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A Novel Biosensor Based on Acetylecholinesterase/Chitosan-Graphene Oxide Modified Electrode for Detection of Carbaryl Pesticides

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A sensitive, fast and cheap sensor for quantitative determination of carbaryl pesticide using amperometric acetylcholinesterase biosensor based on graphene oxide-chitosan hybrid film was reported. This biosensor was fabricated by firstly depositing chitosan-graphene oxide hybrid film on glassy carbon electrode and subsequently assembling acetylcholinesterase on chitosan-graphene oxide hybrid film. The chitosan-graphene oxide hybrid film exhibited a good biocompatibility with acetylcholinesterase and effective immobilization of acetylcholinesterase. The resulted biosensor showed a good electrocatalytic activity toward oxidation of thiocholine, which was a product from the hydrolysis of acetylthiocholine catalyzed by acetylcholinesterase. The inhibition of carbaryl on the activity of acetylcholinesterase was proportional to carbaryl concentration in the range from 0.005 to 0.4 μM and from 1.0 to 5.0 μM, respectively. The detection limit was calculated to be about 4.0 nM. The biosensor provided a new promising tool for pesticide analysis.

Key Words: Acetylcholinesterase, Graphene, Chitosan, Carbaryl, Biosensor.

INTRODUCTION

Carbaryl insecticide, one of the carbamate pesticides, has been extensively used in agriculture owing to its high insecticidal activity[1,2]. However, it presents a serious risk to human health due to its inhibitory effect on acetylcholinesterase[3-5], a key enzyme for the nerve transmission. Thus, a fast and sensitive detection of pesticide residues in food has become more and more important. Traditional analytical methods[6], such as gas chromatography or high-performance liquid chromatography often coupled with mass-selective detectors, are obviously time-consuming and expensive. These methods are still performed in laboratory, but they are not suitable for a fast home-detection.

Enzymatic method[7-9], the most effective method has been used as an alternative to traditional methods for carbaryl detection. Among these, amperometric acetylcholinesterase (AChE) biosensor[10,11] based on the inhibition of acetylcholinesterase by carbaryl pesticide[12] has shown good result for carbaryl analysis. The acetylcholinesterase immobilized on an electrode surface can catalyze the hydrolysis of acetylthiocholine (ATCl) to produce an electro-active product of thiocholine, which shows an irreversible oxidation peak at about 0.68 V[13,14] as a marker for carbaryl detection. The inhibition of carbaryl on acetylcholinesterase results in decrease of produced thiocholine and accordingly the oxidation current decreases. The carbaryl can be detected by measuring the decline of oxidation current of thiocholine. The reactions involved with the detection process are shown as followed[13,14].

$$(CH_3)_3N^+CH_2CH_2SC(O)CH_3 \xrightarrow{H_2O,\ AChE} \underset{\text{Acetylthiocholine}}{} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH$$

$$\underset{\text{Thiocholine}}{2(CH_3)_3N^+CH_2CH_2SH} - 2e^- \longrightarrow (CH_3)_3N^+CH_2CH_2SSCH_2CH_2N^+(CH_3)_3 + 2H^+ \quad (1)$$

The sensor's sensitivity depended strongly on the distribution and immobilization of acetylcholinesterase on electrode and the property of materials used to immobilize acetylcholinesterase.

Graphene, a single layer of carbon atoms in a closely packed honeycomb two-dimensional lattice, has attracted tremendous attention because of its excellent electrical properties and the high specific surface area of 400 m^2/g up to 1500 m^2/g for enzyme adsorption on electrodes[15-19], especially graphene oxide (GO) or chemically derived graphene owing to its abundant groups, such as epoxide, hydroxyl and carboxylic groups and the high water solubility[20-26]. The remarkable surface area and its well electrocatalytic and electrochemical properties have led to an explosion of research in the field of

electrochemical sensors. Chitosan (CHIT) contains a large number of -NH_2 and -OH and is preferable to maintain the high biological activity of the immobilized biomolecules[27,28]. Chitosan has been widely used as a modifying reagent to functionalize graphene or graphene oxide for sensing application due to its excellent biocompatibility, nontoxicity, cheapness, easy-handling and high mechanical strength[29,30].

