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Amperometric acetylthiocholine sensor based on acetylcholinesterase immobilized on nanostructured polymer membrane containing gold nanoparticles

Ivaylo Marinov, Yavor Ivanov, Katya Gabrovska, Tzonka Godjevargova*

University "Prof.Dr.A.Zlatarov", Department of Biotechnology, Prof Y. Yakimov Str. 1, 8010 Bourgas, Bulgaria

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ABSTRACT

Poly(acrylonitrile-methylmethacrylate-sodium vinylsulfonate) membranes were chemically modified and loaded with gold nanoparticles. Acetylcholinesterase was immobilized on the prepared membranes in accordance with two distinctive procedures, the first of which involved immobilization of the enzyme by convection, and the other by diffusion. The prepared enzyme carriers were used for the construction of amperometric biosensors for detection of acetylthiocholine.

Two sets of experiments were carried out. The first set was designed so that to evaluate the effects of the gold nanoparticle deployment and the immobilization procedures over the biosensor effectiveness. The other set of experiments was conducted in order to determine the influence of the individual components of the enzyme mixture, containing gold nanoparticles, acetylcholinesterase, bovine serum albumin and glutaraldehyde, over the current output of the constructed acetylthiocholine biosensors. The optimum composition of the mixture was determined to be as follows: enzyme, 0.1 U ml^{-1}; gold nanoparticles, 0.50 ml (per 1 ml enzyme mixture); albumin, 0.5% and glutaraldehyde, 0.7%.

On the basis of the experimental results, the most efficient enzyme membrane was selected and used for the preparation of an acetylthiocholine biosensor. Its basic amperometric characteristics were investigated. A calibration plot was obtained for ATCh concentration ranging from 10 to 400 μM. A linear interval was detected along the calibration curve from 10 to 170 μM. The sensitivity of the constructed biosensor was calculated to be 0.066 μA μM^{-1} cm^{-2}. The correlation coefficient for this concentration range was 0.996. The detection limit with regard to ATCh was calculated to be 1.80 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 for Paraoxon was determined, 7.39×10^{-11} g l^{-1}, as well as the concentration interval (10^{-10} to 10^{-7} g l^{-1}) within which the biosensor response was linearly dependant on Paraoxon concentration.

Finally the storage stability of the enzyme carrier was traced for a period of 50 days. After storage for 20 days the sensor retained 75% of its initial current response and after 30 days −25%.

1. Introduction

Organophosphate pesticides (OP) irreversibly inactivate acetylcholinesterase (AChE), which is essential to nerve function in insects, humans, and many other animals. Organophosphate pesticides affect this enzyme in varied ways, and thus in their potential for poisoning.

Organophosphate pesticides degrade rapidly by hydrolysis on exposure to sunlight, air, and soil, although small amounts can be detected in food and drinking water. Their ability to degrade made them an attractive alternative to the persistent organochloride pesticides, such as DDT, aldrin and dieldrin. Although organophosphates degrade faster than the organochlorides, they have greater acute toxicity, posing risks to people who may be exposed to large amounts. That is why rapid determination and reliable quantification of OP have become of great importance. Analytical methods such as gas chromatography or high-performance liquid chromatography have been widely used for the determination of OP compounds [1–3]. These methods are still applicable when high accuracy and differentiation of the individual OP compounds are needed. However, for in situ determination of OP, where the overall OP quantity is of importance, electrochemical biosensors are preferred due to their good sensitivity, rapid response and portability [4–6]. A variety of biosensors have been constructed based

* Corresponding author. Tel.: +359 56 858353; fax: +359 56 880249.
E-mail address: godjevargova@yahoo.com (T. Godjevargova).

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on the immobilization of different enzymes [7,8]. Among these, the amperometric biosensors based on the inhibition of immobilized AChE have shown satisfactory results for pesticide analysis [9–11]. The usual immobilization methods include direct physical adsorption onto a solid support [12], crosslinking [13], encapsulation into a hydrogel or a sol–gel film [14,15] and covalent binding [16]. 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 of polyacrylonitrile (PAN) 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 enzyme active centers. And least, but not last, modified PAN membranes would provide a favorable microenvironment for the enzyme molecules, thus prolonging the enzyme storage life, not to mention the convenience of storing only the membranes, and not whole electrodes.

The main disadvantage of PAN membranes comes from their electric 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 metal nanoparticles as mediators of the electron transfer from the enzyme molecules to the electrode surface. Gold nanoparticles (GNPs), in particular, are preferred because of their biocompatibility which makes them an ideal support for the immobilization of a number of biologically active substances such as enzymes [17–19], antibodies [20,21], and DNA [22]. Another reason to choose GNPs is their conductivity properties which make them suitable for enhancing the electron transfer between the enzyme active centers and electrodes acting as electron transfer "wires" [23].

The present work is a continuation of our previous investigations and is focused on the optimization of acetylcholinesterase immobilization on the replaceable nanostructure membrane and the construction of a biosensor for organophosphorus pesticide detection. The objective is to examine the effects of the enzyme mixture in the membrane pores, containing GNPs, albumin, enzyme and glutaraldehyde on the response of electrochemical biosensor. The optimized enzyme carrier, uniting the advantages of chemically modified PAN membranes and the electron conducting properties of GNPs, was tested for its applicability as an amperometric ATCh and OP biosensor. The new biosensor exhibits low detection limit, fast response time, high sensitivity and has relatively good storage stability.

