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Amperometric biosensor based on a site-specific immobilization of acetylcholinesterase via affinity bonds on a nanostructured polymer membrane with integrated multiwall carbon nanotubes

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ABSTRACT

Acetylcholinesterase (AChE) was immobilized on chemically modified poly-(acrylonitrile-methyl-methacrylate-sodium vinylsulfonate) membranes in accordance with three different methods, the first of which involved random enzyme immobilization via glutaraldehyde, the second one—site-specific enzyme immobilization via glutaraldehyde and Concanavalin A (Con A) and the third method—modified site-specific enzyme immobilization via glutaraldehyde in the presence of a mixture of multiwall carbon nanotubes and albumin (MWCNs + BSA), glutaraldehyde and Con A. Preliminary tests for the activity of immobilized AChE were carried out using these three methods. The third method was selected as the most efficient one for the immobilization of AChE and the prepared enzyme carriers were used for the construction of amperometric biosensors for the detection of acetylthiocholine (ATCh).

A five level three factorial central composite design was chosen to determine the optimal conditions for the enzyme immobilization with three critical variables: concentration of enzyme, Concanavalin A and MWCNs. The design illustrated that the optimum values of the factors influencing the amperometric current were C_E: 70 U mL^{-1}; $C_{Con\,A}$: 1.5 mg mL^{-1} and C_{MWCN}: 11 mg mL^{-1}, with an amperometric current 0.418 μA. The basic amperometric characteristics of the constructed biosensor were investigated. A calibration plot was obtained for a series of ATCh concentrations ranging from 5 to 400 μM. A linear interval was detected along the calibration curve from 5 to 200 μM. The correlation coefficient for this concentration range was 0.995. The biosensor sensitivity was calculated to be 0.065 μA μM^{-1} cm^{-2}. The detection limit with regard to ATCh was calculated to be 0.34 μM. The potential application of the biosensor for detection and quantification of organophosphate pesticides was investigated as well. It was tested against sample solutions of Paraoxon. The biosensor detection limit was determined to be 1.39×10^{-12} g L^{-1} of Paraoxon, as well as the interval (10^{-11} to 10^{-8} g L^{-1}) within which the biosensor response was linearly dependant on the Paraoxon concentration. Finally the storage stability of the enzyme carrier was traced for a period of 120 days. After 30-day storage the sensor retained 76% of its initial current response, after 60 days—68% and after 120 days—61%.

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1. Introduction

Many efforts have been invested in the determination of pesticides because of their highly acute toxicity. In fact, they are inhibitors of the cholinesterase enzymes, involved in muscle contraction and impulse transmission in nervous system. The main risks are associated with both professional exposure (industrial production, agricultural use) and exposure through our daily routines (domestic use, food and fresh water contamination).

It is of great importance to develop rapid, sensitive and reproducible high-throughput screening systems for pesticides. The electrochemical biosensors completely respond to these requirements.

Enzyme immobilization is one of the most important steps involved in the biosensor design. The usual immobilization methods include direct physical adsorption onto a solid support [1], cross-linking [2], encapsulation into a hydrogel or a sol–gel film [3,4] and covalent binding [5]. In these traditional immobilization techniques the enzyme molecules are randomly immobilized either directly on the carrier or via a spacer arm, often through the amino functionality of lysine residues on the protein. Since proteins often contain multiple lysine residues spread all over the molecular surface, different orientations of the enzyme with respect to the carrier occur, some completely blocking the active-site from interaction with substrate. Other significant disadvantages of random enzyme immobilization that can be pointed out are multiple-point bind-

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ing [6], and enzyme denaturation [7–12]. In addition, high enzyme loading is not possible with random immobilization [13].

Intensive efforts during recent years have been focused on the oriented and site-specific immobilization of enzymes, which has become crucial for the rational design of biosensors. A recent trend in this direction is the creation of (bio)affinity bonds between an activated support and a specific group of the protein sequence. This can be achieved by using affinity interactions between functional groups (Concanavalin A (lectins), (strept)avidin, sugars and metal chelates) of an activated electrode surface and an affinity tag (carbohydrate residues, biotin, histidine, cysteine) present or genetically engineered at a specific location in the protein sequence which does not affect the activity or the folding of the protein. This method provides a basis for controlled and oriented immobilization of the enzyme on different supports, opening the way for new approaches to enzyme immobilization. Since genetically engineered enzymes are much more expensive to be used as the "sensing part" of a biosensor, the bioaffinity immobilization method, involving Concanavalin A (Con A) is preferred [13,14–18]. However none of these procedures have been utilized for the manufacturing of an enzyme biosensor with a replaceable membrane from acrylonitrile (AN) copolymer.

Usually all of these methods rely on enzyme immobilization directly onto the electrode surface. Although the proximity between the enzyme molecules and the electroactive surface provides for the swiftness of the biosensor response, it cannot overcome the biofouling of the electrode surface, which would eventually lead to the deactivation of the biosensor or at least to worsening of the electrochemical response. By using a replaceable polymer membrane from AN copolymer as a support for the enzyme immobilization, the necessity of constantly cleaning the electrode surface after each immobilization procedure, could be discarded. After an adequate chemical modification such a polymer membrane could provide various functional groups, thus allowing the selection of the most appropriate enzyme immobilization protocol. Furthermore, a polymer membrane placed on the electrode surface would protect it from any high-molecular contaminants present in the investigated sample, allowing only the low-molecular substrate molecules to reach for the enzyme active centers. And last, but not least, modified AN copolymer membranes would provide a favorable microenvironment for the enzyme molecules, that could prolong the enzyme storage life, not to mention the convenience of storing only the membranes, and not whole electrodes.