In this work, a novel acetylcholinesterase biosensor was constructed based on the CHIT-GO hybrid film deposited on a glassy carbon electrode (GCE). The CHIT-GO film shows a large surface area and good biocompatibility. The resulted AChE/CHIT-GO/GCE showed good electrocatalytic ability to oxidation of thiocholine and accordingly the sensitivity of the biosensor was largely improved. The experimental conditions related to the preparation of CHIT-GO hybrid film and the performance of the resulted biosensors was investigated in detail.

EXPERIMENTAL

Acetylcholinesterase (type C3389, 500 U mg^{-1} from electric eel, acetylcholinesterase), acetylthiocholine chloride (ATCl), chitosan (CHIT, 75 % deacetylation) were purchased from Sigma-Aldrich (St. Louis, USA). Other reagents were purchased from Beijing Chemical Reagent Factory (Beijing, China) and were of analytical reagent grade. All solutions were prepared with ultra-pure water purified by a Millipore-Q System (18.2 MW cm). The solutions were deoxygenated by nitrogen before experiments. Phosphate buffer solution (PBS) was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate.

All electrochemical experiment were carried out with CHI 660C electrochemical workstation (CH Instruments, Shanghai, China), the three-electrode system contained a acetylcholinesterase biosensor as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum disk electrode as the counter electrode, respectively.

Atomic force microscopy measurements were carried out with an AJ-III (Shanghai Aijian Nanotechnology) in tapping mode. Standard silicon (Si) cantilevers (spring constant, 0.6-6 nN/m) were used under its resonance frequency (typically, 60-150 kHz). All atomic force microscopy images were acquired at room temperature under ambient conditions.

Synthesis of CHIT-GO nanocomposites: Graphene oxide (GO) was synthesized according to previous methods[31]. Briefly, graphite powder (1.0 g) was dispersed into 23 mL concentrated H_2SO_4 (18.0 M) in ice bath. Then, $KMnO_4$ (3.0 g) was gradually added into above solution under continuous vigorous stirring below 20 ºC. After that, the ice bath was replaced by an oil bath and the mixture was heated to 35 ºC for 0.5 h under continuous stirring. Then, ultra-pure water was slowly added into above solution, which produced a rapid increase in solution temperature up to a maximum of 100 ºC. The reaction was maintained at 98 ºC for a further 15 min and terminated by sequential addition of more distilled water (140 mL in total) and H_2O_2 (30 %, 10 mL). The solid product was separated by centrifugation at 5000 rpm and washed initially with 5 % HCl until SO_4^{2-} ions were no longer detectable with $BaCl_2$. Finally, the solid product was washed three times with acetone and dried overnight at 65 ºC. CHIT-GO nanocomposites were prepared as followed. Graphene oxide was dissolved in 20 mL of ultra-pure water and treated with ultrasound for 45 min. Chitosan solution of 0.005 g mL^{-1} was prepared by dissolving chitosan in aqueous solution of 2 M acetic acid. Then graphene oxide solution was added into the chitosan solution and stirred for 24 h to produce a homogeneous CHIT-GO solution. The solution was stored at 4 ºC when not in use.

Preparation of biosensor: The glassy carbon electrode was polished with 1, 0.3 and 0.05 μm alumina slurry and then it was washed successively with 2 M NaOH, 1 M H_2SO_4, 95 % ethanol and ultra-pure water by ultrasonic for 5 min. The polished glassy carbon electrode was coated with CHIT-GO solution. After the water was evaporated, it was coated with 5 μL acetylcholinesterase solution with different concentration to obtain the acetylcholinesterase /CHIT-GO/GCE. The obtained biosensor was stored at 4 ºC when not in use.

