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Acetylcholinesterase Biosensor Based on Poly (diallyldimethylammonium chloride)-multi-walled Carbon Nanotubes-graphene Hybrid Film

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Abstract: In this paper, an amperometric acetylcholinesterase (AChE) biosensor for quantitative determination of carbaryl was developed. Firstly, the poly (diallyldimethy-lammonium chloride) -multi-walled carbon nanotubes-graphene hybrid film was modified onto the glassy carbon electrode (GCE) surface, then AChE was immobilized onto the modified GCE to fabricate the AChE biosensor. The morphologies and electrochemistry properties of the prepared AChE biosensor were investigated by using scanning electron microscopy, cyclic voltammetry and electrochemical impedance spectroscopy. All variables involved in the preparation process and analytical performance of the biosensor were optimized. Based on the inhibition of pesticides on the AChE activity, using carbaryl as model compounds, the biosensor exhibited low detection limit, good reproducibility and high stability in a wide range. Moreover, the biosensor can also be used for direct analysis of practical samples, which would provide a new promising tool for pesticide residues analysis.

Keywords: Biosensor; Acetylcholinesterase; Multi-walled carbon nanotubes; Graphene; Poly (diallyldimethylammonium chloride)

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Introduction

Carbaryl, one of the carbamate pesticides, has been widely used in agriculture because of its high effectiveness for insect control [1,2]. However, long-term accumulations in environment, carbaryl will present a serious risk to human health due to its high toxicity to acetylcholinesterase, a key enzyme for the function of the central nervous system in humans [3-7]. Traditional analytical methods, such as liquid chromatographic [8,9], gas chromatography [10] and high performance liquid chromatography (HPLC) [11] are rationally selective and have low limits of detection. However, these methods require extensive sample preparation, specialized analytical equipment, and technical expertise, which are all unsuitable for rapid, immediate and large-scale sample analysis. Thus, the development of efficient, sensitive and simple analytical method for the determination of pesticide residues in food has become increasingly important.

Enzymatic methods have some advantages such as rapid response, time saving, low cost and high sensitivity [12,13]. Among these, amperometric acetylcholinesterase (AChE) biosensors based on the inhibition action of pesticides on AChE have shown satisfactory results, in which the enzymatic activity is employed as an indicator of quantitative measurement of insecticides [14-16]. The performance of the biosensors depends on the effective immobilization of enzyme onto the surface of electrode. To maintain the inherent nature of enzyme, it is necessary to choose adequate supporting materials and immobilization methods [17-20].

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Recently, the use of multi-walled carbon nanotubes (MWCNTs) and graphene (GR) for the fabrication of electrochemical biosensors has attracted considerable attention [21-24]. MWCNTs and GR have advantages in the biosensor field because of their unique physicochemical properties including high surface area, excellent electric conductivity, strong mechanical strength, good adsorptive ability and excellent biocompatibility [25-27]. Moreover, studies have revealed that nanohybrid can often combine the merits of each component and exhibit enhanced properties [28-30]. Previous literatures have proved that MWCNTs-GR hybrid can generate synergy effect, which can greatly accelerate electron-transfer processes and provide a promising electrochemical sensing platform [31-33]. However, both MWCNTs and GR are hydrophobic and tend to form agglomerates, limiting their further biological applications [34,35]. So we need to find effectual dispersants to solve this problem.

Poly (diallyldimethylammonium chloride) (PDDA), a water soluble cationic polyelectrolyte, can improve the dispersibility of MWCNTs in water and form the thin films of MWCNTs for various applications [36-40]. Additionally, PDDA has excellent binding capability with graphene, could maintain the electronic structure of graphene and increase the solubility of graphene [41,42]. Therefore, PDDA can be used for dispersing MWCNTs-GR hybrid to fabricate biosensor. However, to the best of our knowledge, there is no report based on PDDA-MWCNTs-GR hybrid modified electrodes for the quantitative determination of carbaryl.

In this study, the PDDA-MWCNTs-GR hybrid film modified glassy carbon electrode (GCE) was prepared for the electrochemical detection of carbaryl. The high performance biosensor with low detection, wide linear range and good reproducibility was obtained based on the PDDA-MWCNTs-GR hybrid film modified electrode. All variables involved in the experiment and the performance of the biosensor were investigated in detail.