The main disadvantage of AN copolymer membranes comes from their hydrophobic and non-conducting properties. Since the strength and the swiftness of the biosensor response is of a crucial significance, the referred disadvantage could be overcome by using carbon nanotubes (CNs) as mediators of the electron transfer from the enzyme molecules to the electrode surface. Their unique electronic properties suggest that CNs have the ability to promote the electron transfer reactions of biomolecules in electrochemistry [13]. Their mechanical properties, high-aspect ratio, electrical conductivity and chemical stability make CNs perfect for a wide range of applications that include fabrication of electrochemical sensors and biosensors [19].

The present work is focused on the construction of an amperometric biosensor on the basis of site-specifically immobilized AChE on a hybrid polymer membrane with integrated multiwall carbon nanotubes (MWCN). The combination of the highly conductive and electrocatalytic behavior of MWCNs with the controlled site-specific enzyme immobilization should result in a stable and sensitive sensor towards ATCh and Paraoxon (as an inhibitor of AChE). The construction of such biosensors with replaceable hybrid polymer membrane will provide real-time and minimum cost detection of organophosphorus pesticides in situ.

2. Materials and methods

2.1. Materials

Acrylonitrile-methylmethacrylate-sodium vinylsulfonate membranes (PAN) were prepared without support according to a methodology described in [20]. The ternary copolymer (acrylonitrile—91.3%; methylmethacrylate—7.3%, sodium vinylsulfonate—1.4%) was a product of Lukoil Neftochim, Bourgas. Ultrafiltration membranes of acrylonitrile copolymer were measured to be 4 μm thick and could retain substances with molecular weight higher than 60 000 Da.

MWCNs (diameter 2–6 nm; length 0.1–10 μm, >90% purity) and pyridine-2-aldoxime methochloride (PAM) were purchased from Sigma–Aldrich (St. Louis, USA). Acetylthiocholine chloride (ATCh) and AChE (Type C3389, 500 U mg^{-1} from electric eel) were purchased from Sigma–Aldrich and used as received. Bovine serum albumin (BSA), glutaraldehyde (GA) and Concanavalin A (Con A) were also purchased from Sigma–Aldrich. Phosphate buffer solution (PBS, pH 7.6) and other reagents were of analytical reagent grade. All solutions were prepared with double distilled water.

2.2. Instruments

Electrochemical measurements were performed on a PalmSens Electrochemical Instrument (Palm Instruments BV, The Netherlands) with a conventional three-electrode system comprising of platinum wire as a counter electrode, Ag/AgCl reference electrode and a platinum working electrode with attached AChE-immobilized membrane.

2.3. Chemical modification of PAN membranes

Each piece of PAN membrane was immersed in 10% NaOH for 20 min at 40 °C. The membrane unit was then washed with distilled water and placed in 1 M HCl at room temperature for 120 min. The color of the hydrolyzed yellowish red PAN membrane turned into white. Then the modified PAN membranes were immersed in a 10% solution of ethylene-diamine for 1 h at room temperature in order to react with the superficial carboxyl groups via one of the terminal NH_2 groups, leaving the other NH_2 group free.

2.4. Chemical modification of MWCNs

Prior to use, MWCNs were treated with concentrated nitric acid in order to introduce carboxylic groups according to report [21]. The carboxylated nanotubes (MWCN-COOH) were carefully washed with distilled water. Then the MWCN-COOH were treated with a 10% solution of ethylene-diamine for 1 h at room temperature to introduce amino groups, followed by washing with distilled water again. Finally, 0.1 g of aminated nanotubes (MWCN-NH_2) was added to 10 mL of 10% BSA solution. The mixture was ultrasonically dispersed to give a black suspension.

2.5. AChE immobilization onto chemically modified membranes

The enzyme immobilization was carried out by three different methods, described below and represented in Table 1.

2.5.1. Method 1—AChE immobilization using glutaraldehyde

The chemically modified membrane was immersed in 10% glutaraldehyde for 1 h at room temperature. After carefully washing with distilled water the membrane was treated in three different ways:

Method 1a—the activated membrane was immersed in an enzyme solution (70 U mL^{-1}) in PBS (pH 7.6) for 24 h at 4 °C. Membrane 1 was prepared according to this immobilization procedure.

Table 1
Enzyme activity of AChE via different immobilization methods.

No. of membranes (Mb)	Methods	Steps of immobilization methods	Enzyme activity (μmol min^{-1} cm^{-2})
1	Method 1a	Mb + GA + AChE	3.28
2	Method 1b	Mb + GA + EDA + AChE	3.56
3	Method 1c	Mb + GA + BSA + AChE	0.05
4	Method 2a	Mb + GA + ConA + AChE	18.40
5	Method 2b	Mb + GA + ConA + BSA + AChE	19.73
6	Method 2c	Mb + GA + ConA + (MWCN-NH_2 + BSA) + AChE	20.04
7	Method 3	Mb + GA + (MWCN-NH_2 + BSA) + GA + ConA + BSA + AChE	26.52

Method 1b—the activated membrane was immersed in 10% ethylene-diamine solution, then washed and immersed in an enzyme solution (70 U mL^{-1}) in PBS (pH 7.6) for 24 h at 4 °C. Membrane 2 was prepared according to this immobilization procedure.

