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Binding of Acetylcholinesterase to Multiwall Carbon Nanotube-Cross-Linked Chitosan Composite for Flow-Injection Amperometric Detection of an Organophosphorous Insecticide

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Abstract: A novel method for immobilization of acetylcholinesterase (AChE) by binding covalently to a cross-linked chitosan–multiwall carbon nanotube (MWNT) composite is described. In addition a sensitive, fast, cheap and automatizable flow injection detection of an organophosphorous insecticide was developed. The MWNTs were homogeneously distributed in the chitosan membrane which showed a homogeneous porous structure. The immobilized AChE could catalyze the hydrolysis of acetylthiocholine with a K_M^{app} value of 177 μM to form thiocholine, which was then oxidized to produce detectable signal in a linear range of 1.0–500 μM and fast response. MWNTs could catalyze the electrooxidation of thiocholine, thus increasing detection sensitivity. Based on the inhibition of an organophosphorous insecticide on the enzymatic activity of AChE, using Sulfotep as a model compound, the conditions for the flow-injection detection of the insecticide were optimized. Both biocompatibility of chitosan and inherent conductive properties of MWNTs favored the detection of the insecticide from 1.5 to 80 μM along with good stability and reproducibility. 95% reactivation from inhibited AChE could be regenerated by using 2-pyridinealdoxime methiodide within 15 min for 15 times. The detection of Sulfotep samples exhibited satisfactory results. The proposed flow-injection analysis device can be applied to automated determination and characterization of enzyme inhibitors.

Keywords: acetylcholinesterase · biosensors · chitosan · enzyme inhibitors · nanotubes

Introduction

Organophosphorous (OP) compounds are highly toxic—often causing respiratory paralysis and death—and can irreversibly inhibit acetylcholinesterase (AChE) which is essential for the central nervous system. Therefore, the rapid, sensitive, selective and reliable quantification of these compounds is highly essential at a lower cost. Chromatographical techniques have been extensively used for the sensitive detection of OP insecticides, but they require trained staff, complicated sample pretreatments and are often not suitable for field conditions. In recent years biosensors show remarkable advances for the detection of toxic compounds based on enzymatic reactions.

A variety of enzymes such as organophosphorous hydrolase, alkaline phsosphatase, ascorbate oxidase, tyrosinase and acid phosphatase have been employed in the preparation of pesticide biosensors.[1] Based on the inhibition action of pesticides and insecticides on cholinesterases, AChE and butyrylcholinesterase have been widely used due to the stability and sensitivity of the enzymes.[2,3] This method generally uses either single enzyme[4] or bienzyme (AChE and choline oxidase)[3,5,6] systems by monitoring the electrochemical oxidation of thiocholine or *p*-aminophenol and hydrogen peroxide, respectively. Some of the reports on immobilized AChE-based single enzyme system apply voltages of +400 to +700 mV for the oxidation of thiocholine.[7–10] AChE can be immobilized on electrode surface by using a variety of matrices such as cross-linked polymers,[11] cross-linked bovine serum albumin,[12,13] chitosan[2] and cellulose,[14] different support matrices such as nylon,[5,12,15] controlled pore glass,[16] magnetic particles,[10,17] or the strong affinity linking with concanavalin A[18] and metal ions.[19] To reduce the working potential mediators such as 7,7,8,8-tetracyanoquinodimethane (TCNQ)[19–22] have been deposited on surface to shuttle electrons between the thiocholine formed and the electrode. The implementation of TCNQ can

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reduce the applied potential to +100 mV versus Ag/AgCl.[22] This work reports a novel method for the immobilization of AChE by using glutaraldehyde as a cross-linker to bind covalently AChE to a cross-linked chitosan–multiwall carbon nanotube (MWNT) composite (CMC), leading to a stable thiocholine biosensor. The cross-linked chitosan matrix with free -CHO groups is formed by mixing a chitosan solution with excessive glutaraldehyde. The presence of MWNTs reduces the working potential by catalyzing the electrochemical oxidation of enzymatically formed thiocholine.