Experimental

Reagents

Acetylcholinesterase (Type C3389, 500 U/mg from electric eel), acetylthiocholine chloride (ATCl), Poly (diallydimethylammonium chloride) (PDDA) (35%, w/w in water) and pralidoxime iodide were purchased from Sigma (St. Louis, USA). Carboxylated MWCNTs were obtained from Nanotech Port Co. (Shenzhen, China). GR was from Nanoon (Hebei, China). 0.1 M pH 7.0 phosphate buffer solution (PBS) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Carbaryl was standard product, ethanol and other reagents were of analytical grade. The carbaryl solution was prepared by a step-dilution method.

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 electrochemical workstation (Shanghai Chenhua Co., China).

Preparation of PDDA-MWCNTs-GR hybrid

PDDA-MWCNTs-GR hybrid was prepared according to the literatures with minor modifications [33,43]. 2 mg of MWCNTs and GR (3:1, MWCNTs:GR) were dispersed into 4 ml of 0.25% PDDA aqueous solution (containing 0.5 M NaCl) by sonication. The resulting suspension was centrifuged at 14000 rpm and washed three times with water. Finally, the obtained hybrid was dispersed into some water to form a concentration of 1 mg/ml and the resulting solution was sonicated for 5 min before use.

Configuration of the biosensor

First, the bare GCE was polished to a mirror-like surface with 0.3 µm and 0.05 µm alumina powder, respectively, and washed through sonication with doubly distilled water, ethanol and doubly distilled water for 3 min and dried with nitrogen stream. Then the clean GCE was coated with 7.0 µl PDDA-MWCNTs-GR suspension and dried at room temperature. Finally, the PDDA-MWCNTs-GR/GCE was coated with 5.0 µl AChE solution (100 mU) to obtain the AChE/PDDA-MWCNTs-GR/GCE. The obtained biosensor was stored at 4℃ when not in use. The stepwise fabrication process of the biosensor was shown in Fig. 1.

Measurement procedure

The AChE/PDDA-MWCNTs-GR/GCE biosensor was employed for the determination of carbaryl using differential pulse voltammetry (DPV) method. The performance of the biosensor was tested by its DPV response in pH 7.0 PBS solution containing 1.0 mM ATCl. Then the electrode was rinsed with water and incubated in pH 7.0 PBS solution containing desired concentration of carbaryl for 12 min. Finally, it was transferred into the 1.0 mM ATCl solution for DPV measurement in the same condition. The inhibition rate of of carbaryl was calculated as follows:

$$\text{Inhibition}(\%) = (I_{\mathrm{P,control}} - I_{\mathrm{P,exp}})/I_{\mathrm{P,control}} \times 100\%$$

Where, $I_{\mathrm{P,control}}$ was the peak current of ATCl on AChE/PDDA-MWCNTs- GR/GCE without carbaryl

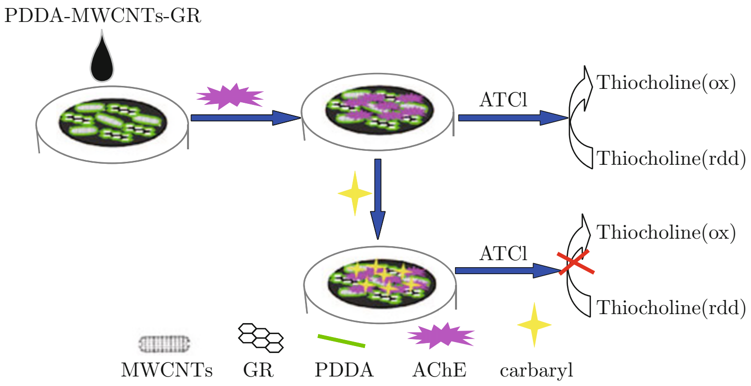


Fig. 1 The fabricating procedures of the biosensor.

inhibition and $I_{P,exp}$ was the peak current of ATCl on AChE/PDDA-MWCNTs-GR/GCE with carbaryl inhibition.

After inhibited by carbaryl, the AChE/PDDA-MWCNTs-GR/GCE was reactivated by immersing into 5.0 mM pralidoxime iodide for 12 min, then transferred into pH 7.0 PBS containing 1 mM ATCl for DPV analysis of the electrochemical response. The reactivation efficiency was calculated as follows:

$$R(\%) = (I_r - I_{P,exp})/(I_{P,control} - I_{P,exp}) \times 100\%$$

Where, I_r was the peak current of 1 mM ATCl on AChE/PDDA-MWCNTs -GR/GCE after 5.0 mM pralidoxime iodide reactivation.