Method 1c—the activated membrane was immersed in 10% BSA solution, then washed and immersed in an enzyme solution (70 U mL^{-1}) in PBS (pH 7.6) for 24 h at 4 °C. Membrane 3 was prepared according to this immobilization procedure.

2.5.2. Method 2—AChE immobilization using glutaraldehyde and Con A

The procedure included the activation of the amino groups with 10% glutaraldehyde, as described in Section 2.5.1, followed by cross-linking of Con A on the activated support. This was achieved by immersing the activated membrane in a Con A solution (1.5 mg mL^{-1}) for 1 h at room temperature. After carefully washing with distilled water the membrane was treated in three different ways:

Method 2a—the membrane was immersed in an enzyme solution (70 U mL^{-1}) in PBS (pH 7.6) for 24 h at 4 °C. Membrane 4 was prepared according to this immobilization procedure.

Method 2b—The next step was to block the non-reacted glutaraldehyde and the adsorption sites by incubation in a BSA solution (10%, in PBS). After being carefully washed with distilled water the membrane was immersed in an enzyme solution (70 U mL^{-1}) in PBS (pH 7.6) for 24 h at 4 °C. Membrane 5 was prepared according to this immobilization procedure.

Method 2c—The next step was to block the non-reacted aldehyde groups and the adsorption sites by incubating the membrane in a mixture of modified MWCN and BSA (6 mg MWCN-NH_2 in 1 mL 10% BSA solution). Finally, the immobilization of the enzyme was performed by immersing the membrane in enzyme solution (70 U mL^{-1}) in PBS (pH 7.6) for 24 h at 4 °C. Membrane 6 was prepared according to this immobilization procedure.

The immobilization technique for methods 2b and 2c was presented in Fig. 1A and B, respectively.

2.5.3. Method 3—AChE immobilization using glutaraldehyde, mixture of MWCN + BSA and Con A

This immobilization technique involved seven steps (as presented in Table 1): this procedure included in the activation of the amino groups with glutaraldehyde (10%, PBS), described in Section 2.5.1. The next step was to immerse the membrane in a mixture of modified MWCN and BSA (from 2 to 10 mg MWCN-NH_2 in 1 mL 10% BSA solution). Then the membrane was immersed in a glutaraldehyde solution (10%, PBS) once more. After being carefully washed with distilled water the activated membrane was immersed in Con A solution, then the non-reacted aldehyde groups and the adsorption sites were blocked by incubating the membrane in a solution of BSA (10%, PBS). This was followed by immersing the membrane in an enzyme solution (10–130 U mL^{-1}) in PBS (pH 7.6), as described in Section 2.5.2. Membrane 7 was prepared according to this immobilization procedure. The immobilization technique for methods 3 was presented in Fig. 1C.

2.6. Enzyme activity determination

The AChE activity was determined according to the Ellman method [22]. The reaction mixture contained 100 μL of 2 mM ATChI as substrate and 100 μL of 2 mM DTNB as chromogen added in 1.8 mL of 0.1 M phosphate buffer, an appropriate amount of the enzyme. This mixture was kept for 10 min at 30 °C for free AChE and 35 °C for immobilized AChE. The enzyme reaction product interacted with DTNB and the intensity of the obtained yellow solution, was determined spectrophotometrically at 412 nm.

2.7. Electrochemical measurements of the nanostructured AChE membranes

Each enzyme carrier was attached to a platinum working electrode, using a plastic ring, with the non-selective side (contained pores larger than pores of the selective layer) of the membranes facing the platinum surface of the electrode, which was then placed in an electrochemical cell containing 40 mL 0.1 M PBS solution under stirring at 38 °C. A potential of 0.8 V was applied to the working electrode and the electrochemical current was awaited

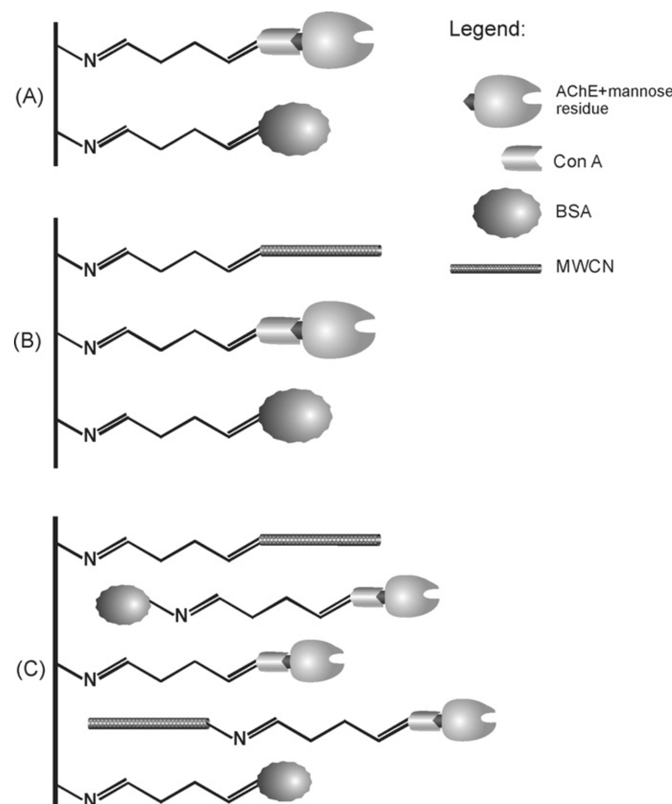

Fig. 1. Immobilization methods of enzyme onto modified polymer membranes: (A) immobilization via glutaraldehyde (method 1), (B) immobilization via glutaraldehyde and Con A (method 2), (C) immobilization via glutaraldehyde, mixture of MWCN + BSA, glutaraldehyde and Con A (method 3).

to become stationary. Then a series of 100 μL from a 2 mM solution of ATCh were added to the cell and the resulting current was recorded.