Since their discovery, carbon nanotubes (CNTs)[23] have attracted considerable interest, because of their interesting properties,[24] as they have for example emerged as highly conductive (fast electron transfer) nanomaterials. The ability of CNTs to promote the electron transfer of NADH and hydrogen peroxide suggests great protential for the dehydrogenase- and oxidase-based amperometric biosensors.[24,25] These materials have been employed for electrocatalytic oxidation of glucose, cytochrome c, thymine, ascorbic acid and nitric oxide.[26–30] In view of their advantages AChE and choline oxidases have been covalently co-immobilized on MWNTs for the preparation of OP-pesticides biosensors.[6] Other OP biosensors have also been constructed, for example, by adsorption of AChE on MWNTs modified thick film.[4] In order to stabilize the bioactivity of the immobilized enzymes some biocompatible materials such as chitosan, an aminopolysaccharide, have been have been used as support matrices.[2,31] The combination of highly conductive and electrocatalytic behaviors of CNTs with the good biocompatibility of chitosan led to a stable and sensitive glucose biosensor.[31] In this work the proposed biosensor based on the immobilization of AChE on CMC showed good stability and high sensitivity for both thiocholine and Sulfotep, a model compound of OP insecticides, which could be employed for flow-injection analysis of Sulfotep.

The combination of biosensors with flow-injection analysis makes it possible to control all the stages of the reagent additions, measure the enzyme activity, improve the sample throughput and achieve the completely automated determination along with sensitive detection limits, quick response and repeated use of the immobilized enzyme.[9] This technique has used for OP insecticides monitoring by immobilizing AChE on a gold-coated nylon mesh by a self-assembled monolayer of cystamine preadsorbed on the gold surface[15] and a platinum electrode by entrapment in a photocrosslinker polymer[32] with the detection limits of 50 nM (defined as the concentration of inhibitor required to obtained a 5% of inhibition) and in micromolar range, respectively. The inhibited AChE on the gold-coated nylon mesh could be reactivated by immersion in a solution of 2-pyridinealdoxime methiodide (2-PAM) for at least 6 h.[15] Here, a more sensitive, faster and cheap method for flow-injection detection of organophosphorous insecticides as shown in Figure 1 was developed with a detection limit of 1.0 nM at a 10% inhibition for Sulfotep; the inhibited AChE could be regenerated for 15 cycles by using 2-PAM within 15 min.

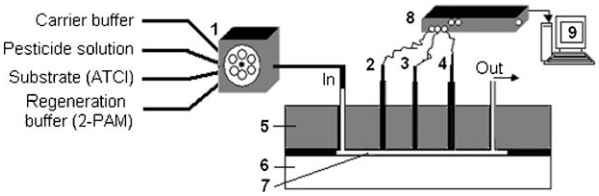

Figure 1. Schematic diagram of the flow-injection detection system. 1) Peristalytic pump, 2) auxiliary electrode, 3) working electrode, 4) reference electrode, 5, 6) upper and bottom portions of flow cell, 7) flow chamber, 8) electrochemical detection instrument, 9) computer.

Results and Discussion

Electrochemical behavior of AChE/CMC/GCE: The cyclic voltammograms of 1.0 mM ATCl at different electrodes are shown in Figure 2. AChE/glutaraldehyde-chitosan/GCE showed an irreversible oxidation peak at +710 mV, while no detectable signal was observed at CMC/GCE. Both

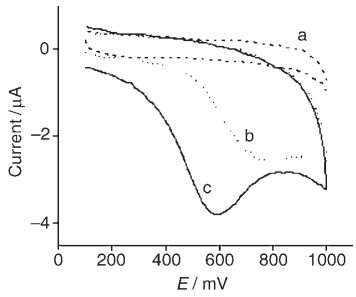

Figure 2. Cyclic voltammograms at 100 mV s^{-1} of 100 mM pH 7.4 PBS containing 0.1 M KCl and 1.0 mM ATCl at a) CMC/GCE, b) AChE/glutaraldehyde-chitosan/GCE and c) AChE/CMC/GCE; GCE: glass carbon electrode.