Preparation and determination of real samples

For the analysis of carbaryl in real samples, cleaned cabbage, garland chrysanthemum, leek and pakchoi were washed three times with double-distilled water and chopped. Then 5 g of each sample was sprayed with different concentrations of carbaryl and stored at 4°C for 24 h, then mixed with 10 ml mixed solution of acetone/pH 7.0 PBS (1:9, v:v). After that, the mixture was sonicated for 15 min and centrifuged (10 min, 10,000 rpm). The DPV analysis was performed directly in the acquired supernatants without extraction or preconcentration. The concentration of carbaryl in the samples can be obtained from the calibration curve.

Results and discussion

Characterization of MWCNTs, GR and PDDA-MWCNTs-GR hybrid

The morphologies of only MWCNTs and GR film together with PDDA-MWCNTs-GR hybrid film were characterized with scanning electron microscope (SEM). As seen from Fig. 2(a), PDDA-MWCNTs presented a large number of filamentous structure. Figure 2(b) showed a general view of PDDA-GR which clearly illustrated the flake-like shapes of graphene. Figure 2(c) showed a typical SEM image of PDDA-MWCNTs-GR hybrid film in which MWCNTs were uniformly dispersed throughout GR film. We can found that the number of junctions and extent of stacking between MWCNTs in the hybrid film were clearly reduced compared to the only MWCNTs film (Fig. 2(a)). The usual aggregation and stacking between individual GR were significantly inhibited in the PDDA-MWCNTs-GR hybrid film due to the introduction of MWCNTs. These interesting properties could enhance the surface area and porosity of PDDA- MWCNTs-GR hybrid film, which are very beneficial to the further application in bioseneor field [44,45].

Electrochemical impedance analysis

The stepwise assembly of the biosensor was characterized by electrochemical impedance spectroscopy (EIS), and the results were shown in Fig. 3. The

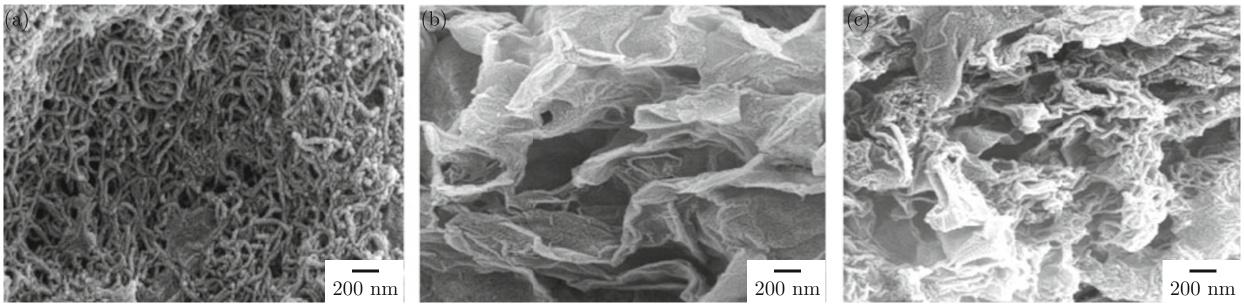


Fig. 2 SEM images of (a) PDDA-MWCNTs; (b) PDDA-GR; and (c) PDDA-MWCNTs-GR.