2.8. Factor design

Based on the results obtained by the classical method, three critical variables were selected for the optimization of the biosensor by RSM. A five level four factorial CCRD was employed in this study for the factors A: C_E, B: C_{MWCN}, C: $C_{Con\,A}$ with an alpha value of ±1.414. The relationship between the variation of the response, Y_c—amperometric current I (μA) and the variation of factors A, B and C, were represented by a second-order mathematical model using the following equation,

$$\begin{aligned} Y_c = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \text{ (Intercept and main effects)} \\ & + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 \text{ (Interactions)} \\ & + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_2 X_3 \text{ (Quadratic effects)} \end{aligned}$$

where Y_c was the response calculated by the model and X_1, X_2 and X_3 were the coded variables corresponding to factors A, B and C, respectively. Coding was required, since the factors were expressed in different units and β_0 represented the regression coefficient at the center. β_1, β_{11} and β_{12} were coefficients estimated by the model, which represented the linear quadratic and interactive effects of X_1, X_2 and X_3 factors on the response, respectively. The treatment combinations of CCRD were allocated in three blocks and each block had ten runs. The first two blocks each had eight factorial points and two center points. The last block had eight axial points and two center points. Thus, in total, the experimental set up consisted of thirty trials and the value of the dependent response was the mean of triplicates.

2.9. Data analysis

The data from the experiments performed were analyzed and interpreted using Design-Expert software package (Version 7.0.3, Stat – Ease Inc., Minneapolis, USA). Three main analytical steps: analysis of variance (ANOVA), a regression analysis and the plotting of response surface were performed to establish an optimum condition for biosensor preparation.

2.10. Inhibition measurements

The degree of inhibition (I%) of the organophosphorus insecticide on the enzymatic activity of immobilized AChE was measured as a relative decrease of the amperometric response after a contact of the enzyme carrier with Paraoxon. The initial amperometric response I_0 of 100 μL 50 mM ATCh was first measured. After washing the membrane with 0.1 M PBS (pH 7.6), it was incubated in a Paraoxon solution with a given concentration for 20 min. This was again followed by washing the membrane with PBS and measuring the response to 100 μL 50 mM ATCh as I_t. The inhibition I% was calculated according to Eq. (1):

$$I\% = \frac{I_0 - I_t}{I_0} \times 100 \quad (1)$$

2.11. Reactivation of the immobilized AChE

After the inhibition measurements each membrane was reactivated in a 5 mM solution of pyridine-2-aldoxime methochloride (PAM) in PBS for 30 min. This was followed by a thorough washing of the membrane with PBS solution.

2.12. SEM analysis of the nanostructured membranes

SEM studies were carried out on gold sputtered membrane samples using JEOL JSM-5510 Scanning Electron Microscope. The samples for SEM studies were prepared by soaking the membranes in isopropanol overnight, then in hexane for 10 h and subsequent vacuum drying at 40 °C for three days. The cross-sections of samples were prepared by fracturing in liquid nitrogen.

2.13. Treatment of experimental data

Each experimental point in the figures for biosensor performing is the average of 6 independent experiments carried out under the same conditions. The experimental error never exceeded 4.4%.

3. Results and discussion

3.1. Effect of AChE immobilization technique on the enzyme activity

Preliminary tests for the immobilization of AChE were carried out using three different methods with some variants (Table 1).

The experiments were designed so that to provide initial information on which of those seven immobilization techniques would yield maximum enzyme activity and would be the most appropriate for the construction of a biosensor. During this stage qualitative estimations were done by spectrophotometric measurements of the activity of the enzyme molecules, attached to PAN membranes functionalized via different methods.

First of all a comparison was made between the enzyme activities of randomly AChE immobilized via glutaraldehyde alone (Table 1, Membranes 1 and 2) and site-specifically AChE immobilized via glutaraldehyde and Con A (Table 1, Membranes 4, 5, 6 and 7). As can be seen from Table 1, the enzyme activity of AChE immobilized via GA + Con A is substantially greater than the activity of AChE immobilized solely via GA. Site-specifically immobilized enzyme arrays have a much higher activity per mg bound enzyme relative to randomly immobilized enzyme. Thus, the activity randomly immobilized enzyme saturates at a low level of immobilized enzyme compared to site-specifically immobilized enzyme. The site-specific orientation provides the clear advantages over randomly immobilized enzymes higher activity, higher loading and an active-site structure similar to that of soluble enzyme.

Two variants of method 1 were applied when immobilizing AChE via GA—direct covalent bonding (Membrane 1) and using a spacer molecule—ethylene-diamine (Membrane 2). The results revealed that the enzyme activities of the prepared membranes were very similar. The first challenge in the immobilization protocol was to prevent and/or to eliminate the adsorption of the enzyme onto the surface of the modified membranes. In order to prevent the enzyme adsorption the glutaraldehyde-activated membrane (Membrane 3) was immersed in a 10% solution of BSA. The role of BSA was to block the adsorption sites and to react with the cross-linking agent. Then the membrane was immersed in an enzyme solution. The enzyme activity of this membrane (Membrane 3) is very low in comparison with AChE chemically immobilized on Membranes 1 and 2. This could be explained with BSA efficiently blocking the adsorption sites on the support.

