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Immobilization of acetylcholinesterase on new modified acrylonitrile copolymer membranes

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ABSTRACT

The three new dual-layer matrices (polyacrylonitrile (PAN) membranes coated with physically bound chitosan (CHI)—PANCHI-A and chemically bound chitosan—PANCHI-B and PANCHI-C) for immobilization of acetylcholinesterase (AChE) were obtained. The chemical-modified PAN membrane (PAN-NaOH + ethylenediamine (EDA)) was used as a base for the prepared dual-layer membranes. For chemical chitosan bound membrane, chitosan was tethered onto the membrane surface to form a dual-layer biomimetic membrane in the presence of glutaraldehyde (GA). The basic characteristics (amount of amino groups, hydrophilicity and transport characteristics) of the chitosan-modified membranes were investigated. The SEM analyses were shown essential morphology change in the different chitosan membranes.

The relative activities and V_{max} of the covalently immobilized enzyme on PANCHI-B and PANCHI-C membranes were higher than that on PANCHI-A membrane and chemical-modified membrane with NaOH + EDA. K_m values for the different modified membranes are lower for the chitosan-treated membranes. The pH and temperature optimum of immobilized enzyme were determined. The bound enzymes on PANCHI-B and PANCHI-C have higher thermal and storage stability in comparison with AChE on PANCHI-A membrane and free enzyme.

1. Introduction

Compared with conventional chemical catalysts enzymes as biocatalysts exhibit a number of features that make their use advantageous. Most solubilized enzymes are very unstable and they retain their activity only for a short period of time. Immobilization of an enzyme in a state close to its natural environment usually result in more stable and efficient enzyme [1–3]. Obviously, the support used in enzyme immobilization is important since its interaction with the enzyme may have an influence on the stability and kinetics. The synthetic polymers such as polyacrylonitrile (PAN) [4–6], can be used for enzyme immobilization, since it has good mechanical stability and easy of preparation in different geometrical configurations. Unmodified polyacrylonitrile membranes are not suitable as carrier for enzyme immobilization, because they do not have active groups. Several methods are used for the modification of the polyacrylonitrile membrane to introduce active groups. One of the way to improve these properties is the modification of membrane with different chemical reagents [7,8]. Another way to prepare a modified membrane is by coating the polyacrylonitrile membrane with chitosan (CHI). Chitosan is a natural polyelectrolyte containing free amino groups ($pK_a = 6.5$) and is suitable for the convenient preparation of films [9]. Due to its biocompatibility, chitosan has been extensively used for the immobilization of biomolecules [10], but it features bad mechanical stability. Application of this natural macromolecules for preparation of chitosan-coated polysulfone membranes and immobilization of the polyphenol oxidase has been reported by Edwards et al. [11]. Musale et al. [12] reported the formation of PAN/chitosan composite ultrafiltration membranes and their application only for membrane processes. Gabrovska et al. [13] use unmodified PAN membrane coating with physical bound chitosan for urease immobilization. The chitosan coating has bad stability, because the chitosan was bound physically. Ye et al. [14] have tethered chitosan on poly(acrylonitrile-*co*-maleic acid) hollow fiber membrane surface to receive a membrane support for lipase immobilization. In this case the chitosan was chemically bound by 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxyl succinimide. The tethered chitosan on the polyacrylonitrile-based copolymer membrane surface is more stable.

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Studying the acetylcholinesterase (AChE) immobilization is very important, because immobilized AChE can be used for construction of biosensor for pesticide determination [15–18].

In this investigation, the application of modified PAN membranes with glutaraldehyde (GA) chemically tethered chitosan for covalent immobilization of acetylcholinesterase was studied. The basic characteristics of the immobilized enzyme were investigated in comparison with the free enzyme.

2. Experimental

2.1. Materials

Acrylonitrile–methylmethacrylate–sodium vinylsulfonate membranes (PAN), with cut-off 60 kDa toward dextran, were supplied by Ecofilter, Bulgaria. The ternary copolymer (acrylonitrile, 91.3%; methylmethacrylate, 7.3%, sodium vinylsulfonate, 1.4%) was a product of Lukoil Neftochim, Bourgas, Bulgaria. Ultrafiltration membrane of acrylonitrile copolymer retaining substances with molecular weight higher than 60 000 Da was used.