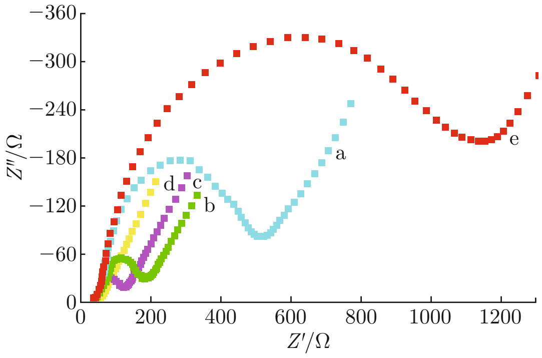


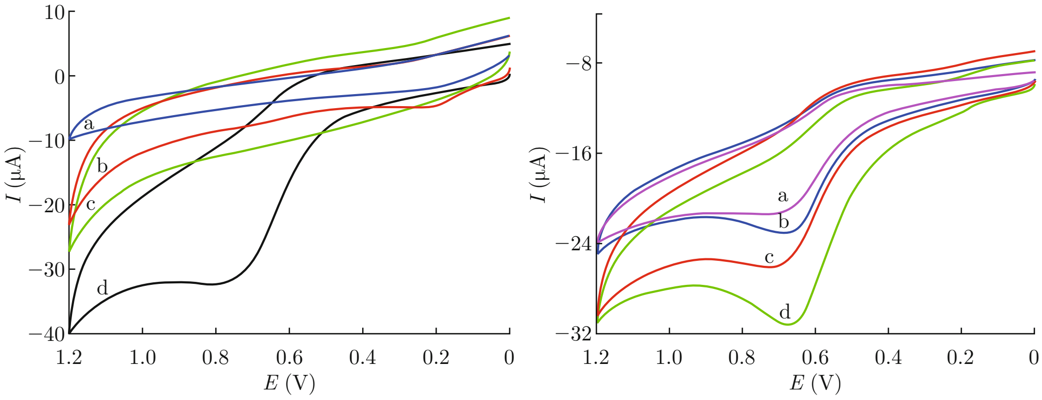
Fig. 3 EIS of (a) bare GCE; (b) PDDA-GR/GCE; (c) PDDA-MWCNTs/GCE; (d) PDDA-MWCNTs-GR/GCE; (e) AChE/PDDA-MWCNTs-GR/GCE recorded in pH 7.0 PBS containing 5.0 mM $[\mathrm{Fe(CN)_6}]^{3-/4-}$and 0.1 M KCl.

electron transfer resistance (Rct) of the bare GCE was 486 Ω (curve a), by contrast, the Rct of PDDA-GR/GCE (curve b) and PDDA-MWCNTs/GCE (curve c) decreased dramatically to about 180 Ω and 120 Ω, respectively. This phenomenon suggested that PDDA-GR and PDDA-MWCNTs hybrid greatly improved the conductivity of the modified electrode due to their high surface area and excellent electric conductivity. For PDDA-MWCNTs-GR/GCE (curve d), the Rct was 50 Ω, further decreased compared with curve b and curve c. The reason might be that the nano-hybrid of MWCNTs and GR could significantly reduce the aggregation and stacking between MWCNTs or GR, which resulted in enhanced surface area and wide porous structure of PDDA-MWCNTs-GR hybrid. When AChE was immobilized onto the surface of PDDA-MWCNTs-GR/GCE (curve e), the Rct remarkably increased to 1100 Ω due to the increase of the thickness of the interface, which insulated the conductive support and reduced the electron transfer between anionic $[\mathrm{Fe(CN)_6}]^{3-/4-}$ and GCE [46-48].

Cyclic voltammetric behavior of ATCl

Figure 4(A) showed the cyclic voltammograms (CVs) of different fabricated electrodes in absence and presence of ATCl in pH 7.0 PBS. No peak was observed at bare GCE (curve a) and AChE/PDDA-MWCNTs-GR/GCE (curve b) in PBS without ATCl. CV of AChE/PDDA-MWCNTs-GR/GCE (curve d) showed an irreversible oxidation peak in the presence of 1.0 mM ATCl, whereas no detectable signal was observed for PDDA-MWCNTs-GR/GCE (curve c). Conspicuously, this peak is ascribed to the oxidation of thiocholine, hydrolysis product of ATCl, catalyzed by immobilized AChE. The peak current produced by the oxidation of thiocholine was used as an indicator for quantitative measurement of the enzyme activity.

To further study the different modified materials of electrode, the CVs of AChE/GCE (curve a), AChE/PDDA-GR/GCE (curve b), AChE/PDDA-MWCNTs/GCE (curve c) and AChE/PDDA-MWCNTs-GR/GCE (curve d) in the presence of 1.0 M ATCl in pH 7.0 were showed in Fig. 4(B). AChE/GCE (curve a) showed a small peak current, which may be due to the low enzyme loading arising from the weak combination between AChE and GCE. Compared with AChE/GCE, the peak currents increased sharply of the electrode with PDDA-GR or PDDA-MWCNTs. It was estimated that PDDA-GR and PDDA-MWCNTs had the ability to promote the electron transfer. A significant increase of peak current was obtained at AChE/PDDA- MWCNTs-GR/GCE (curve d), which was attributed to the synergistic effect between PDDA-GR and PDDA-MWCNTs.