AChE/glutaraldehyde-chitosan/GCE and AChE/CMC/GCE did not show any detectable response in absence of ATCl (not shown). Obviously, the peak came from the oxidation of hydrolysis product, thiocholine, of acetylthiocholine (ATCl), catalyzed by the immobilized AChE. At AChE/CMC/GCE the oxidation peak increased greatly and shifted negatively to +590 mV due to the presence of MWNTs in the composite, which possessed inherent conductive properties[33] and catalytic behavior towards the oxidation of thiocholine. The electrocatalytic action of MWNTs was also observed for the oxidation of some compounds containing a thiol moiety, such as cysteine and glutathione.[4,34] A high background current was observed when the MWNTs were directly coated on the electrode surface and then covered with cross-linked chitosan membrane, instead of entrapment in cross-linked chitosan membrane. Following experiments were carried out by using AChE/CMC/GCE configuration.

Microscopic characterization of CMC membrane: Figure 3 shows the SEM and TEM images of the different membranes. The cross-linked chitosan membrane showed a ho-

mogenous porous structure (Figure 3a), and MWNTs were almost homogeneously distributed in the membrane to form a fluey structure (Figure 3b), which indicates that both the membrane and the composite on GCE were crack-free. The homogenous porous structure of the cross-linked chitosan membrane favored the entrapment of MWNTs in the membrane. The fluey structure of the composite was beneficial to the loading or covalently linking of AChE to the free -CHO groups in CMC and the approach of substrate and inhibitor to the immobilized enzyme, which increased the sensitivity of the biosensor for detection of both ATCl and OP insecticides.

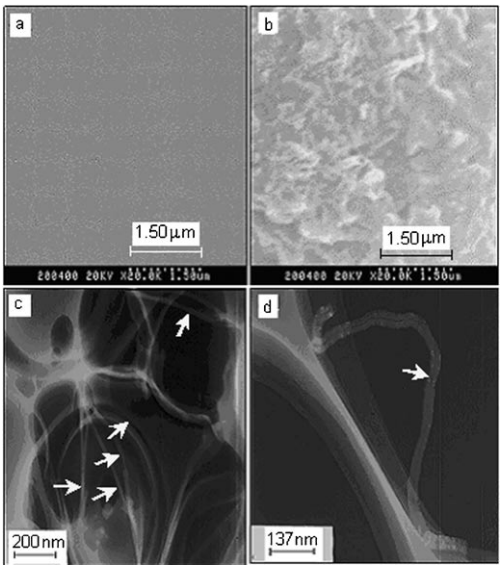

Figure 3. SEM images of a) cross-linked chitosan and b) CMC membranes and TEM images of c) CMC and d) single CNT.

The TEM images indicated that the entrapment of MWNTs in the membrane did not change the structure and morphology of MWNTs. Thus this matrix displayed excellent conductive properties.

Applied potential and buffer pH for amperometric detection: The dependence of the steady-state current on the applied potential is shown in Figure 4. The steady-state current quickly increases with increasing positively applied potential from +300 to +550 mV and reaches a maximum current at +600 mV; this indicates that the more positive applied potential facilitates the oxidation of the thiocholine produced from the enzymatic reaction. The applied potential of +600 mV was close to the

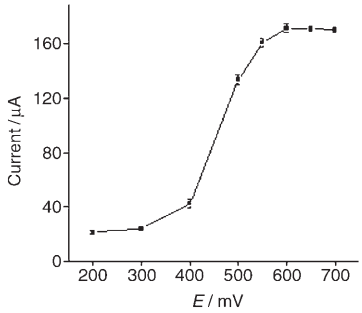

Figure 4. Effect of applied potential on amperometric response of biosensor prepared at the contents of 0.45 (w/v), 0.26 (w/v) and 2.4% (v/v) for chitosan, MWNTs and glutaraldehyde in 100 mM pH 7.4 PBS containing 0.1 M KCl and 0.6 mM ATCl.

anodic peak potential of cyclic voltammogram of thiocholine in the same system at 100 mV s^{-1}. Subsequently, we used +600 mV as the applied potential for following amperometric measurements.