During the enzyme immobilization via GA + Con A, several variants of method 2 were used as well: method 2a, Membrane 4—without blocking the free aldehyde groups (from GA) remaining after the covalent bonding of Con A; methods 2b and 2c, Membranes 5 and 6—with blocking of the residual aldehyde groups. The group blocking was realized in two ways—via BSA (Membrane 5) and via a mixture of BSA and MWCN-NH_2 (Membrane 6). The objective of the included aminated carbon nanotubes in Membrane 6 was to block

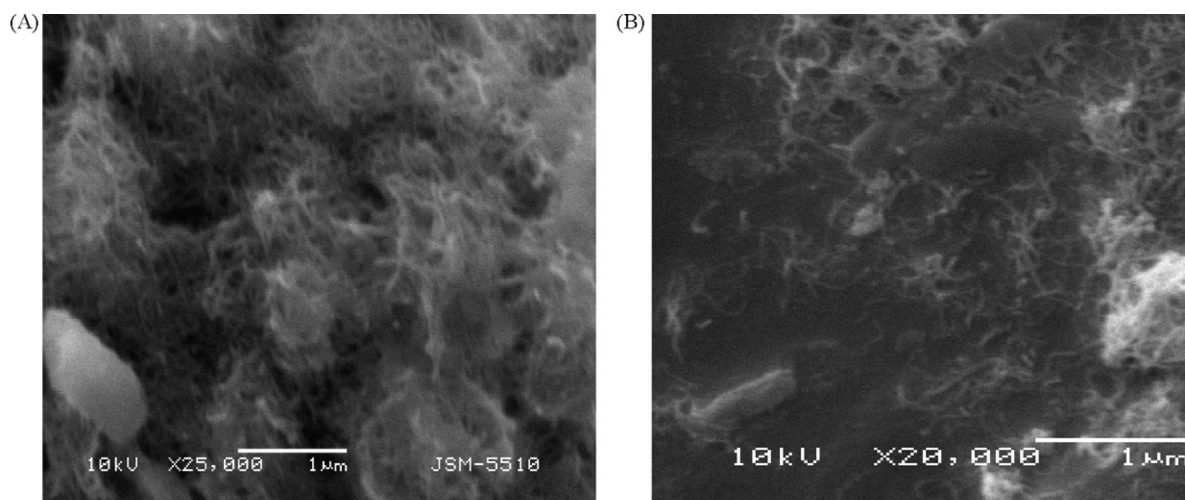

Fig. 2. SEM photographs of the membranes prepared in accordance with the two different immobilization techniques: (A) method 3: Mb + GA + (MWCN-NH_2 + BSA) + GA + ConA + BSA + AChE; (B) method 2c: Mb + GA + ConA + (MWCN-NH_2 + BSA) + AChE.

some of the residual aldehyde groups as well and to enhance the electrical conductivity of the polymer membrane, which is essential for the biosensor performance. The addition of BSA provided a favorable microenvironment for the enzyme molecules.

The method 3 was used for the preparation of Membrane 7. It was designed so that a greater amount of MWCN-NH_2 and enzyme to be immobilized on the polymer carrier. This was achieved by immersing the GA-activated membrane in a mixture of BSA and MWCN-NH_2 before adding Con A. Then the following steps were used for the enzyme immobilization: covalent bonding via glutaraldehyde again; bonding of Con A; blocking free aldehyde groups with BSA and finally—the immobilization of AChE. Thus, a significant increase of the amount of bound BSA and MWCN-NH_2 was observed on Membrane 7 (method 3) in comparison with Membrane 6 (method 2c) which was proved by SEM analysis of the two membranes (Fig. 2). As can be seen from Fig. 2A Membrane 7 contains a higher amount of MWCN-NH_2 than Membrane 6 (Fig. 2B). The amount of bound protein by method 3 was achieved to be twice as high (0.878 mg cm^{-2}) as the amount of bound protein achieved by the other methods (0.33–0.45 mg cm^{-2}).

When comparing all the immobilization methods, it was found that the highest enzyme activity was exerted by AChE immobilized according to method 3 (Membrane 7), followed by method 2c (Membrane 6) and method 2b (Membrane 5). As can be seen from the results, the blocking of the residual free aldehyde groups reduced significantly the random immobilization, thus emphasizing the specific bonding between Con A and AChE and yielding a substantially higher activity. The addition of preliminary aminated MWCNs, before Con A (Membrane 7), resulted in an enhanced AChE activity, higher enzyme loading capacity and an improved electrical conductivity of the membrane. On the basis of the results, method 3 (Membrane 7) was selected as the most efficient method for the immobilization of AChE and the rest of the experimental work was conducted using this method.

3.2. Optimization of the AChE biosensor preparation, using glutaraldehyde and a mixture of MWCN + BSA and Con A

On the basis of the obtained results, three critical variables were selected for the biosensor optimization by RSM. The experimental domain depicting the levels for each selected factor is given in Table 2. The experiments were performed to obtain a quadratic model with three independent variables as shown in Table 2. Since the predicted values obtained from the model fitting technique using Design-Expert v.7.0.3 were found to correlate to the observed values, the quadratic polynomial model was seen to be highly significant to represent the actual relationship between the response (amperometric current) and the significant variables.

Table 2
Experimental range of variables for the central composite design.