(A) (B)

Fig. 4 (A) CVs of (a) bare GCE; (b) AChE/PDDA-MWCNTs-GR/GCE in the blank PBS (0.1 M, pH 7.0); and (c) PDDA-MWCNTs-GR/GCE; (d) AChE/PDDA- MWCNTs-GR/GCE in 0.1 M PBS (pH 7.0) containing 1.0 mM ATCl. (B) CVs of (a) AChE/GCE; (b) AChE/PDDA-GR/GCE; (c) AChE/PDDA-MWCNTs /GCE; (d) AChE/PDDA-MWCNTs-GR/GCE in the presence of 1.0 mM ATCl in 0.1 M PBS (pH 7.0).

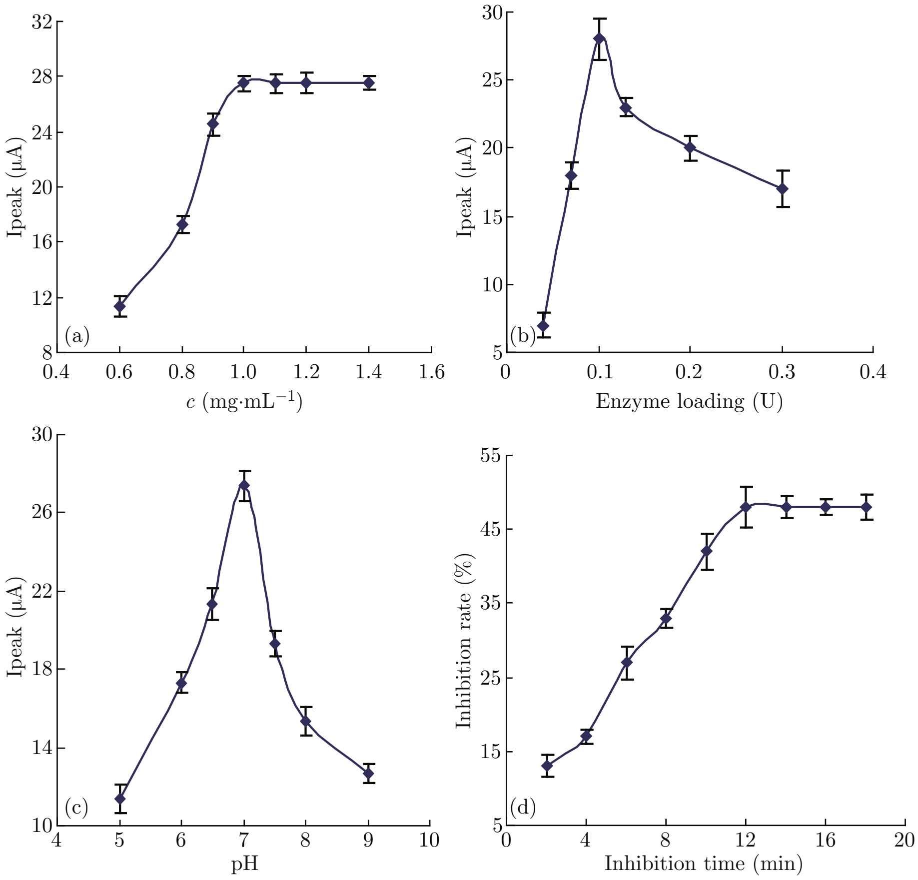


Fig. 5 (a) Effect of the PDDA-MWCNTs-GR concentration on the amperometric response; (b) Effect of enzyme amount on the amperometric response; (c) Relationship between inhibition rate and inhibition time; (d) Effect of the detection solution pH on the amperometric response.

Optimization of experimental parameters

Influence of PDDA-MWCNTs-GR concentration

The concentration of PDDA-MWCNTs-GR hybrid played an important role in achieving good analytical performance. The response currents of the enzyme electrode were investigated in 0.1 M pH 7.0 PBS containing 1.0 mM ATCl. As shown in Fig. 5(a), the signal current increased gradually with the increase of PDDA-MWCNTs-GR concentration, and when it increased to 1 mg/ml, no obvious enhancement of current was observed. Thus, 1 mg/ml PDDA-MWCNTs-GR was selected to prepare the biosensor.