2. Materials and methods

2.1. Materials

Acrylonitrile-methylmethacrylate-sodium vinylsulfonate membranes (PAN) were prepared without support according to a methodology described in Ref. [24]. 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.

Acetylthiocholine chloride (ATCh) and AChE (Type C3389, 500 U/mg from electric eel) were purchased from Sigma–Aldrich (St. Louis, USA) and used as received. Bovine serum albumin (BSA), glutaraldehyde (GA), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), pyridine aldoxime methochloride (PAM), mercaptopropionic acid (MPA), Paraoxon and a colloid solution of GNPs with concentration of 5.74×10^{12} particles per milliliter and average size of 9.7 nm 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 PalmSense Electrochemical Instrument (Palm Instruments BV, The Netherlands) with a conventional three-electrode system comprising of platinum wire as an auxiliary electrode, Ag/AgCl electrode as reference and AChE-immobilized membrane attached to a platinum electrode as a working electrode.

2.3. Methods

2.3.1. Chemical modification of PAN membranes

2.3.1.1. Introducing carboxyl functional groups. 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. The membranes processed according this modification procedure were used as enzyme carriers in the immobilization technique, described in Section 2.3.2.1.

2.3.1.2. Introducing amine functional groups. Already modified PAN membranes (according to Section 2.3.1.1) 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. Those membranes were used as enzyme carriers in the immobilization technique, described in Section 2.3.2.2.

2.3.2. AChE immobilization onto chemically modified membranes

2.3.2.1. Covalent immobilization of AChE with EDC (Method 1). This immobilization procedure involved loading of GNPs into the pores of the modified membranes through convection. This was realized according to Fig. 1A following the designated steps:

(1) A circular piece of modified membrane was placed in an ultrafiltration cell with the larger pores facing upward, after which a given volume of the GNP solution (5.74×10^{12} particles per milliliter) was added and the cell was pressurized to 0.3 MPa (nitrogen atmosphere) and kept this way until all the liquid passed through the membrane.
(2) After the GNP loading the membrane was immersed in a 1 mM ethanolic solution of mercaptopropionic acid (MPA) for 20 h at 4 °C in order to introduce carboxyl groups on the surface of the GNPs.
(3) After that the membrane was immersed in 1 mM solution of EDC for 80 min at 4 °C.
(4) AChE was covalently immobilized by immersing the membrane into a 106.5 U ml^{-1} enzyme solution prepared in 100 mM phosphate buffer (pH 7.6) for 20 h at 0 °C. Finally, the membrane unit with the immobilized enzyme was washed with bi-distilled water and 0.1 M solution of phosphate buffer, pH 7.6.