The bioactivity of the immobilized AChE depends on the solution pH.[3] The optimal pH is usually in the range of 7.0 to 7.5.[4,5,8,20] Thus the effect of pH was examined in the range of pH 6 to 8. The results are listed in Table 1, which show that the optimal pH value is 7.4. Thus, pH 7.4 was selected for amperometric detection.

Optimization of enzyme electrode preparation: As shown in Table 1, the amperometric response of obtained biosensor increased, with an increasing content of chitosan in the mixture for preparation of CMC; the response then decreased with a maximum value occurring at the content of 0.45% (w/v). A lower content of chitosan made the membrane more fragile, although a higher content of chitosan decreased the concentration of free -CHO in the membrane. The fragility of the film made the sensor response unstable due to easy detachment of the film during the experiments and washing. Both factors also decreased the loading of enzyme. Furthermore, the membrane formed at high content of chitosan also possibly led to a barrier to enzyme for linkage, substrate for enzymatic hydrolysis and produced

Table 1. Effects of pH of detection solution, and the contents of chitosan, MWNTs, and glutaraldehyde for biosensor preparation on amperometric response of the obtained biosensor to 600 μM ATCl at +600 mV (bold values refer to optimal conditions).

Parameter	Content, response and SD [nA]						
pH of detection solution	6	6.5	7	**7.4**	7.8	8	
response ($n=5$)	139±5	212±6.3	236±6.9	**241±6**	226±7.1	189±8.7	
chitosan (w/v)	0.31	**0.45**	0.67	0.9	1.12		
response ($n=3$)[a]	113±3	**173±3.2**	160±3.5	125±4.5	95±4.1		
MWNTs (w/v)	0.032	0.065	**0.13**	0.2	0.26		
response ($n=3$)[b]	132±5.3	165±4.5	**201±4.6**	184±3.6	172±6.8		
glutaraldehyde (v/v)[c]	0.32	0.65	0.9	**1.26**	1.56	1.85	2.4
response ($n=5$)[d]	166±8.0	196±8.4	227±7.6	**241±6.3**	228±6.4	198±6.9	179±5.1

[a] Obtained at the MWNTs content of 0.26 (w/v) and glutaraldehyde content of 2.4 (v/v). [b] Obtained at the chitosan content of 0.45 (w/v) and glutaraldehyde content of 2.4 (v/v). [c] Volume ratio of 25% glutaraldehyde to 0.5% chitosan solution. [d] Obtained at the chitosan content of 0.45 (w/v) and MWNTs content of 0.13 (w/v).

thiocholine for oxidation, thus decreased the sensor response.

Similarly with an increasing content of MWNTs in the membrane, the current increased and then decreased at the content of 0.13% (w/v). This was possibly due to the decrease of biocompatibility of the formed membrane, which decreased the enzymatic activity of the immobilized enzyme, thus decreasing the response of the enzymatic product. The response also changed with the glutaraldehyde content due to the fact that the amount of enzyme covalently bound to electrode surface was mainly regulated by the amount of aldehyde groups in cross-linked chitosan membrane. The maximum response occurred at the glutaraldehyde content of 1.26% (v/v). Thus, the CMC was prepared with the optimal chitosan, MWNTs and glutaraldehyde contents at 0.45% (w/v), 0.13% (w/v) and 1.26% (v/v), respectively, in following experiments.