PAN membranes were modified by using sodium hydroxide and ethylenediamine. Chitosan, with MW = 10 and 400 kDa was purchased from Fluka (AG, Switzerland).

Acetylcholinesterase from *Electrophorus electricus* (E.C. 3.1.1.7, 403 U mg^{-1}, Sigma, USA) was covalently immobilized onto the modified PAN membranes by means of glutaraldehyde (Fluka). The AChE activity was determined using acetylthiocholine iodide (ATChI) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) from Sigma, Germany.

All reagents were analytical grade.

2.2. Methods

2.2.1. Modifications of PAN membranes

2.2.1.1. Chemically modified membrane (PAN-NaOH + EDA). Membrane with 100 cm^2 surface area was immersed in 100 cm^3 15% NaOH for 60 min at 50 °C. The membrane was then washed with distilled water and placed in 1 M HCI at room temperature for 120 min. The color of the hydrolyzed yellowish red PAN membrane had turned into white. After that, the membrane was immersed in 100 cm^3 10% solution of ethylenediamine for 60 min at room temperature [19]. The chemically modified membrane (PAN-NaOH + EDA) was obtained in two steps:

- Modification with NaOH—the product is polyacrylamide and polyacrylic acid (Fig. 1, step 1).
- Modification with EDA—the carboxylic groups of polyacrylic acid turns to amino groups (Fig. 1, step 2).

2.2.1.2. Modified membrane by physically bound high molecular weight (HMW: 400 kDa) chitosan (PANCHI-A membrane). Preparation of PANCHI-A membranes was obtained by filtering the chitosan solution (0.25%, w/w) in aq. acetic acid (25%, w/w) through the PAN-NaOH + EDA base membrane at 2×10^5 Pa for 10 s using stirred cell (Sartorius, 600 rpm). The composite layer of chitosan acetate was then converted to chitosan by filtering NaOH (1 M in ethanol–water mixture, 1:1) through the membranes for 3 min. These membranes were washed with ethanol–water (1:1) mixture by filtering it through the membrane for 2 min. After that the membrane was immersed in ethanol–water (1:1) mixture for 24 h and then washing with ethanol–water mixture three times. The procedure was followed by curing the membranes at 60 °C and washing with ethanol–water mixture [11].

2.2.1.3. Modified membrane by chemical bound low molecular weight (LMW: 10 kDa) chitosan (PANCHI-B membrane) and high molecular weight (HMW: 400 kDa) chitosan (PANCHI-C). The PAN-NaOH + EDA membrane according to Section 2.2.1.1 was immersed into 10% GA for 1 h. After that the membrane was washed with distilled water. Then the membrane was immersed in chitosan solution (15 mg ml^{-1} in acetic acid buffer solution, 50 mM, pH 5.0) and stirred gently for 24 h at room temperature. Two kinds of chitosan with different molecular weight were used (MW = 400 and 10 kDa). Finally, the modified membrane was taken out, washed several times with 1.0% acetic acid solution and then washed with distilled water to remove the free chitosan adsorbed on membrane surface, this obtained the dual-layer membrane.

PANCHI-B and PANCHI-C membranes with covalently bound chitosan were prepared in two steps:

1. Modification of PAN-NaOH + EDA membrane with glutaraldehyde to produce aldehyde groups, needed for the chemical binding of chitosan on the membrane surface (Fig. 1, step 3). For amino groups in the modified membranes, there are a pendant structure between one aldehyde groups in the glutaraldehyde molecule and one amino group in the PAN-NaOH + EDA membrane.
2. The activated membrane with glutaraldehyde was treated with chitosan solution (Fig. 1, step 4). For the D-glucosamine unit in the chitosan molecule, there are (a) pendant structure due to the reaction of another aldehyde group in the glutaraldehyde molecule with one amino group in the chitosan molecule and (b) unreacted amino groups in the chitosan film.