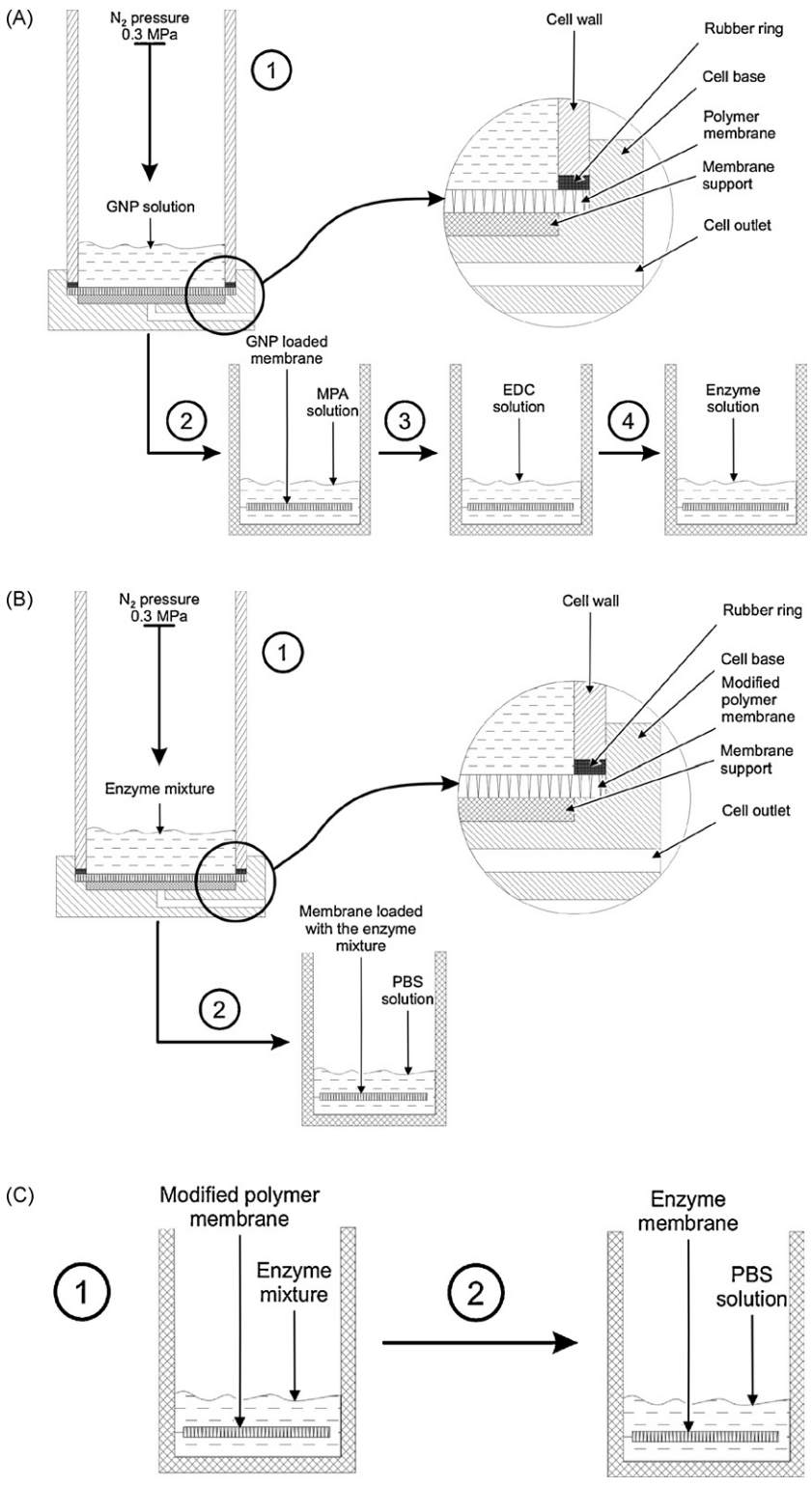

Fig. 1. Enzyme immobilization onto a modified polymer membrane: (A) by pressurized loading of GNPs and covalent bonding with EDC (Method 1); (B) by pressurized loading of an enzyme mixture and crosslinking the enzyme with glutaraldehyde (Method 2); (C) by immersing the membrane in an enzyme mixture and crosslinking the enzyme with glutaraldehyde (Method 3).

Membrane 1 was prepared according to this immobilization procedure.

2.3.2.2. Immobilization of AChE via GA crosslinking. Pressurized loading of an enzyme mixture into a modified membrane (Method 2): This immobilization technique involved two steps (as described in Fig. 1B):

(1) A preliminary preparation of a mixture of enzyme, GNPs, BSA and GA with a final volume of up to 1 ml. The mixture pre-

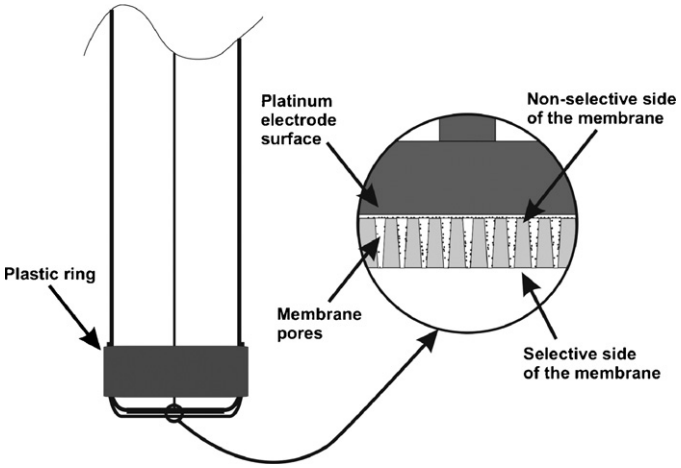

Fig. 2. Enzyme biosensor—an assembly of a platinum working electrode and an enzyme membrane.

pared thereby, was loaded into the pores of a membrane carrier through convection under 0.3 MPa (nitrogen atmosphere) at room temperature.
(2) Finally, the membrane unit with the immobilized enzyme was washed with bi-distilled water and 0.1 M solution of phosphate buffer, pH 7.6.

Membrane 2 was prepared according to this immobilization method.

Diffusion of enzyme mixture components into a modified membrane (Method 3): This immobilization method comprised two steps (as described in Fig. 1C):

(1) A preliminary preparation of a mixture of enzyme, GNPs, BSA and GA with a final volume of up to 1 ml. The mixture was placed in a flask and the modified membrane was immersed in it for 24 h at 4 °C in order to let the mixture components diffuse into the pores of the membrane.
(2) The prepared enzyme membrane was washed with bi-distilled water and 0.1 M solution of phosphate buffer, pH 7.6.

Membranes from 3 to 13 were prepared according to this immobilization method.

2.3.3. Electrochemical measurements of the nanostructured AChE membranes

Each enzyme carrier (Membranes 1–13) was attached to a platinum working electrode, using a plastic ring, with the non-selective side of the membranes facing the platinum surface of the electrode (Fig. 2), 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 to become stationary. Then 200 μl of 2 mM ATCh solution were added to the cell and the resulting current was recorded.

2.3.4. Determination of the apparent (K_m^{app})

Values of K_m^{app} for AChE were calculated from the Lineweaver–Burk plots using electrochemical data extracted from the calibration curves of the constructed ATCh biosensors at 38 °C, with ATCh concentrations ranging from 10 to 400 μM.

2.3.5. 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):

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

2.3.6. Reactivation of the immobilized AChE

After the inhibition measurements each membrane was reactivated in a 5 mM solution of PAM in PBS for 30 min. This was followed by a thorough washing of the membranes with PBS solution.

2.3.7. 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.

2.3.8. Treatment of experimental data

Each experimental point in the figures 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 response of the biosensor

The enzyme membranes prepared according to Method 1 (Membrane 1), Method 2 (Membrane 2) and Method 3 (Membrane 3) described in Section 2, were all used for the construction of biosensors, the basic electrochemical characteristics of which were further investigated. Results were obtained for all the three carriers representing different immobilization methods and each set of results was summarized from six individual amperometric experiments. Fig. 3 depicts the effect of the immobilization technique on the electric current generated by each of the biosensors.