Calibration curve for ATCl: Under the optimal conditions the calibration curve of the biosensor was obtained by successive additions of the substrate into a stirred cell. With the increasing concentration of ATCl the amperometric response increased linearly in the range of 1.0–500 μM ($R=$ 0.997) and then reached a plateau value (Figure 5), which

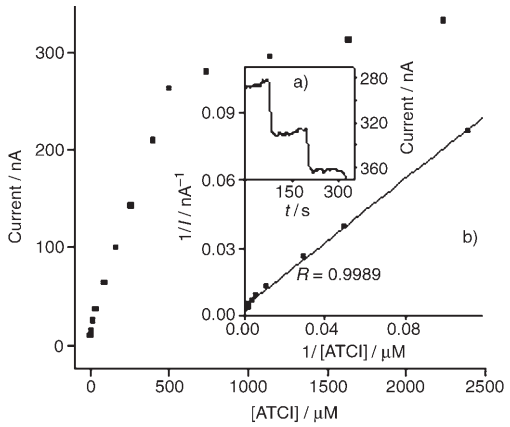

Figure 5. Calibration plot of the biosensor for ATCl. Inset: a) typical steady-state response of the biosensor in 100 mM pH 7.4 PBS containing 0.1 M KCl upon additions of ATCl and b) Lineweaver–Burk plot.

reflects a typical Michaelis–Menten process. The linear slope (sensitivity) was 0.497 nA μM^{-1}. The biosensor achieved 95% of the steady-state current in 15 s after addition of substrate (inset a in Figure 5), indicating a fast response due to the fluey structure of formed CMC. The ATCl concentration of 500 μM was selected for flow-injection detection of the OP insecticide Sulfotep for obtaining the maximum response.

The apparent Michaelis–Menten constant (K_m^{app}) value was calculated to be 177 μM according to the Lineweaver–Burk equation (inset b in Figure 5).[35] The K_m^{app} value was comparable with that of 100 μM for the free enzyme.[18] It was lower than that of 660 μM for AChE adsorbed on CNTs,[4] 220 μM for immobilized AChE by affinity binding with concanavalin A,[18] and 450 μM for AChE immobilized by affinity linkage of metal-chelate Ni–nitrilotriacetic acid,[19] indicating a better affinity of the AChE bound covalently to CMC.

Flow-injection analysis of biosensors for ATCl: The main factor that affects the analytical performance of the biosensor for flow-injection detection of the substrate is its flow rate. This work examined the effect of flow rate on amperometric response in the range of 0.15 to 1.0 mL min^{-1}. As shown in Figure 6 with an increasing flow rate of the ATCl

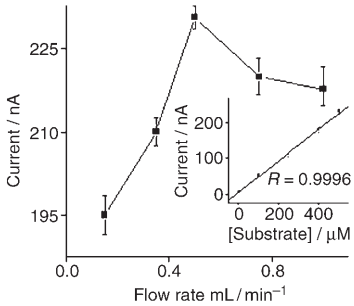

Figure 6. Effect of flow rate of 0.5 mM ATCl solution on current response. Inset: plot of current response versus ATCl concentration at a flow rate of 0.5 mL min^{-1}.

solution the amperometric response increased and then decreased. A maximum value occurred at the flow rate of 0.5 mL min^{-1}, which was chosen as the optimal rate for detection of ATCl. At 0.5 mL min^{-1} the amperometric response was proportional to the concentration of ATCl from 5.0 to 500 μM (inset in Figure 6). The analysis time for one ATCl sample was 2 min.

The reproducibility of the current response for biosensor was examined at different ATCl concentrations. The relative standard deviation was 3.0, 2.8, 3.6 and 2.6% at 5.0, 100, 400 and 500 μM, respectively, for three successive assays. The fabrication reproducibility of three sensors, made at the same electrode independently, showed an acceptable reproducibility with a relative standard deviation of 3.9% for the current determined at 500 μM ATCl.

The stability of the biosensor with an AChE activity of 10 mIU was checked by amperometric detection in a flow cell at regular intervals of time over a period of two months. After the biosensor was stored at 4°C under dry conditions its response was stable in a 10 day-storage period, and then decreased to 50% after one month. When the biosensor was preserved at −20°C it retained 95% of its initial current response after a storage period of 42 days. The response decreased to 72% after two month. Whereas the biosensor based on the direct adsorption of AChE on MWNTs could stabilize for only seven days at 4°C even at high AChE concentration (132 mIU).[4] The enhanced stability of the proposed biosensors was mainly due to the biocompatible nature of chitosan.