2.2.2. SEM analysis of the membranes

SEM studies were carried out on gold sputtered membrane samples using Philips SEM 515 scanning electron microscope. Membrane samples for SEM studies were prepared by soaking in isopropanol overnight, then in hexane for 10 h and subsequently vacuum-dried at 40 °C for 3 days. The cross-sections of samples were prepared by fracturing in liquid nitrogen [11].

2.2.3. Immobilization of AChE on modified membranes

The modified membrane (10 cm^2) was immersed in 10% solution of glutaraldehyde for 1 h at 4 °C. Then the membrane was washed with bi-distilled water and after that was immersed in 0.05% solution of in 0.1 M sodiumphosphate buffer of pH 7.0. The membrane was kept in 0.05% enzyme solution for 20 h at 4 °C. Finally, the membrane with the immobilized enzyme was washed with bi-distilled water and 0.1 M solution of phosphate buffer.

2.2.4. Analytical methods

The amounts of functional (NH_2–) groups in the modified membranes were measured by residual titration in heterogeneous medium [20].

The amount of bound protein for different composite membranes was determined by the modified method of Lowry. The method is based on spectrophotometric measurement of the blue coloring resulting from the cupric ions binding to peptide bonds in alkali medium and from the reaction of the aminoacidous residues with Folin reactant [21].

The AChE activity was determined according to the Ellman method [22] using ATChI as substrate and 5,5′-dithiobis-(2-nitrobenzoic acid) as chromogen. The sequence of the reactions cascade is illustrated in Fig. 2. The amount of the enzyme reaction product, proportional to the amount of hydrolyzed ATChI degraded and, therefore, to the intensity of the yellow solution, was determined spectrophotometrically at 412 nm.

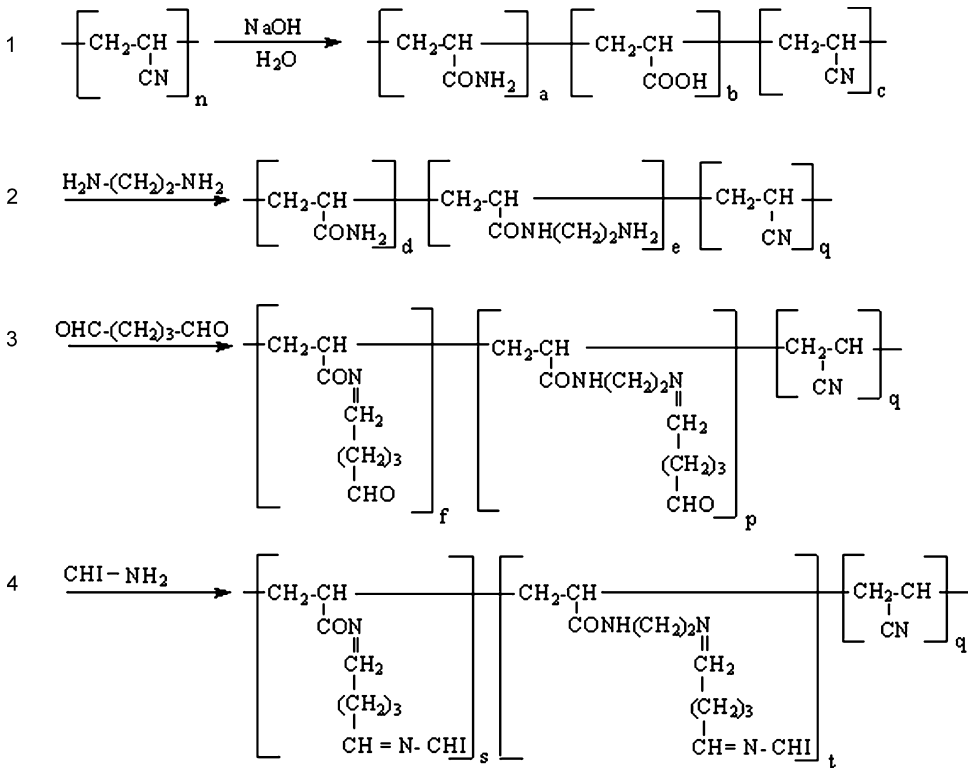


Fig. 1. Modification of PAN membrane.