Variable	Range of variables		
	Low (−1)	Mid (0)	High (+1)
C_E (U mL^{-1})	10	70	130
$C_{Con\ A}$ (mg mL^{-1})	0.5	1.5	2.5
C_{MWCNs} (mg mL^{-1})	1	6	11

The variation of the amperometric current from 0.224 to 0.418 μA indicated that interactions among the factors played a more significant role than the effect of individual factors alone.

$$
\begin{aligned}
I = {} & 0.0032345C_E + 0.0187259C_{MWCN} + 0.1735944C_{Con\ A} \\
& - 1.272 \times 10^{-5}{C_E}^2 - 0.000277{C_{MWCN}}^2 - 0.051191{C_{Con\ A}}^2 \\
& - 0.00012C_E C_{MWCN} - 0.000203C_E C_{Con\ A} \\
& - 0.000578C_{MWCN} C_{Con\ A}
\end{aligned}
$$

The ANOVA for quadratic regression model (Table 3) showed that the model was highly significant with an F value of 62.77, as is evident from Fisher's F-test along with a very low probability value (P model > F = 0.0001). The determination coefficient (R^2) of the model was 0.984 meaning that 98.4% of the variability in

Table 3
ANOVA with the data in Table 2.

Source	DF	Sum of squares	F ratio	Prob > F	Mean square
Model	9	1.9720	62.77	<0.0001	0.2191
C_E	1	0.03913951	11.2128	0.0036	
C_{MWCN}	1	0.00904878	2.5923	0.1248	
$C_{Con\ A}$	1	0.03650989	10.4595	0.0046	
${C_E}^2$	1	0.01330040	3.8103	0.0667	
${C_{MWCN}}^2$	1	0.00030822	0.0883	0.7698	
${C_{Con\ A}}^2$	1	0.02206924	6.3225	0.0216	
C_E C_{MWCN}	1	0.01732563	4.9635	0.0389	
C_E $C_{Con\ A}$	1	0.00210780	0.6039	0.4472	
C_{MWCN} $C_{Con\ A}$	1	0.00012045	0.0345	0.8547	
C total	27	2.0348686			
Error	18	0.0628	0.00349		
R^2 = 0.984					

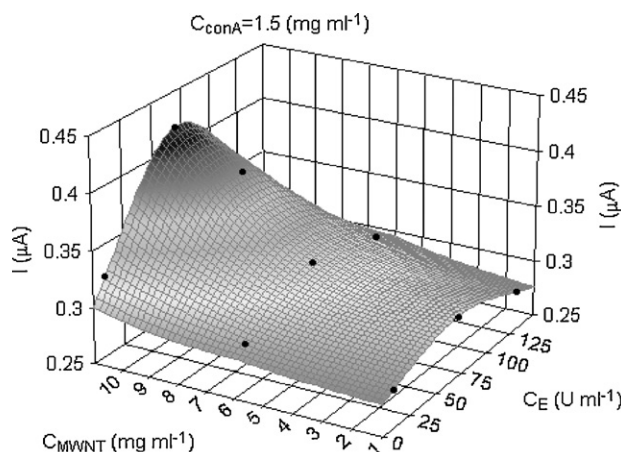

Fig. 3. Response surface plot showing the effect of C_{MWCN} and C_E and their mutual effect on the amperometric current at $C_{Con\,A}$ = 1.5 mg mL^{-1}.

the response could be accounted by the model. The analysis of the design showed a high degree of fitting between predicted and experimental data, which indicated that the model was suitable to represent the real relationship among the selected factors. The insignificant lack of a fit test also indicated that the model was suitable to represent the experimental data and the final predictive equation was as follows:

It was clear that the three linear coefficients and three quadratic coefficients were highly significant (P < 0.05) from the model and among the three variables, C_E and $C_{Con\,A}$ were the most significant for the amperometric current, while C_{MWCN} had exerted a less significant effect on the amperometric current. The high value of 11.21, in addition to a least probability value (<0.0036) for C_E, and value of 10.46 in addition to a least probability value (<0.0046) for $C_{Con\,A}$, indicated that C_E and $C_{Con\,A}$ variables played a very prominent role in the amperometric current generation, when compared to C_{MWCN} variable.

The cumulative effect of C_{MWCN} and C_E concentrations on the biosensor response at Con A concentration of 1.5 mg mL^{-1} is shown in the response surface plot of Fig. 3. The amperometric current was 0.418 μA, when the immobilization mixture contained 11 mg mL^{-1} aminated MWCNs and 70 U mL^{-1} AChE. Decreasing the concentrations of MWCN-NH_2 and AChE drastically reduced the amperometric current levels, confirming that those two components had significant influence on the biosensor performance.

The interactive effect of C_E and $C_{Con\,A}$ on the amperometric current is shown in Fig. 4. The maximum amperometric current was exerted at the following concentrations: C_E—70 U mL^{-1} and $C_{Con\,A}$—1.5 mg mL^{-1}. A further increase of the enzyme and Con A concentrations led to the decrease of the amperometric current. The design illustrated that the optimum values of the factors influencing the amperometric current were C_E—70 U mL^{-1}; $C_{Con\,A}$—1.5 mg mL^{-1} and C_{MWCN}—11 mg mL^{-1}, with an amperometric current of 0.418 μA.

3.3. Amperometric response of the ATCh sensor

Fig. 5 depicts a current-concentration plot of the investigated biosensor obtained by measuring the generated current at a potential of 0.8 V after a definite number of successive additions of 100 μL 2.0 μM ATCh to a 40 mL PBS into a stirred cell. On addition of an aliquot of ATCh to the buffer solution, the oxidation current increased steeply to reach a stable value. The enzyme electrode achieved 95% of the steady-state-current in 10 s. This rapid response was due to the MWCNs promoting the electron transfer.