Flow-injection detection of Sulfotep: One of the most influencing parameters in pesticide analysis is the incubation time for the inhibition. With increasing incubation period the percentage of the inhibition also increases.[5] This work used a flow-stop method[10] for the inhibition of the enzyme. The Sulfotep sample was injected at 0.5 mL min^{-1} and then stopped in the cell to bind the pesticide with enzyme active site, which led to the inhibition. The incubation time required for the inhibition was checked at different time intervals from 5 to 20 min. With an increasing incubation time the inhibition increased and reached a maximum value after the incubation time of 14 min. Thus, including the analysis time, 14 min was used for flow-stop assay of Sulfotep.

Following the incubation and washing steps the amperometric response of 500 μM ATCl was detected. The response decreased with an increasing Sulfotep concentration (inset Figure 7). At concentrations higher than 90 nM 100% inhibition occurred. The logarithmic plot of I versus Sulfotep concentration ranging from 1.5 to 80 nM showed a good linearity (Figure 7). The detection limit was calculated to be 1.0 nM at 10% inhibition.

The presence of MWNTs greatly enhanced the oxidation

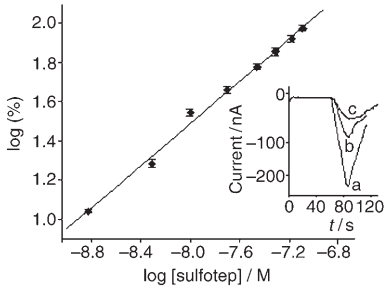

Figure 7. Logarithmic plot of I [%] versus Sulfotep concentration for flow-injection detection. Inset: amperometric responses at a) 0, b) 20 and c) 50 nM Sulfotep.

current of the enzymatically formed product. As shown in Table 2, the amperometric response of the biosensor in the flow-injection analysis of 0.5 mM ATCl concentration was 2.1 times that of the modified electrode without MWNTs. Upon injection of the same concentration of Sulfotep the decrease of amperometric response at the biosensor was also about twice that without MWNTs. The decreasing rate of the amperometric response was faster than that without MWNTs. Thus the MWNTs improved the sensitivity of the biosensor obtained.

Table 2. Ampermetric responses [nA] and relative standard deviations (RSD%, $n=3$) in flow-injection analysis of 500 μM ATCl at +600 mV at different concentrations of Sulfotep.

Concentration of Sulfotep [nM]	Without CNT/chitosan	Chitosan–MWNTs composite
0	110.1 (± 2.9)	231.0 (± 1.2)
1.5	98.5 (± 3.7)	205.6 (± 1.1)
5	92.9 (± 4.1)	187.0 (± 1.1)
10	83.3 (± 3.4)	150.0 (± 1.3)
20	66.2 (± 3.3)	125.4 (± 1.0)
35	54.9 (± 2.2)	95.0 (± 1.0)
50	39.0 (± 2.5)	67.5 (± 1.6)
66	28.7 (± 2.5)	38.8 (± 2.1)
80	16.4 (± 3.5)	15.4 (± 1.6)

The reproducibility for the Sulfotep detection was evaluated first. The relative standard deviations for inhibition detection at the Sulfotep concentrations of 5.0 and 66 nM were 3.3 and 0.9%, respectively, for six independently made sensors; these results show a good reproducibility. To validate the practicability of the proposed method, real samples were tested. Water from Yangzhi river and tap was filtered with a 0.2 μm filter and the pH adjusted to 7.0–9.0. These water samples were then probed with a known concentration of Sulfotep and measured with the proposed method. The results are shown in Table 3, which were in good agreement with the given concentration with an average recovery of 99.5% ($n=18$). This indicates that this method could be used for assay of real samples.