The highest amperometric current was exhibited by Membrane 3, which involved a diffusion of the prepared enzyme mixture (GNP, AChE, BSA and GA) into the membrane. The weakest current was generated by Membrane 1, where GNPs were loaded into the membrane pore through convection, followed by a covalent immobilization of AChE with EDC. The results were also confirmed by the linear intervals and the corresponding slopes (presented in Table 1) of the calibration curves, obtained by successive additions of 200 μl 2 mM solution of ATCh.

As can be seen from Table 1 Membrane 3 outstands with the greatest slope value for the corresponding linear interval. Furthermore Membrane 3 exerted the greatest substrate affinity, as confirmed by the relevant K_m^{app} value in Table 1. The lower slope value and the narrower substrate concentration interval of linearity, characterizing Membranes 1 and 2 could be explained with the formation of two separate layers within the membrane pores—an inner layer of GNPs situated near the selective side of the membrane, and an outer enzyme layer. It is highly probable that not all of GNPs were chemically bound to enzyme molecules and not all of them were contacting with the platinum surface of the electrode at the same time, thus not contributing to the strength of the biosensor response.

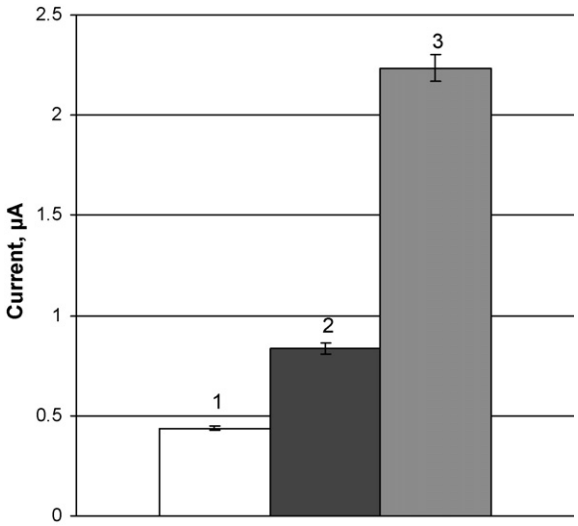

Fig. 3. Effect of the immobilization procedures on the response of the prepared ATCh biosensors with Membranes 1–3: (1) GNP loaded through convection and AChE immobilized via EDC; (2) GNP, AChE, BSA and GA loaded through convection; (3) GNP, AChE, BSA and GA loaded through diffusion.

Table 1
Basic characteristics from the calibration plots of the acetylthiocholine biosensors with nanostructured membranes, prepared in accordance with the immobilization Methods 1–3.

Immobilization method	Slope, μA μM^{-1}	Linear interval, μM	K_m^{app}	R^2
1	0.032	10.0–67.6	724.3	0.996
2	0.021	10.0–181.8	574.5	0.996
3	0.052	10.0–173.5	251.5	0.996

In order to understand the behavior of the enzyme carriers, SEM photographs were taken of slices and surfaces from Membranes 1 and 3 prepared according to the two described immobilization methods. Fig. 4A reveals clusters of GNPs at the end of the membrane pores (from the selective side of Membrane 1) and their sparse presence on the pore walls. Fig. 4B displays the surface of Membrane 1, which structure was obviously disrupted by the GNP loading under pressure. These two facts could certainly explain the hindered mass-flow of the substrate molecules to the enzyme active sites to the platinum surface of the working electrode, thus reducing considerably the response of the biosensor.

On the other hand, when GNPs were allowed to diffuse freely (Membrane 3), their presence becomes clearly visible inside the membrane pores (Fig. 4C) as well as on the membrane surface (Fig. 4D). Although GNPs were not quite homogeneously distributed on the membrane surface, the disruption of the latter was avoided and the mass-flow of the substrate molecules to the enzyme active sites was not disturbed, allowing the biosensor to generate a strong response.

The better amperometric characteristics of Membrane 3 led to the conclusion that the rest of the experiments should be based on the second immobilization procedure, which involved diffusion of a preliminary prepared enzyme mixture into the pores of a modified carrier and simultaneous crosslinking with GA.

3.2. *Effect of the enzyme mixture composition on response of AChE biosensors*

A series of experiments were conducted in order to study how the response of the constructed AChE biosensors was affected by the concentration variation of each mixture component—GNPs, BSA, enzyme and GA. In all the experiments below the final volume of the prepared enzyme mixture was 1 ml.