The relative activity was determined as the ratio between the specific activity of a bound enzyme and the specific activity of the same free enzyme, multiplied by 100.

2.2.5. Storage stability

Activities of free and immobilized AChE after storage in phosphate buffer solution at 4 °C and were determined by measuring at 412 nm. The measurements were performed at intervals of week within a period of 60 days.

2.2.6. Reusability assay

The reusability of bound AChE was examined by conducting the activity measurement of bound AChE at 30 °C at time intervals of 30 min. After each activity measurement, the bound AChE

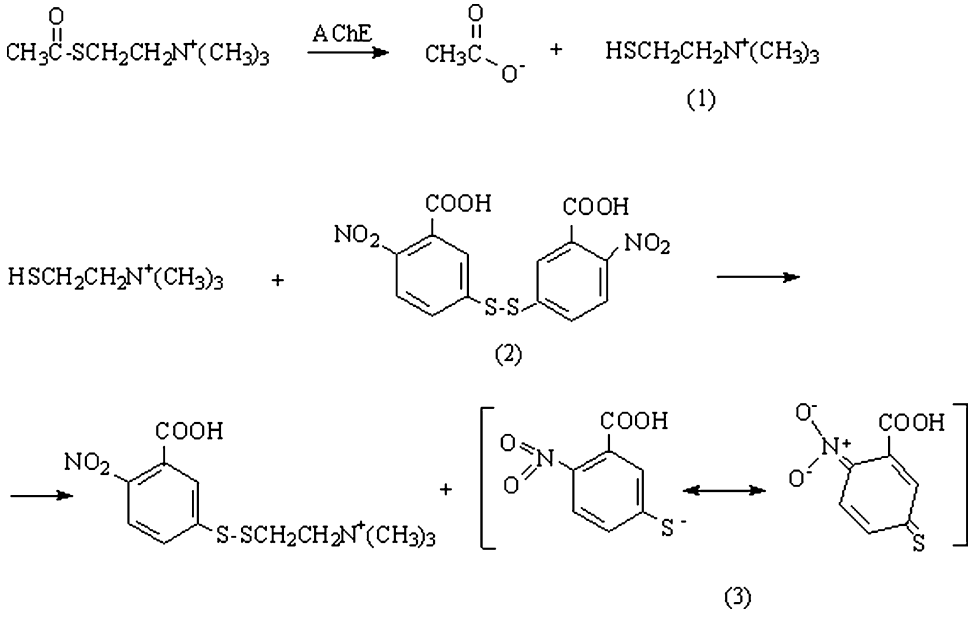


Fig. 2. AChE catalyzed hydrolysis of acetylthiocholine and thiocholine (1) reaction with Ellman reagent (2) with yellow anion (3) production.

was washed several times in 0.1 M sodiumphosphate buffer with corresponding optimum of pH.

3. Results and discussion

3.1. Physico-chemical characteristics of the modified membranes

The modified membrane by physically bound high molecular weight (HMW: 400 kDa) chitosan (PANCHI-A membrane) was obtained by filtering the chitosan solution through the PAN-NaOH + EDA base membrane and then by NaOH treatment. The modified membranes by chemical bound low molecular weight (LMW: 10 kDa) chitosan (PANCHI-B membrane) and high molecular weight (HMW: 400 kDa) chitosan (PANCHI-C) were obtained by chitosan tethering onto the membrane surface to form a dual-layer biomimetic membrane in the presence of glutaraldehyde. To optimize the extent of chitosan tethered, two kinds of chitosan with different molecular weight were used and the results were compared. For chitosan with molecular weigh of 10 000 Da, it was found that the extent of chitosan tethered was 0.19 g g^{-1} dry membrane, while that was 0.13 g g^{-1} dry membrane for chitosan with molecular weight of 400 000 Da. These results indicated that the decrease of chitosan molecular weigh can largely enhance the tethering extent of chitosan. It is well known that carrying out chemical reactions directly between polymers given their steric constraints, meets some difficulties in achieving good efficiency. The steric hindrance of chitosan limits its tethering on the membrane surface. And such hindrance is more serious for high molecular weight chitosan than that with low molecular weight.