Reactivation of AChE: AChE, which has been irreversibly inhibited by organophosphorous pesticides, can be completely reactivated by using nucleophilic compounds such as 2-PAM.[16,21,31,32] 5.0 mM 2-PAM prepared in PBS was used for the reactivation. After 2-PAM flowed through the cell at 0.5 mL min^{-1} for different periods of time, the reactivation efficiency R was estimated. With increasing reactivation time R increased and reached a constant value after 15 min (Figure 8). After exposing the enzyme to 5, 50 and 80 nM of Sulfotep 97, 95 and 94% of the enzymatic activity could be regained within 15 min, respectively. The time was slightly longer than that reported by Andreescu et al.,[36] who obtained 90% recovery within 5 min using 1 mM 2-PAM. This was possibly due to the less toxicity of parathion methyl and dichlorovos than Sulfotep;[37] though it was still much faster than that of 6 h for the reactivation of AChE inhibited by carbaryl.[15] With the reactivation procedure this biosensor could be repeatedly used for 15 cycles with an acceptable reproducibility.

Table 3. Measurement of Sulfotep in tap and river water using proposed biosensor ($n=3$).

concentration of Sulfotep spiked [nM]	1.5	27	50
detected concentration in tap water [nM]	1.42(±0.04)	27.4(±0.1)	49.2(±0.15)
recovery [%]	95	101	98
detected concentration in river water [nM]	1.53(±0.05)	26.8(±0.18)	50.8(±0.1)
recovery [%]	102	99	102

Conclusion

This work proposes a simple and efficient method for immobilization of acetylcholinesterase on an electrode surface and

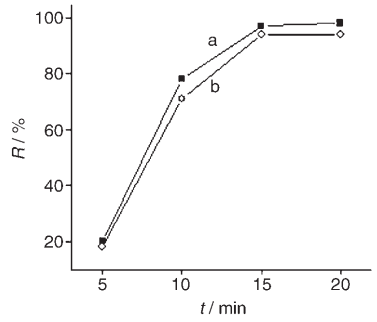

Figure 8. Effect of flow time on reactivation of inhibited AChE at a) 5 and b) 80 nM Sulfotep.

develops a sensitive, fast, cheap and automatizable method for the detection of both acetylthiocholine and organophosphorous insecticides. MWNTs can catalyze the oxidation of thiocholine formed from the interface enzymatic hydrolysis reaction. The cross-linked chitosan–multiwall carbon nanotube composite constructed for immobilization of AChE shows a fluey structure with a homogeneous distribution of MWNTs, which favors loading and covalently linking of AChE to the composite and the attachment of the substrate and inhibitor to the immobilized enzyme. This in turn improves the affinity of the immobilized enzyme, increases the sensitivity and facilitates the amperometric response of the biosensor for ATCl and OP insecticides. The proposed biosensors possess a good electrode-to-electrode reproducibility and stability. The developed flow-injection detection method shows low detection limit, good precision and accuracy. The inhibited AChE can be completely reactivated in a relatively short time and repeatedly used for 15 cycles. Furthermore the proposed flow-injection analysis device can be easily automatized and miniaturized and be extended for the detection of other toxic compounds against to AChE and characterization of enzyme inhibitors.

Experimental Section

Materials: Acetylcholinesterase (EC 3.1.1.7, 1540 IU mg^{-1} from electric eel), acetylthiocholine chloride (ATCl), 2-pyridinealdoxime methiodide, chitosan (85% deacetylation), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and glutaraldehyde (25%) were purchased from Sigma-Aldrich (St. Louis, USA) and used as received. Multiwall carbon nanotubes (MWNTs, length 1–2 μm, external diameter 10–20 nm, and surface area 40–300 m^2 g^{-1}) were procured from Shenzhen Nanotech Port Co. (China). Sulfotep ($C_8H_{20}O_5P_2S_2$, *O,O,O′O′*-tetra-ethyldithiopyrophosphate, 99.0% pure, M_W 322.31) was obtained from Sigma-Aldrich (Laborchemikalien GmbH, Seelze). All other reagents used were of analytical reagent grade.