Influence of enzyme amount

Figure 5(b) showed the influence of enzyme amount on the response of biosensor. With the increasing of AChE loading amount, the peak current increased sharply and reached the maximal value at 0.1 U. After that, the amperometric response decreased gradually as the amount of AChE further increased. Therefore, 0.1 U of AChE was used in the subsequent experiment.

Influence of detection solution pH

The sensitivity and stability of the biosensor depended greatly on the pH of electrolyte solution. Figure 5(c) showed the plot of peak current of the biosensor versus different pH in 0.1 M PBS in the presence of 1.0 mM ATCl. The maximum peak current appeared at pH 7.0, which was closed to that previous report for free AChE [49], suggesting the immobilization did not change the fundamental microenvironment of AChE. Thus, pH 7.0 was chosen for the subsequent experiment.

Influence of incubation time on inhibition

The incubation time was an important factor for capturing carbaryl. As shown in Fig. 5(d), the steady-state current decreased greatly with the increase of incubation time. When the immersing time was longer than 12 min, the inhibition curve trended to a stable value. However, the inhibition of carbaryl could not reach 100%, which indicated that the binding sites between pesticides and enzymes could reach an equilibration

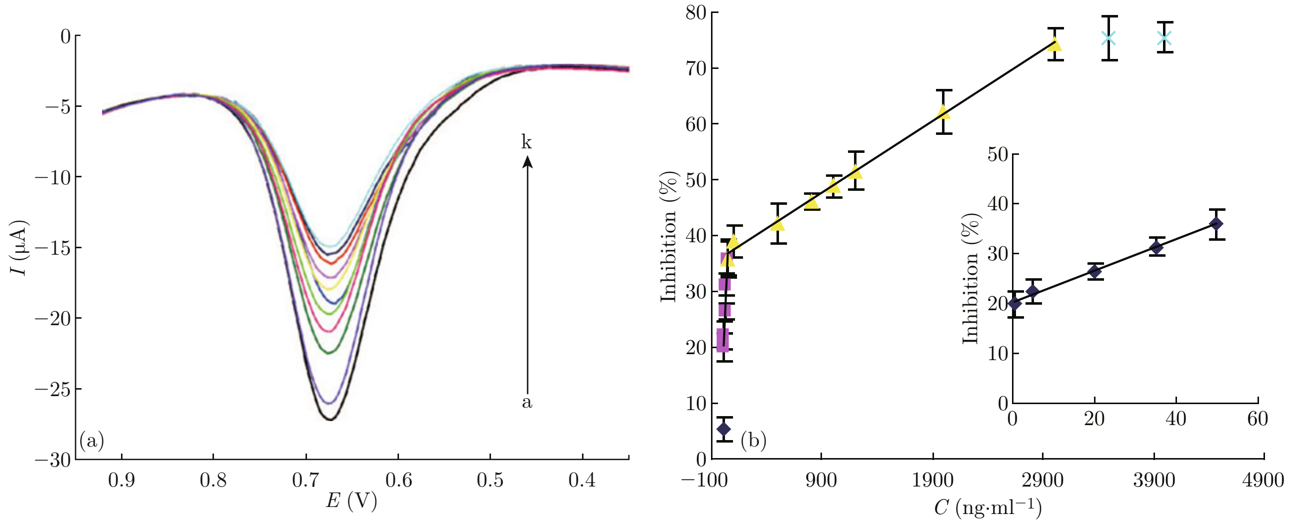


Fig. 6 (a) The DPVs of AChE/PDDA-MWCNTs-GR/GCE after inhibition by carbaryl of different concentrations (from a to k): 0, 0.1, 0.5, 20, 35, 50, 100, 500, 800, 1000, 1200 ng/ml under the optimal conditions; (b) Relationship between inhibition rates and carbaryl concentrations. Inset: linear relationship between low inhibitions and low concentrations.