Measurement procedure: The prepared AChE/CHIT-GO/GCE was first activated in 0.2 M phosphate buffer solution solution (containing 0.4 mM ATCl) by cyclic voltammetric sweeping from 0 V to 1.0 V until stable curve were obtained. Then, the pretreated AChE/CHIT-GO/GCE was immersed into 0.2 M phosphate buffer solution containing carbaryl for 10 min for the inhibition of carbaryl on acetylcholinesterase. Finally, the AChE/CHIT-GO/GCE was transferred into the electrochemical cell of 10 mL 0.2 M phosphate buffer solution (containing 0.4 mM ATCl) to study the electrochemical response by cyclic voltammograms (CVs). The inhibition of carbaryl was calculated as followed[32]:

$$\text{Inhibition}\,(\%) = \frac{i_{p,\,control} - i_{p,\,exp}}{i_{p,\,control}} \times 100$$

where $i_{p,\,control}$ is the peak current at the AChE/CHIT-GO/GCE and $i_{p,\,exp}$ is the peak current at the AChE/CHIT-GO/GCE with carbaryl inhibition.

RESULTS AND DISCUSSION

Atomic force microscopy is an effective tool to observe surface topography. Fig. 1a was the atomic force microscopy image of the bare glassy carbon electrode, showing a smooth and homogeneous surface and its root mean square roughness (RMs) was about 2.09 nm. After the CHIT-GO sheets were formed on the glassy carbon electrode and they uniformly dispersed on this surface (Fig. 1b). The root mean square roughness of the CHIT-GO hybrid film modified glassy carbon electrode became 2.67 nm, which could provide a significant increase of effective electrode surface for immobilization of biomolecules. As shown in Fig. 1d, some dots appeared after acetylcholinesterase assembled on CHIT-GO/GCE. The root mean square roughness of AChE/CHIT-GO/GCE was 3.22 nm, suggesting that the small acetylcholinesterase was well immobilized on the surface of CHIT-GO/GCE. For comparison, the acetylcholinesterase was directly immobilized on polished glassy carbon electrode surface. As shown in Fig. 1c, acetylcholinesterase molecules aggregated on bare glassy carbon electrode surface. The results showed that the CHIT-GO not only immobilized acetylcholinesterase but also resulted in uniform distribution.

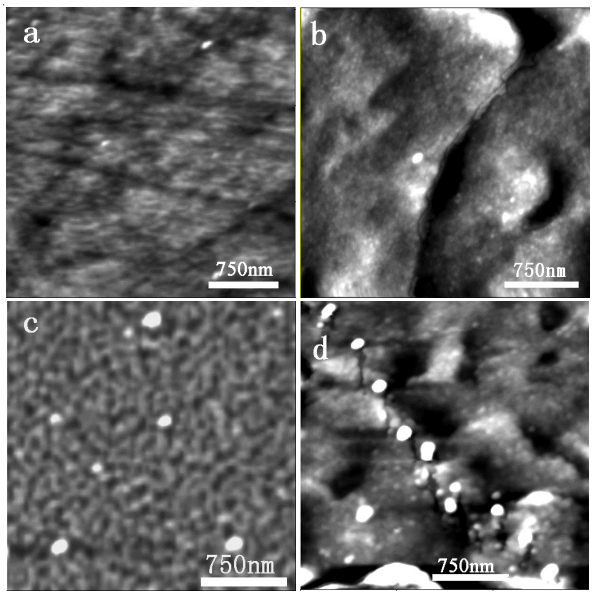

Fig. 1. AFM images of bare GCE (a), CHIT-GO/GCE (b), AChE/GCE (c) and AChE/CHIT-GO/GCE (d)