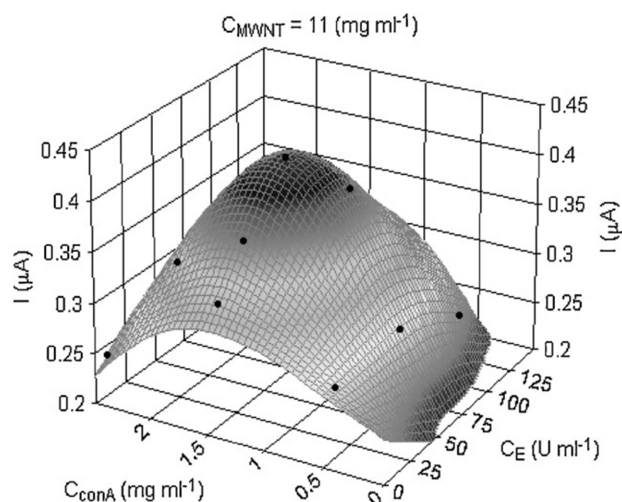

Fig. 4. Response surface plot showing the effect of C_E and $C_{Con\,A}$ and their mutual effect on the amperometric current at C_{MWCN} = 11 mg mL^{-1}.

3.4. Calibration plot of ATCh sensor

The current–concentration response curve of the biosensor was obtained by successive additions of the substrate into a stirred cell. With the increasing concentration of ATCh the amperometric response increased linearly in the range from 5 to 200 μM (Fig. 6). The linear regression equation is I (μA) = 0.6705 + 0.051[ATCh] (μM) with correlation coefficient of 0.995 (n = 28). Then the amperometric response tended to a plateau value (Fig. 6), showing a typical Michaelis–Menten process. The detection limit was 0.34 μM at a signal-to-noise ratio of 3. Thus, 200 μM ATCh was selected for the detection of Paraoxon.

The performance of the constructed biosensor is comparable to the results reported by other authors (see Table 4).

3.5. Reproducibility and stability of the ATCh sensor

The inter-assay precision, or fabrication reproducibility was estimated by determining the response to 100 μL 2 mM solution of ATCh of six different electrodes and the relative standard deviation was found to be 2.43%. The intra-assay precision of the sensors was evaluated by assaying one enzyme electrode for six replicate determinations and the relative standard deviation was calculated

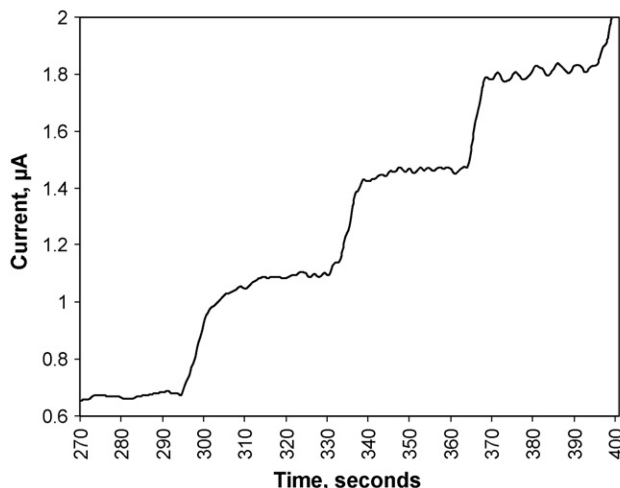

Fig. 5. Response time of the ATCh biosensor.

Table 4
Performance comparison of different ATCh biosensors.

No	Biosensor	Range of linearity (μM)	Detection limit (μM)	Response time (s)	References
1	GCE with MWCN–cross-linked chitosan composite	2–20 20–400	0.10	10	[23]
2	GCE with gold nanoparticles embedded in sol–gel film	10.0–1000	1.0	10	[24]
3	GCE with MWCN-cross-linked chitosan composite	1.0–500	–	15	[25]
4	GCE with MWCN-chitosan matrix	1–6	0.10	–	[26]
5	Screen-printed electrode by affinity bonds using Concanavalin A	10–110 mmol L^{-1}	–	–	[27]
6	Pt electrode with gold nanoparticles in PAN membrane	10–170	1.80	10	[28]
7	Pt electrode with MWCN in PAN membrane by affinity bonds using Con A	5–200	0.34	10	In our paper

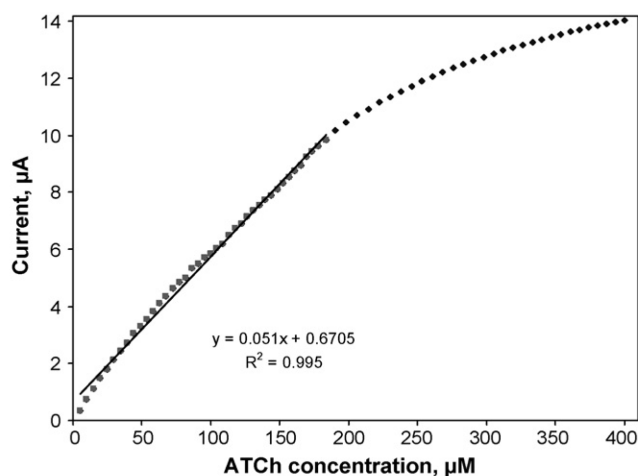

Fig. 6. Calibration curve for ATCh biosensor.

to be 1.12% for an ATCh concentration of 100 μL 2 mM solution of ATCh. These results were indicative of an acceptable reproducibility regarding ATCh determination.