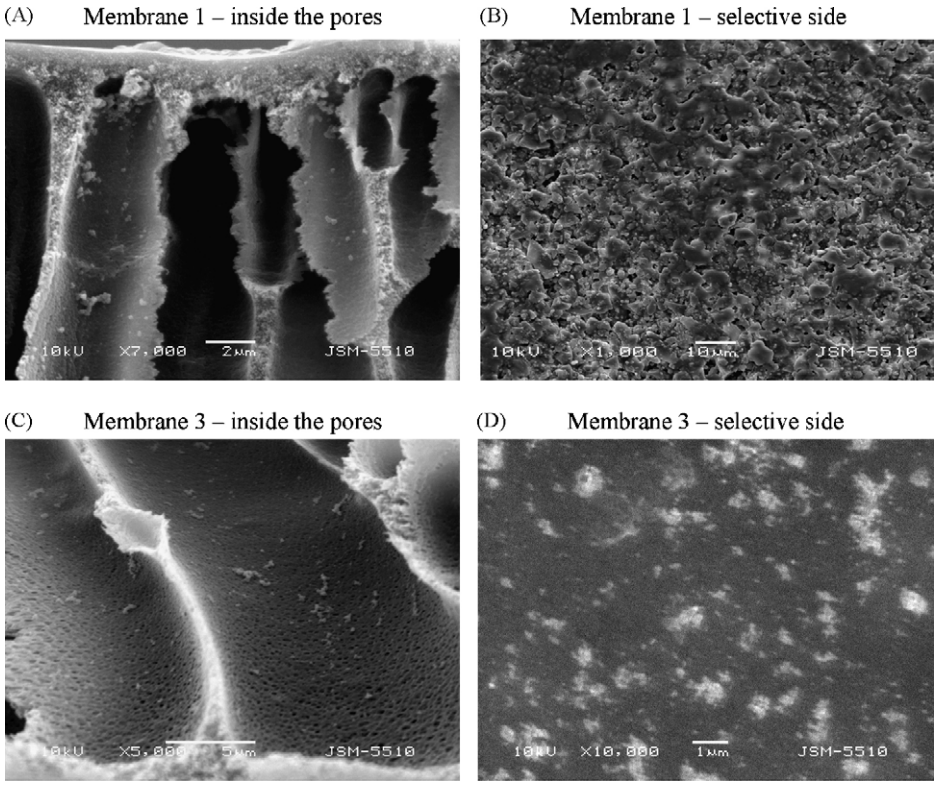

Fig. 4. SEM photographs of the membranes prepared in accordance with the different immobilization techniques: (A) and (B) GNP loaded through convection and AChE immobilized via EDC; (C) and (D) GNP, AChE, BSA and GA loaded through diffusion.

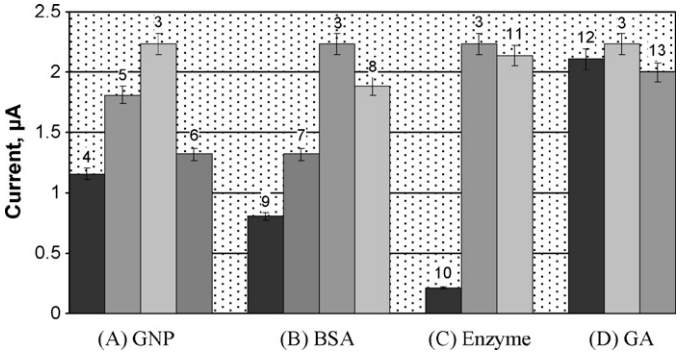

Fig. 5. Effect of the enzyme mixture composition on the response of the prepared ATCh biosensors (with Membranes 3–13); the enzyme mixture composition is displayed in Table 2. (A) GNP; (B) BSA; (C) Enzyme; (D) GA.

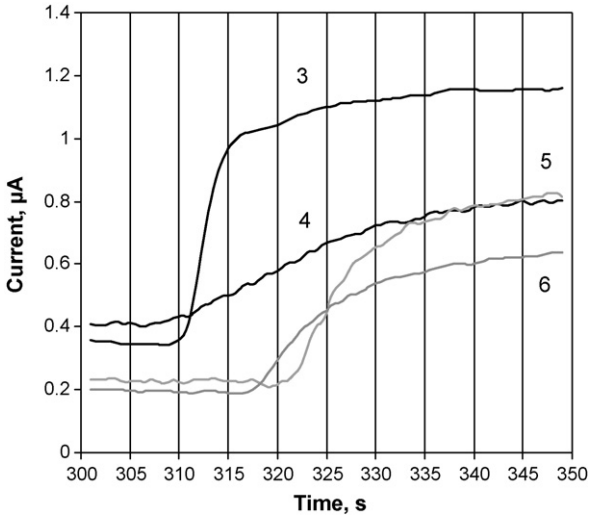

Fig. 6. Effect of GNP amount on the response time of the prepared ATCh biosensors with Membranes 3–6: 3, 0.5 ml GNPs; 4, without GNPs; 5, 0.25 ml GNPs; 6, 0.75 ml GNPs.

3.2.1. *Effect of GNP quantity in the enzyme mixture*

Each of the newly prepared carriers were immersed in enzyme mixtures containing different volumes of GNP solution per 1 ml enzyme mixture: Membrane 4: without GNPs, Membrane 5: 0.25 ml, Membrane 3: 0.50 ml and Membrane 6: 0.75 ml GNPs, keeping the concentration of the other components constant: 0.5% BSA, 0.1 U/ml AChE, 0.7% GA. The results are displayed in Fig. 5A, where the numbers from 3 to 13 designate the investigated enzyme membranes.

A noticeable rise of the amperometric current was observed with the increase of the carrier GNP loading—the lowest current was exhibited by Membrane 4 (without GNPs), and the highest by Membrane 3 (with 0.50 ml GNPs). However, after reaching an optimum amount of GNPs the amperometric signal tended to decrease, probably due to diffusion limitations impeding the mass-transfer of the substrate molecules to the enzyme active sites.

The effect of GNP quantity on the linear intervals and the corresponding slopes could be traced through the results in Table 2. The best electrochemical characteristics were exhibited by the enzyme carrier with 0.50 ml GNPs. A fact, worth noting, is that even Membrane 4 (without GNPs) displayed satisfactory amperometric characteristics.