Electrochemical behaviours of AChE/CHIT-GO/GCE: Fig. 2 showed the cyclic voltammograms of different electrodes in absence and presence of acetylthiocholine in 0.2 M phosphate buffer solution (pH 7.0) at a scan rate of 40 mV s^{-1}. No obvious peak was observed at bare glassy carbon electrode (curve c) and CHIT-GO/GCE (curve d) in 0.2 M phosphate buffer solution (pH 7.0) containing 0.4 mM acetylthiocholine. In the absence of acetylthiocholine (curve e), there was no obvious peak on AChE/CHIT-GO/GCE. When 0.4 mM acetylthiocholine was added into 0.2 M phosphate buffer solution (pH 7.0), the irreversible oxidation peak at 0.7 V was observed (curve a). Obviously, this peak might result from the oxidation of thiocholine, hydrolysis product of acetylthiocholine catalyzed by immobilized acetylcholinesterase[13,14]. Compared with AChE/GCE (curve b), the oxidation current of thiocholine at AChE/CHIT-GO/GCE was larger, suggesting that CHIT-GO was a good matrix for acetylcholinesterase immobilization.

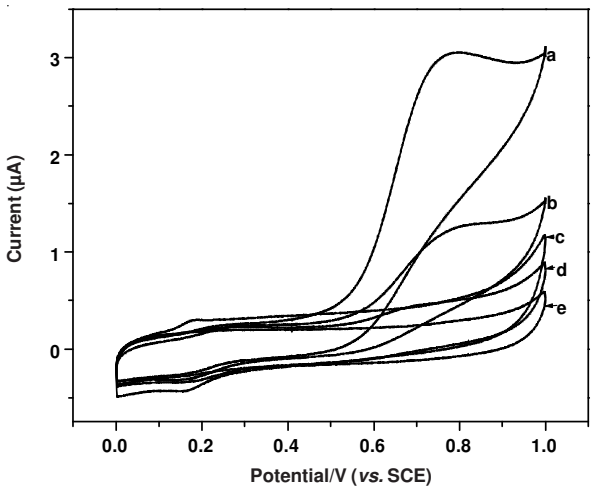

Fig. 2. Cyclic voltammograms of the AChE/CHIT-GO/GCE (a, e), AChE/GCE (b), GCE (c) and CHIT-GO/GCE (d) in 0.2 M PBS solution (pH 7.0) in the presence (a, b, c, d) and absence (e) of 0.4 mM ATCl. Scan rates: 40 mV s^{-1}

Parameters optimizing for the biosensor performance: The carbaryl could be accurately determined by measuring the decline of catalytic current of thiocholine at the AChE/CHIT-GO/GCE after the inhibition of carbaryl on acetylcholinesterase resulted in decrease of produced thiocholine. Some factors involved with the formation of CHIT-GO sheets and the activity of acetylcholinesterase might affect the performance of the biosensor. These factors were investigated in the following discussion.

The thickness of the CHIT-GO hybrid film deposited on glassy carbon electrode depended strongly on the volume of CHIT-GO. Thus, the concentration of CHIT-GO on the performance of biosensor in the absence of carbaryl was firstly investigated and the result was shown in Fig. 3a. There was a noticeable increase in the current response with the increase of concentration of CHIT-GO and reached the maximal value at 0.4 g mL^{-1}. After that, the current decreased gradually as the concentration of CHIT-GO further increased. This phenomenon might be ascribed to the following reason. With the increase of CHIT-GO concentration, it's better to immobilize acetylcholinesterase , but excess CHIT-GO might result in a compact film on glassy carbon electrode surface to block the electron transfer in reverse.

The amount of acetylcholinesterase immobilized on the electrode surface was another important factor relating to the performance of the biosensor. Fig. 3b displayed the plot of the concentration of acetylcholinesterase *versus* the amperometric response of the biosensor. With the increasing of the concentration of acetylcholinesterase , the peak current increased gradually and reached the maximal value at 20.0 U mL^{-1}. After that, the amperometric response decreased gradually as the concentration of acetylcholinesterase further increased. This turning point might be ascribed to the fact that a large number of acetylcholinesterase would block the electron transfer.