When the enzyme electrode was not in use, the enzyme membrane was stored at 4 °C in PBS, pH 7.6. The obtained biosensor showed the following storage stability (Fig. 7): after 30 days of storage the sensor retained 76% of its initial current response, after 60 days—68% and after 120 days—61%. This retention of AChE activity indicated that the enzyme composite included in the prepared polymer carriers provided a biocompatible microenvironment around the enzyme; this was enough to effectively stabilize its biological activity for a longer period.

Du et al. [23,24] achieved 70% retention of the initial AChE activity after storage for 30 days by immobilizing it on GNPs-cross-linked chitosan composite. Bucur et al. [27] immobilized the AChE via affinity bonds using Concanavalin A. The prepared biosensor

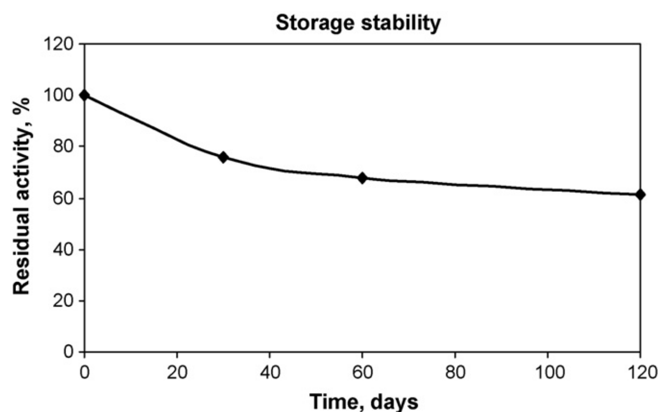

Fig. 7. Stability of the AChE biosensor response for a 120-day period of storage.

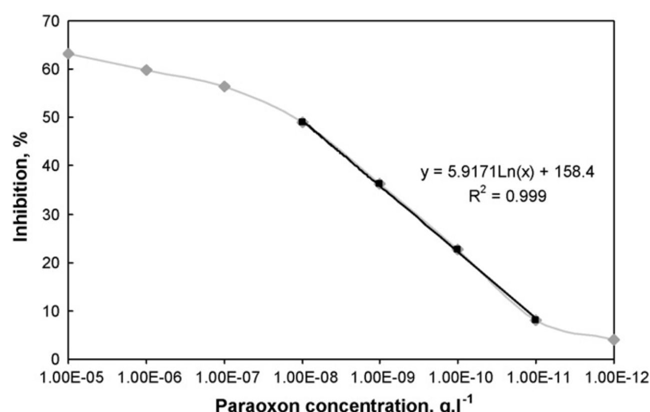

Fig. 8. Inhibition dependence of the AChE biosensor on Paraoxon concentration.

retained 43% after 20 days and 27% of its initial current response after a month of storage.

4. Pesticide detection

As a final step of the experimental work the prepared biosensor was applied to detect organophosphate pesticide in sample solutions. In this case, Methyl-Paraoxon was used as the enzyme inhibitor. The optimum times were determined for an enzyme carrier to be incubated in a pesticide solution as well as in a reactivation solution—20 and 30 min, respectively.

A series of experiments were carried out, involving measurements of the biosensor signal before and after the incubation of the carrier in a pesticide solution with a definite concentration. The relation between the inhibition percentage (I%) and the corresponding Paraoxon concentration (ranging from 10^{-12} to 1×10^{-5} g L^{-1}) is presented in Fig. 8. It was observed that for a concentration of pesticide from 10^{-11} to 10^{-8} g L^{-1} the inhibition increased in a linear manner, with percentages of inhibition between 8 and 49%. The equation of the linear portion of the inhibition curve was $y = 5.917 \ln(x) + 158.4$. The correlation coefficient was $R^2 = 0.999$. The standard deviation and the mean of the biosensor response to a standard solution of ATCh were calculated on the basis of eight separate amperometric measurements. The results were used to calculate the detection limit of the biosensor, which was estimated to be 1.39×10^{-12} g L^{-1}.

The enzyme membrane exhibited relatively high reactivation (90.2%) after nine consecutive inhibition experiments in pesticide solutions with different concentrations (from 1×10^{-5} to 1×10^{-12} g L^{-1}) followed by reactivation of the enzyme membrane in a 5 mM PAM solution for 30 min.

5. Conclusion

An important advantage, as believed, of the constructed biosensor, is that the enzyme carrier is a separate element that could be

incubated in a pesticide solution and reactivated in PAM solution afterwards aside from the working electrode, which is therefore available to be assembled with another enzyme membrane and used for pesticide measurements. This is especially useful for the detection of irreversible enzyme inhibitors, because of the easier replacement of the enzyme membrane and utilization of a single working electrode. Another advantage of the PAN polymer carrier is the presence of selective and non-selective sides due to the asymmetry of the membrane pores. The enzyme molecules trapped into the pores of the non-selective membrane side cannot be washed away and are being protected from any electrochemical interference present in the solution during the measurement procedures. Another very important advantage of the constructed biosensor is the oriented site-specific immobilization of AChE which ensures high enzyme activity and therefore an enhanced biosensor performance. On the other hand, MWCNTs in polymer membrane enhance the electron transfer from the enzyme molecules to the electrode surface and insure rapid biosensor response.

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