Since the response time of a biosensor is important from a practical point of view, a comparison was made in order to estimate the effect of GNP quantity on the response time of the prepared biosensors. As can be seen from Fig. 6 Membrane 3 (containing an optimal amount of GNPs) showed a response time of approximately 5 s. In comparison, Membrane 4 (without GNPs) exhibited approximately four times longer response time, which again serves as a confirmation of the importance of GNPs for the electron transfer and the swiftness of the biosensor response.

3.2.2. *Effect of BSA concentration in the enzyme mixture on response of the AChE biosensor*

In order to establish the optimum BSA concentration, the protein amount was varied (Membrane 3: 0.5%, Membrane 9: without BSA, Membrane 7: 0.05% and Membrane 8: 2.5%), while the concentrations of the other components were kept constant: 0.1 U/ml AChE, 0.7% GA, 0.50 ml GNPs. The highest amperometric signal was generated by Membrane 3, containing 0.5% BSA (Fig. 5B). Membrane 9 generated three times lower amperometric current (the enzyme carrier was prepared without BSA). The experimental results revealed a certain tendency of increasing the biosensor signal with the increase of the BSA quantity. This tendency is visible from Table 2 data as well. The greater the BSA quantity, the higher the slope of the linear interval, extracted from the calibration plots of the relevant enzyme carriers, and the higher the biosensor sensitivity. The positive influence of BSA over the biosensor parameters could be explained with the favorable microenvironment created by the BSA molecules around AChE, due to their hydrophilicity and biocompatibility with AChE. Similar results regarding the positive effect of an optimum BSA concentration over the AChE stability and activity were reported by Laschi et al. [25].

3.2.3. *Effect of enzyme quantity in the mixture on response of the AChE biosensor*

In order to establish the effect of the enzyme concentration on the amperometric signal three enzyme carriers were prepared, containing different enzyme amounts (Membrane 3: 0.1 U/ml AChE,

Table 2
Basic characteristics from the calibration plots of acetylthiocholine biosensors with nanostructured membranes, processed in enzyme mixtures with different composition.

Membrane no.	Enzyme mixture composition				Slope, μA μM^{-1}	Linear interval, μM	K_m^{app}	R^2
	Enzyme, U ml^{-1}	GNPs, ml	BSA, %	GA, %				
3	0.1	0.50	0.5	0.7	0.052	10.0–173.5	251.5	0.996
4	0.1	–	0.5	0.7	0.035	10.0–76.9	450.9	0.996
5	0.1	0.25	0.5	0.7	0.047	10.0–156.7	443.9	0.998
6	0.1	0.75	0.5	0.7	0.037	10.0–86.1	334.3	0.996
7	0.1	0.50	0.05	0.7	0.040	10.0–104.3	349.9	0.996
8	0.1	0.50	2.5	0.7	0.058	10.0–113.2	790.0	0.998
9	0.1	0.50	–	0.7	0.024	10.0–86.1	1248.4	0.995
10	0.01	0.50	0.5	0.7	0.006	10.0–48.8	257.1	0.991
11	0.2	0.50	0.5	0.7	0.066	10.0–122.1	419.5	0.996
12	0.1	0.50	0.5	0.5	0.056	10.0–76.9	281.9	0.994
13	0.1	0.50	0.5	1.4	0.054	10.0–86.1	299.6	0.993

Membrane 10: 0.01 U/ml AChE and Membrane 11: 0.2 U/ml AChE), while the concentrations of the other components were kept constant (BSA: 0.5%, GNPs: 0.50 ml, GA: 0.7%). As can be seen from Fig. 5C the increase of the enzyme quantity was followed by an increase of the amperometric current up to an optimum amount of 0.1 U/ml AChE, after which the current started to decrease. The calibration curves, corresponding to each of the three enzyme carriers (Membranes 3, 10 and 11), were used to define the relevant linear intervals and slopes, the values of which are displayed in Table 2. Membrane 3 could be clearly distinguished by the largest linear interval and one of the highest slopes.

3.2.4. Effect of glutaraldehyde concentration in the enzyme mixture on response of the AChE biosensor

GA was used as a crosslinking agent. When preparing the enzyme mixture, BSA was added in the first place, and then GA in different concentrations: Membrane 12: 0.5%, Membrane 3: 0.7%, and Membrane 13: 1.4%. The concentrations of the other components were kept constant: 0.1 U/ml AChE, 0.50 ml GNPs, 0.5% BSA. After that optimum concentrations of GNPs and enzyme were added to the mixture. The remaining free aldehyde groups covalently bonded to amino-groups from the enzyme molecules. The results from amperometric current measurements (Fig. 5D) did not distinguish clearly any of the prepared carriers with respect to glutaraldehyde concentration (Table 2). Since Membrane 3 performed better than the other enzyme carriers, the optimum glutaraldehyde concentration was selected to be 0.7%.

3.3. Apparent Michaelis–Menten constants K_m^{app}

K_m^{app} is an important quantity, describing enzyme affinity toward a given substrate. To examine this further, K_m^{app} for AChE immobilized on the prepared membranes was calculated from the Lineweaver–Burk plots using electrochemical data extracted from the calibration curves of the constructed ATCh biosensors. K_m^{app} values obtained from the different types of enzyme carriers were presented in Table 2. The change in the GNP content in the membranes did not appear to change K_m^{app} significantly in each biosensor. The smaller K_m^{app} value for Membrane 3 (251.50 μM) demonstrated that AChE immobilized on this carrier possessed one of the highest substrate affinity. BSA concentration in the enzyme mixture exerted a considerable influence over the K_m^{app} values, since BSA molecules had probably improved the hydrophilicity of the enzyme microenvironment. This relation was confirmed by the results showing that K_m^{app} values decreased with the increase of the carrier hydrophilicity thus improving the enzyme affinity toward ATCh. Most of the membranes exhibited low K_m^{app} values, since they had been prepared with an optimum BSA concentration, except Membranes 7–9. It was not unexpected for Membrane 9 to be distinguished with the highest K_m^{app} value (1248.36 μM), since it had been prepared without BSA.