The bioactivity of the immobilized acetylcholinesterase depended greatly on the pH of electrolyte solution. Fig. 3c showed the plot of amperometric response of the biosensor *versus* different pH in 0.2 M phosphate buffer solution (6.0-8.0) in the presence of 0.4 mM acetylthiocholine. The AChE/CHIT-GO/GCE showed the optimal electrocatalytic activity in buffer solution of pH 7, which was close to that reported for soluble enzyme, suggesting the immobilization did not change the fundamental microenvironment of acetylcholinesterase in a solution. Thus, the optimum pH of 7 was selected in this work for the determination of carbaryl.

Effect of carbaryl concentration on activity of immobilized acetylcholinesterase: As shown in Fig. 4, with the increasing of the carbaryl concentration in the immersing solution, a noticeable decrease in voltammetric signal at AChE/CHIT-GO/GCE was observed. The decrease of peak current mainly resulted from a decrease of the activity of immobilized acetylcholinesterase and accordingly resulted in the decrease of produced thiocholine. The carbaryl as one of the carbamate pesticides exhibited high toxicity to inhibit irreversibly the activity of acetylcholinesterase . Thus, the thiocholine from the hydrolysis of acetylthiocholine catalyzed by acetylcholinesterase also decreased. When the concentration of carbaryl

was over 5 mM, the inhibition of carbaryl on the activity of acetylcholinesterase trended to a maintained constant value, indicating its binding interaction with active target group in acetylcholinesterase could reach saturation, at which the maximum inhibition occurred.