Preparation of biosensors: Prior to immobilization of the enzyme a glassy carbon electrode (GCE, 3.0 mm diameter) was polished with 0.3 and 0.05 mm alumina slurry (Behler), respectively, and rinsed thoroughly with double distilled water. It was then successively ultrasonicated in ethanol and double distilled water. 5.0 IU mL^{-1} AChE solution was prepared in 0.1 M phosphate buffer solution (pH 7.4, PBS). MWNTs were sonicated in water bath for 20 min and ethanol bath for another 30 m in, and dried at 80 °C for 30 min. Chitosan (0.5% w/v) solution was prepared by dissolving 50 mg chitosan in 2 M acetic acid and then diluting to 10 mL with water. Its pH was adjusted to 4.0–6.0 by using a concentrated NaOH solution.

An aliquot of 25% glutaraldehyde was mixed thoroughly with clear solution of 0.5% chitosan with stirring for 10 min to form cross-linked chitosan with free -CHO groups. Then 1.3 mg MWNTs were added to 1.0 mL of the mixture and sonicated thoroughly until a homogeneous suspension was obtained. 3.0 μL of the homogeneous suspension was coated on a pretreated GCE and allowed for reaction at 25 °C for 4 h to form a membrane of cross-linked chitosan–MWNT composite (CMC). The modified electrode (CMC/GCE) was washed thoroughly with double distilled water to remove the excess glutaraldehyde and coated with 2.0 μL AChE solution (10 mIU), which was incubated at 25 °C for 30 min for covalent linkage of AChE to CMC *via* the free -CHO groups of glutaraldehyde coupled to chitosan molecules. After evaporation of water it was washed with PBS for the removal of unbound AChE to obtain a biosensor (AChE/CMC/GCE). This biosensor was stored at −20 °C when not in use.

Apparatus and electrochemical measurements: Electrochemical measurements were carried out on BAS 100B (BAS Inc., USA) with a three-electrode system comprising a platinum wire as auxiliary, a Ag/AgCl as reference and an enzyme modified GCE as working electrodes. 0.1 M pH 7.4 PBS containing 0.1 μM KCl was used as supporting electrolyte. Amperometric experiments were performed by applying a potential of +600 mV vs Ag/AgCl to a stirring or flow cell at room temperature. After a stable base line was obtained ATCl substrate was added to the detection system. The flow system was equipped with three electrodes and connected to BAS 100B. The total volume of the flow cell including tubing was 500 μL. Sample injection was controlled manually at a flow rate of 0.5 mL min^{-1}. PBS was used as carrier buffer. The flow rate was controlled with a BT00–100 M peristaltic pump (Baoding, China).

Scanning electron microscopic (SEM) and transmission electron microscopic (TEM) images were recorded on a Hitachi X-650 SEM and Jeol-Jem 200CX TEM (Japan), respectively.

Inhibition measurements: The degree of irreversible inhibition (% *I*) of the OP insecticide on the enzymatic activity of immobilized AChE was measured as a relative decrease of the amperometric response after a contact of the biosensor with Sulfotep. The amperometric response I_0 of 500 μM ATCl (250 μL, flow rate 0.5 mL min^{-1}) was first measured. After the electrode was washed with carrier buffer at 1.0 mL min^{-1} for 2 min, 0.5 mL Sulfotep solution was injected and stopped in the cell for 14 min followed by washing with the carrier buffer at 1.0 mL min^{-1} for 2 min. Again the response of 500 μM ATCl was measured as I_t. The inhibition (I%) was calculated with Equation (1) and reported as average of three measurements.

$$I/\% = (I_0 - I_t)/I_0 \times 100 \quad (1)$$

Enzyme reactivation: After the enzyme electrode was exposed to the OP insecticide, it was washed with carrier buffer at 1.0 mL min^{-1} for 2 min and reactivated with 5.0 mM 2-PAM (prepared in PBS) at a flow rate of 0.5 mL min^{-1} for 15 min. After the cell was washed with carrier buffer at 1.0 mL min^{-1} for 2 min, enzyme activity was monitored with the response I_r of 500 mM ATCl. The reactivation efficiency (% *R*) was estimated with Equation (2).

$$R/\% = (I_r - I_t)/(I_0 - I_t) \times 100 \quad (2)$$

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