The amount of crosslinking agent (GA) had influenced carrier hydrophilicity as well. The highest K_m^{app} value among the enzyme carriers with varied GA concentration was observed for Membrane 13 (299.59 μM), which had been prepared with the highest glutaraldehyde concentration.

Scrutinizing the experimental results revealed that K_m values did not demonstrate a completely good correlation with the sensitivity of the constructed biosensors and the linear intervals from the corresponding calibration curves. Apart from the factor "immobilized enzyme", there are other factors that can affect biosensor behavior, e.g. inter- and intra-diffusion of substrates and reaction products, substrate steric and conformational effects, immobilization to a given matrix that may result in enzyme disfiguration, electrode active surface properties that may influence the conductivity, the enzyme amount at the membrane surface and the amplification of the biosensor response by recycling process occurring at the electrode surface.

From the results obtained so far Membrane 3 emerged as one of the enzyme carriers with the highest substrate affinity (251.50 μM). This value of K_m^{app} is lower than the values, reported by other authors: for AChE adsorbed on carbon nanotubes—N,N-dimethylformamide composite film (660 μM) [26] and for AChE adsorbed on polyethyleneimine modified electrode (1500 μM) [27]. Apparently AChE immobilized on the modified polyacrylonitrile membrane exhibits a greater affinity for ATCh, due to the favorable microenvironment of the enzyme molecules, generated by the presence of biocompatible GNPs and hydrophilic BSA molecules.

3.4. Investigation of the characteristics of ATCh biosensor, prepared with Membrane 3

Membrane 3 was selected to be used as the sensing part of the biosensor under investigation. The amperometric response of the biosensor was recorded while a constant volume of ATCh solution was successively added to the volume of the electrochemical cell (Fig. 7). A calibration plot was obtained for ATCh concentration ranging from 10 to 400 μM. A linear interval was detected along the calibration curve from 10 to 170 μM. The sensitivity of the constructed biosensor was calculated to be 0.066 μA μM^{-1} cm^{-2}. The correlation coefficient for this concentration range was 0.996. The standard deviation and the mean of the biosensor response to 200 μl 2 mM standard solution of ATCh were calculated on the basis of 8 separate amperometric measurements. The results were used to calculate ACTh detection limit of the biosensor, which was estimated to be 1.80 μM. The performance of the constructed biosensor is comparable to the results reported by other authors (see Table 3).

3.5. Reproducibility and stability of the ATCh sensor

The inter-assay precision, or fabrication reproducibility was estimated by determining the response to 200 μl 2 mM solution of ATCh of six different electrodes and the relative standard deviation was found to be 3.50%. 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 to be 1.68% for an ATCh concentration of 200 μl 2 mM solution of ATCh. These results were indicative of an acceptable reproducibil-

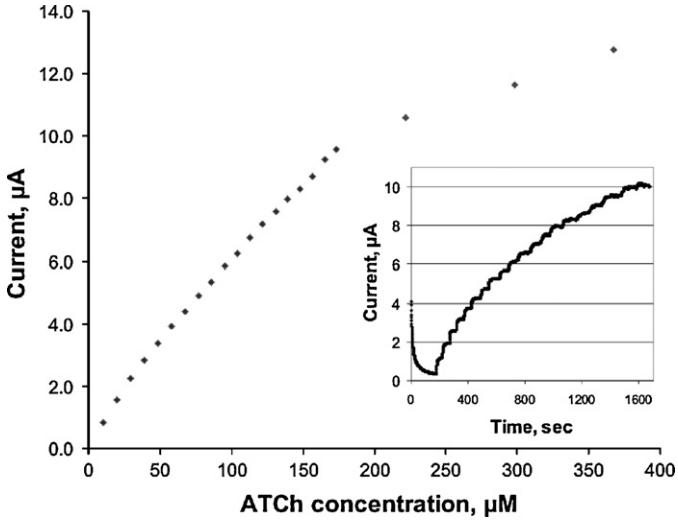

Fig. 7. Calibration curve for ATCh biosensor; E_{app} = 0.80 mV; successive addition of 200 μl 2 mM solution of ATCh in 0.1 M PBS, pH 7.6.

Table 3
Comparison data of amperometric acetylthiocholine sensor performance based on immobilized acetylcholinesterase.

Number	Biosensor	Range of linearity, μM	Detection limit, μM	Correlation coefficient	Response time, s	References
1.	GCE with multiwall carbon nanotube–crosslinked chitosan composite	2–20, 20–400	0.10	0.9959	10	[28]
2.	GCE with gold nanoparticles embedded in sol–gel film	10.0–1000	1.0	0.9989	10	[29]
3.	GCE with multiwall carbon nanotube–crosslinked chitosan composite	1.0–500	–	0.997	15	[30]
4.	GCE with multiwall carbon nanotube–chitosan matrix	1–6	0.10	0.97	–	[31]
5.	Screen-printed electrode by affinity bonds using concanavalin A	10–110 mmol/l	–	0.9842	–	[32]
6.	Gold nanoparticles into membrane	10–170	1.80	0.9960	10	In our paper

ity regarding ATCh determination. Covalent bonding of AChE by using glutaraldehyde was preferred to a physical adsorption of the enzyme because of the hydrophobic nature of the surface of the prepared polymer membranes, which does not provide for a significant enzyme loading and does not prevent the enzyme molecules from leaving the carrier surface, as shown in Ref. [33].