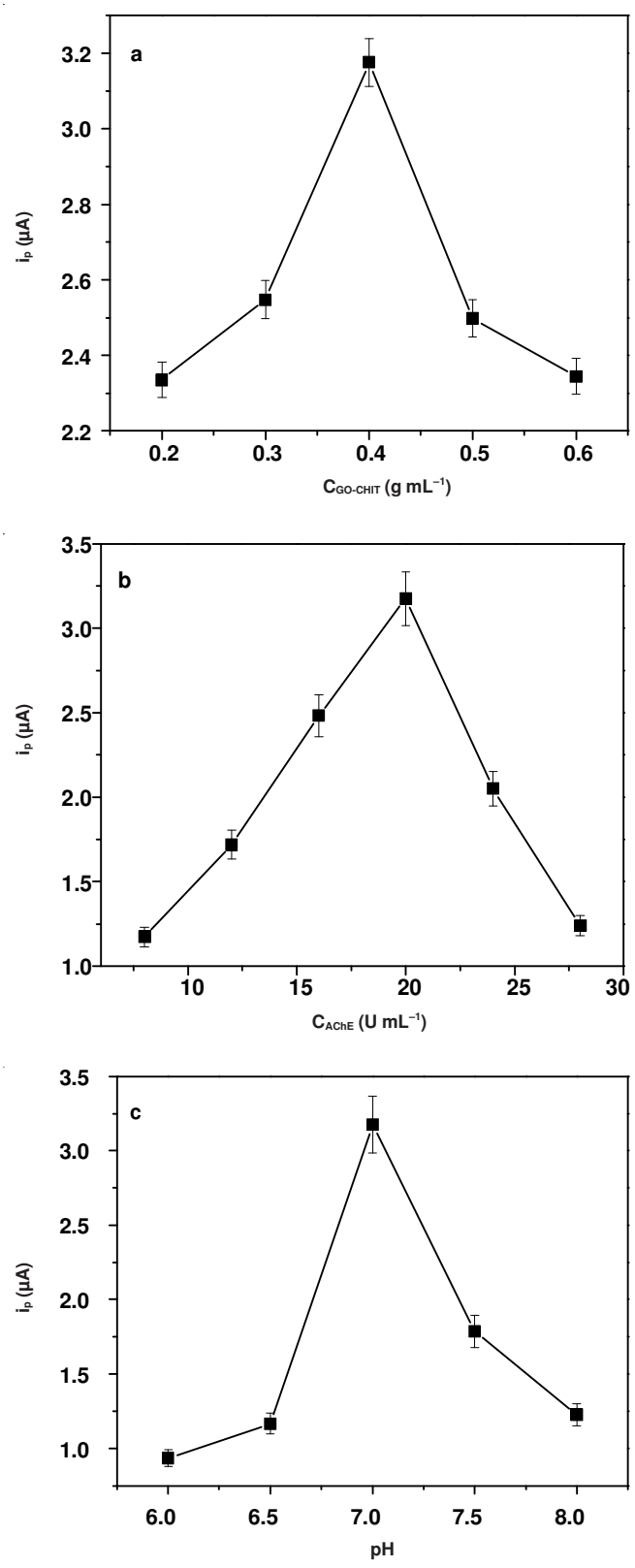

Fig. 3. Effects of concentration of CHIT-GO (a), concentration of AChE (b) and pH of PBS (c) on carbaryl detection

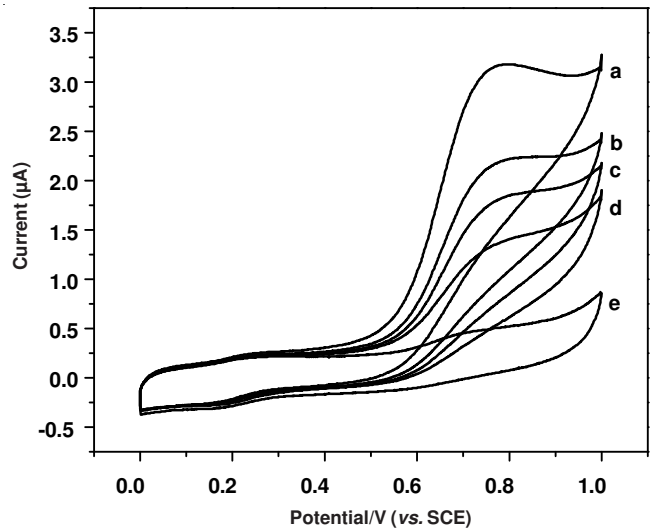

Fig. 4. Cyclic voltammograms of AChE/CHIT-GO/GCE in 0.2 M PBS solution (pH 7.0) containing 0.4 mM ATCl after adding different concentration of carbaryl: 0 (a), 0.2 (b), 0.3 (c), 1.0 (d) and 4.0 (e) μM. Scan rate: 40 mV s^{-1}

Calibration curve: Fig. 5 showed the inhibition of AChE/CHIT-GO/GCE in 0.2 M phosphate buffer solution (pH 7.0) containing 0.4 mM acetylthiocholine after the modified electrode was immersed in a solution containing different concentration of carbaryl for 10 min. As the concentration of carbaryl in the immersing solution increased, the peak current at the AChE/CHIT-GO/GCE decreased gradually. Under the optimal experimental condition, the carbaryl inhibition to AChE/CHIT-GO/GCE was proportional to its concentration in two ranges from 0.005 to 0.4 μM (the inset a in Fig. 5) and 1.0 to 5.0 μM (the inset b in Fig. 5) with the correlation coefficients of 0.9990 and 0.9994, respectively. The detection limit was calculated to be about 4.0 nM.

