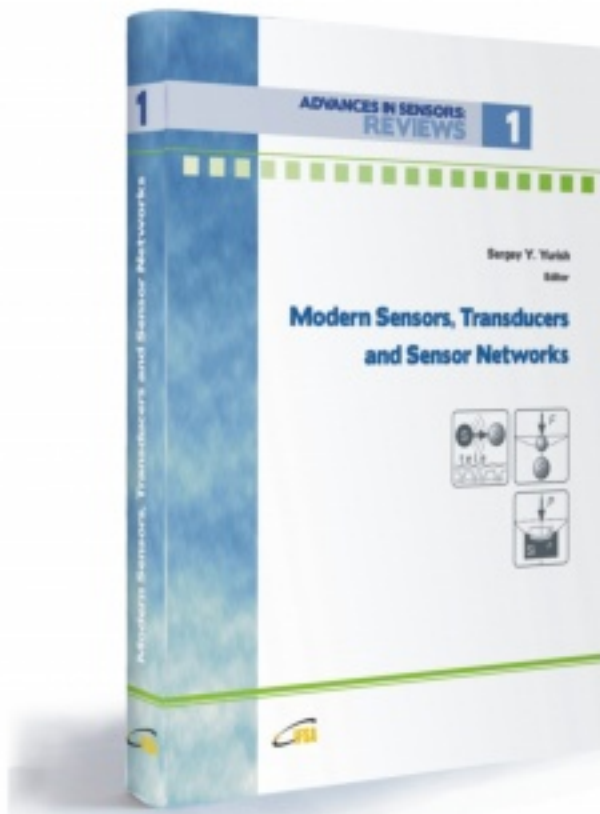
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