When the enzyme electrode was not in use, the enzyme membrane was stored at 4 °C in PBS, pH 7.6. No obvious decrease in response to ATCh was observed in the first 10 days (Fig. 8). After storage for 20 days the sensor retained 75% of its initial current response and after 30 days −25%. This retention of AChE activity indicated that although the enzyme composite included in the prepared polymer carriers provided a biocompatible microenvironment around the enzyme, this was not enough to effectively stabilize its biological activity for a longer period. This was probably due to the fact that membranes were stored in PBS and not under dry conditions as reported by other authors. Du et al. [29] achieved 90% retention of the initial AChE activity by immobilizing it on GNPs distributed on silica sol–gel film. Bai et al. [34] developed GNP-mesoporous silica composite which was used as an immobilization matrix for AChE. The prepared biosensor retained 90% of its initial current response after a month of storage.

3.6. 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, Paraoxon was used as an enzyme inhibitor. The optimum times were determined for an enzyme carrier to be incubated in a pesticide solution as well as in a reactivation PAM-2 solution—20 min and 30 min, respectively.

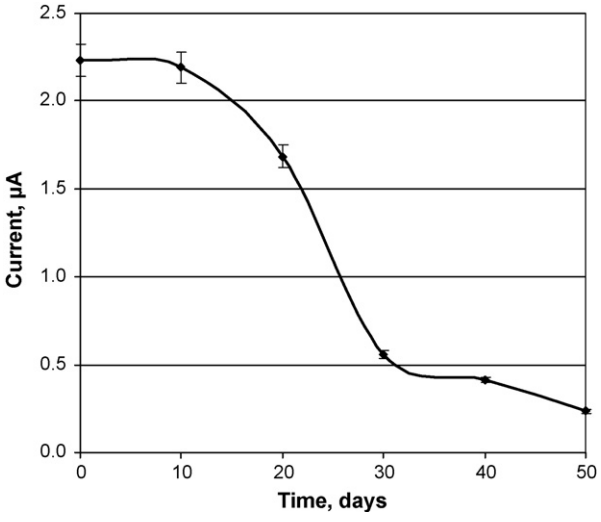

Fig. 8. Storage stability of the ATCh biosensor.

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 10^{-4} g l^{-1}) is presented in Fig. 9. It was observed that for a concentration of pesticide from 10^{-10} to 10^{-7} g l^{-1} the inhibition increased in a linear manner, with percentages of inhibition varying from 10% to 86%. The equation of the linear portion of the inhibition curve was $y = 11.2 \ln(x) + 269$. The correlation coefficient was $R^2 = 0.995$. The standard deviation and the mean of the biosensor response to 100 μl 50 mM standard solution of ATCh were calculated on the basis of 8 separate amperometric measurements. The results were used to calculate the detection limit of the biosensor, which was estimated to be 7.39×10^{-11} g l^{-1}.

After each reactivation, the biosensor response was measured in order to assess the influence of the pesticide inhibition over the biosensor operational stability. The results are displayed in Table 4:

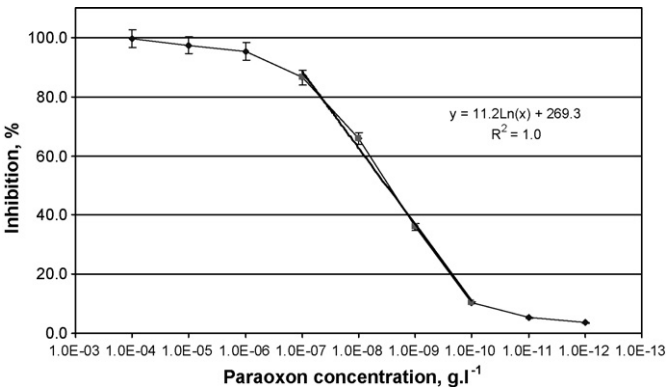

Fig. 9. Inhibition plot of Paraoxon onto a ATCh biosensor.

Table 4
Reactivation of the immobilized enzyme after incubating in a 5 mM solution of PAM in 0.1 M PBS for 30 min.

No.	Pesticide concentration, g l^{-1}	Reactivation, %
1.	10^{-12}	100
2.	10^{-11}	100
3.	10^{-10}	100
4.	10^{-9}	99.6
5.	10^{-8}	97.8
6.	10^{-7}	96.2
7.	10^{-6}	94.0
8.	10^{-5}	92.6
9.	10^{-4}	90.2

As can be seen from Table 4 the enzyme membrane exhibited relatively high reactivation (90.2%) after nine consecutive inhibition experiments in pesticide solutions with different concentrations, followed by reactivation of the enzyme membrane in a 5 mM PAM solution.

3.7. 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 hence available to be assembled with another enzyme membrane and used for further 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 crosslinked with glutaraldehyde into the pores of the non-selective membrane side, which faces the platinum surface of the electrode, cannot be washed away and are being protected from any electrochemical interference present in the solution during the measurement procedures. The high enzyme loading and the presence of GNPs result in a high amperometric current, hence improved sensitivity, a swift biosensor response and good operational and satisfactory storage stability.

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