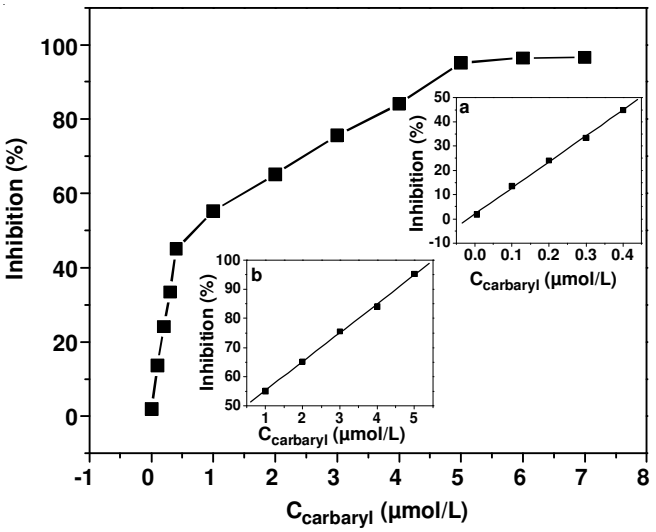

Fig. 5. Inhibition of AChE/CHIT-GO/GCE in 0.2 M PBS (pH 7.0) containing 0.4 mM ATCl after adding different concentration of carbaryl. Inset: calibration curves in the range of 0.005-0.4 μM (A) and 1.0-5.0 μM (B), respectively

To further demonstrate the practicality of the proposed method, the recovery test was studied by adding different

amounts of carbaryl into water samples. Results are summarized in Table-1. The recoveries were from 96.3 to 106 %. The results indicated that the proposed method was highly accurate, precise and reproducible. It could be used for direct analysis of relevant samples.

TABLE-1
RECOVERY STUDIES OF CARBARYL IN WATER SAMPLES

Sample	Taken (μmol L^{-1})	Found (μmol L^{-1})	Recovery (%)
1	0.050	0.0048	98
2	0.100	0.1080	108
3	0.400	0.3800	95
4	1.000	0.9600	96
5	5.000	5.3500	107

Selectivity and stability: The interferences from the other electroactive nitrophenyl derivative such as nitrophenol and other oxygen containing inorganic ions (SO_4^{2-}, NO_3^-) were investigated. No obvious inhibition behaviour could be observed. Thus, the electrode was selective and the electrode could be applied to the determination of carbaryl in practical samples.

When the enzyme electrode was not in use, it was stored in a refrigerator in N_2-saturated desiccator at 4 ºC for 10 days, no obvious decrease in the current response of AChE/CHIT-GO/GCE was observed. After 30-day storage period, the biosensor retained 82.2 % of its initial current response. It indicated that CHIT-GO hybrid interface provided a biocompatible microenvironment around the acetylcholinesterase to stabilize its biological activity to a large extent.

Conclusion

In summary, a simple method to immobilize acetylcholinesterase on CHIT-GO sheets modified glassy carbon electrode was proposed and a sensitive amperometric biosensor for fast determination of carbaryl pesticide was developed. Under the optimal parameters, the AChE/CHIT-GO/GCE exhibited a good electrochemical response for carbaryl owing to its good biocompatibility with acetylcholinesterase toward oxidation of thiocholine, which was a product from the hydrolysis of acetylthiocholine catalyzed by acetylcholinesterase. The inhibition of carbaryl on the activity of acetylcholinesterase was proportional to carbaryl pesticide concentration in the range from 0.005 to 0.4 μM and from 1.0 to 5.0 μM with the correlation coefficients of 0.9990 and 0.9994, respectively. The detection limit was estimated to be about 4.0 nM. The developed biosensor provides a new promising tool for pesticide analysis.