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

Electrode	Liner range	Detection limit	References
AChE-CdS-G-CHIT/GCE	2-2000 ng/ml	0.7 ng/ml	[51]
AChE/PB-CHIT/GCE	2-80 ng/ml, 200-1000 ng/ml	0.6 ng/ml	[52]
AChE/carbon paste electrodes	1-15 μg/ml	0.4 μg/ml	[53]
AChE-CHIT/Au	0.005-0.1 μg/ml, 0.5-5 μg/ml	0.003 μg/ml	[54]
GC/MWCNT/CoPc	66-1322 ng/ml	1.1 ng/ml	[55]
GC/PANI/MWCNT/AChE	1.98-9.92 μg/ml	0.28 μg/ml	[56]
AChE/PDDA-MWCNTs-GR/GCE	0.8-50 ng/ml, 50-3000 ng/ml	0.13 ng/ml	This work

state [50]. Thus, the time of 12 min was selected as the optimum inhibition time.

Detection of carbaryl

Figure 6(a) showed the DPVs of the biosensor before and after incubation with carbaryl of different concentrations under the optimal conditions. It was evident that the peak current decreased drastically with the increasing concentration of carbaryl. It might be due to the fact that carbaryl exhibited fairly high toxicity and performed irreversible inhibition action on AChE, which reduced the enzymatic activity to its substrate. Under the optimal conditions, the inhibition of carbaryl on AChE/PDDA- MWCNTs-GR/GCE was proportional to its concentrations in two ranges, from 0.5 to 50 ng/ml and from 50 to 3000 ng/ml (Fig. 6(b)). The regression equations were $I\% = 0.313c$ (ng/ml) + 20.228 ($R = 0.9971$) and $I\% = 0.0128c$ (ng/ml) + 36.185 ($R = 0.9968$). The detection limit was calculated to be 0.13 ng/ml ($S/N = 3$).

A comparison of analytical characteristics of AChE/PDDA-MWCNTs-GR/GCE with other reported biosensors for the detection of carbaryl was summarized in Table 1.

Precision, stability and reactivation

The inter-assay precision was estimated by comparing the responses of 5 different electrodes to 1.0 mM ATCl after being treated with 10 ng/ml carbaryl solutions for 12 min. Similarly, the intra-assay precision of the biosensor was evaluated by analyzing carbaryl levels for 5 replicate measurements at one enzyme electrode. The RSD values of inter-assay and intra-assay were found to be 4.1% and 4.7%, respectively, indicating acceptable precision and replicability. The enzyme electrode was stored at 4℃ in dry condition for 7 days, and no obvious decrease in the response of ATCl was observed. After a 30-day storage period, the biosensor still retained 87% of its initial current response, indicating satisfactory stability.

According to previous reports, the inhibited AChE could be reactivated by oximes, such as pralidoxime iodide [57,58]. After reactivation with 5.0 mM pralidoxime iodide solution for 12 min, the activity of regenerated AChE could reach to 93% of its original value, indicating acceptable reproducibility.

Real samples analysis

In order to evaluate the practicality of the proposed biosensor, the recovery tests were studied by adding different amounts of carbaryl into real samples, cabbage, garland chrysanthemum, leek and pakchoi. Results were summarized in Table 2. The recoveries were from 92.3% to 107%, suggesting that the proposed biosensor could be used for direct analysis of practical samples.

Table 2 Recovery studies of carbaryl in real samples

Sample	Taken (ng/ml)	Found (ng/ml)	Recovery (%)
cabbage	10	9.31	93.1
	100	97.1	97.1
garland chrysanthemum	10	10.5	105
	100	92.3	92.3
leek	10	10.7	107
	100	106	106
pakchoi	10	9.53	95.3
	100	106	106

Conclusion

In this work, the AChE/PDDA-MWCNTs-GR/GCE biosensor had been successfully fabricated for the detection of carbaryl. The nano-hybrid of MWCNTs-GR could significantly reduce the aggregation and stacking between MWCNTs and GR, which resulted in enhanced surface area and wide porous structure of the hybrid. At the same time, PDDA increased the solubility of MWCNTs-GR hybrid. Based on these good properties, the AChE/PDDA-MWCNTs-GR/GCE exhibited excellent performances for the detection of carbaryl, such as wide linear detection range, low detection limit, high sensitivity, good stability and acceptable reproducibility. In addition, the biosensor could be applied for direct analysis of practical samples. Therefore, we believe the PDDA-MWCNTs-GR hybrid biocompatible film will pave the way to the new AChE-based inhibitor biosensor for carbamate pesticides' determination.

Acknowledgements

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