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REFERENCES

1. D.W. Miwa, G.R.P. Malpass, S.A.S. Machado and A.J. Motheo, *Water Res.*, **40**, 3281(2006).
2. F. Arduini, F. Ricci, C.S. Tuta, D. Moscone, A. Amine and G. Palleschi, *Anal. Chim. Acta*, **580**, 155 (2006).
3. A. Vakurov, C.E. Simpson, C.L. Daly, T.D. Gibson and P.A. Millner, *Biosens. Bioelectron.*, **20**, 2324 (2005).
4. D. Du, S.Z. Chen, J. Cai and A.D. Zhang, *Talanta*, **74**, 766 (2008).
5. N. Sattarahmady, H. Heli and A.A. Moosavi-Movahedi, *Biosens. Bioelectron.*, **25**, 2329 (2010).
6. S.Q. Liu, L. Yuan, X.L.Yue, Z.Z. Zheng and Z.Y. Tang, *Adv. Power Technol.*, **19**, 419 (2008).
7. C. Chouteau, S. Dzyadevych, C. Durrieu and J.M. Chovelon, *Biosens. Bioelectron.*, **21**, 273 (2005).
8. D. Du, W.J. Chen, W.Y. Zhang, D.L. Liu, H.B. Li and Y.H. Lin, *Biosens. Bioelectron.*, **25**, 1370 (2010).
9. Z. Zheng, Y. Zhou, X. Li, S. Liu and Z. Tang, *Biosens. Bioelectron.*, **26**, 3081 (2011).
10. A.P. Periasamy, Y. Umasankar and S.M. Chen, *Sensors*, **9**, 4034 (2009).
11. Y. Song, M. Zhang, L. Wang, L. Wan, X. Xiao, S. Ye and J. Wang, *Electrochim. Acta*, **56**, 7267 (2011).
12. D. Du, X. Huang, J. Cai and A.D. Zhang, *Biosens. Bioelectron*, **23**, 285 (2007).
13. G.D. Liu and Y.H. Lin, *Anal. Chem.*, **78**, 835 (2006).
14. D. Du, X.X. Ye, J. Cai, J. Liu and A.D. Zhang, *Biosens. Bioelectron*, **25**, 2503 (2010).
15. L. Liu, S. Ryu, M.R. Tomasik, E. Stolyarova, N. Jung, M.S. Hybertsen, M.L. Steigerwald, L.E. Brus and G.W. Flynn, *Nano Lett.*, **8**, 1965 (2008).
16. I. Calizo, A.A. Balandin, W. Bao, F. Miao and C.N. Lau, *Nano Lett.*, **7**, 2645 (2007).
17. A.K. Geim and K.S. Novoselov, *Nature*, **446**, 183 (2007).
18. J.C. Meyer, A.K. Geim, M.I. Katsnelson, K.S. Novoselov, T.J. Booth and S. Roth, *Nature*, **446**, 60 (2007).
19. M. Ishigami, J.H. Chen, W.G. Cullen, M.S. Fuhrer and E.D. Williams, *Nano Lett.*, **7**, 1643 (2007).
20. Y. Fang, S. Guo, C. Zhu, Y. Zhai and E. Wang, *Langmuir*, **26**, 11277 (2010).
21. C. Shan, H. Yang, D. Han, Q. Zhang, A. Ivaska and L. Niu, *Biosens. Bioelectron*, **25**, 1070 (2010).
22. M. Zhou, Y. Zhai and S. Dong, *Anal. Chem.*, **81**, 5603 (2009).
23. C. Shan, H. Yang, J. Song, D. Han, A. Ivaska and L. Niu, *Anal. Chem.*, **81**, 2378 (2009).
24. R.S. Dey and C.R. Raj, *J. Phys. Chem. C*, **114**, 21427 (2010).
25. W. Hong, H. Bai, Y. Xu, Z. Yao, Z. Gu and G. Shi, *J. Phys. Chem. C*, **114**, 1822 (2010).
26. L. Cao, Y. Liu, B. Zhang and L. Lu, *ACS Appl. Mater. Interf.*, **2**, 2339 (2010).
27. J.M. Gong, T. Liu, D.D. Song, X.B. Zhang and L.Z. Zhang, *Electrochem. Commun.*, **11**, 1873 (2009).
28. D. Du, S.Z. Chen, D.D. Song, H.B. Li and X. Chen, *Biosens. Bioelectron*, **24**, 475 (2008).
29. X. Kang, J. Wang, H. Wu, I.A. Aksay, J. Liu and Y. Lin, *Biosens. Bioelectron.*, **25**, 901 (2009).
30. H. Xu, H. Dai and G. Chen, *Talanta*, **81**, 334 (2010).
31. W.S. Hummers Jr. and R.E. Offeman, *J. Am. Chem. Soc.*, **80**, 1339 (1958).
32. F. Ricci, F. Arduini, A. Amine, D. Moscone and G. Palleschi, *J. Electroanal. Chem.*, **563**, 229 (2004).