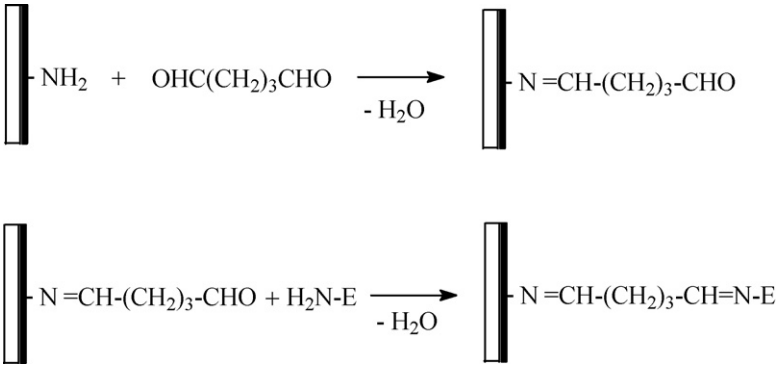
The basic characteristics of the three chitosan-modified membranes were determined. For comparison the characteristic of initial (PAN) and chemical-modified membranes (PAN-NaOH + EDA) were investigated. The amount of amino groups and hydrophilicity of the five types of membranes were presented in Table 1. It was found, that PANCHI-B membrane was contained the highest amount of amino groups (mgequiv. for g of modified membrane) and the PAN-NaOH + EDA membrane the lowest amount. The modifications increased the hydrophilicity (water content per gram of membrane) of the initial membrane, which was 67% (Table 1). The additional modification improved membrane hydrophilicity and it was higher in the membrane, which had higher degree of chitosan tethered, corresponding to higher amount of amino groups (Table 1). Every experimental point represents the average value of five experiments performed under the same conditions. The experimental errors never exceeded 0.5%.

The effect of the chemical modifications on the transport characteristics was studied. Table 1 shows the water flow of the modified membranes measured by an ultrafiltration cell (Sartorius, Germany) with nitrogen at 2×10^5 Pa. The modified membranes showed lower transport characteristics compared to the initial membrane. The reason for these results was the fact, that the HMW chitosan solution is more viscous and it has given denser film on the membrane surface and probably the smaller pores were closed.

SEM of the cross-section and SEM of the surface of unmodified and modified PAN membranes with NaOH + EDA and covalently bound chitosan were presented in Fig. 3. Fig. 3d, f, and h shows how the membrane morphology of the membrane surface has changed for the chitosan modification and NaOH modification compared to initial PAN membrane (Fig. 3a and b). The morphology change in the different chitosan membranes is essential (Fig. 3f and h).

3.2. Biochemical characteristics of immobilized enzyme

AChE was covalently immobilized onto all kinds of modified membranes using glutaraldehyde. The proposed method involves the following reactions:



where

The activity of immobilized AChE on the five types of membranes was determined. In this work, it can be seen that the relative activity of the immobilized enzyme on the dual-layer biomimetic membranes were higher (about 83%) than that on the membrane modified with NaOH + EDA (50%). Natural macromolecules, such as chitosan, cellulose, agarose and carragenan, show excellent biocompatibility and hydrophilicity, which usually lead to high relative activity for enzymes immobilized on them. Therefore, the immobilized enzyme on the dual-layer biomimetic membranes has a high relative activity compared to that on the PAN-NaOH + EDA, membrane, since the chitosan layer on support surface could reduce some non-biospecific enzyme–support interactions and create a biocompatible microenvironment for the immobilized enzyme. Comparison of the relative activity results for immobilized AChE on different dual-layer membranes was shown, that the highest relative activity has the immobilized AChE on PANCHI-B membranes. The reason for this probably is the highest amount of amino groups in this membrane.

The amount of bound protein on investigated membranes was determined (Table 1). It was found that the amount of bound protein depended on the content of amino groups on the membrane. Furthermore, compared to that on the PAN-NaOH + EDA membrane, there was an increase of the amount of bound protein on the dual-layer membrane. This phenomenon might be largely attributed to the presence of a large number of amino groups on the chitosan layer, which could provide many potential reaction sites for

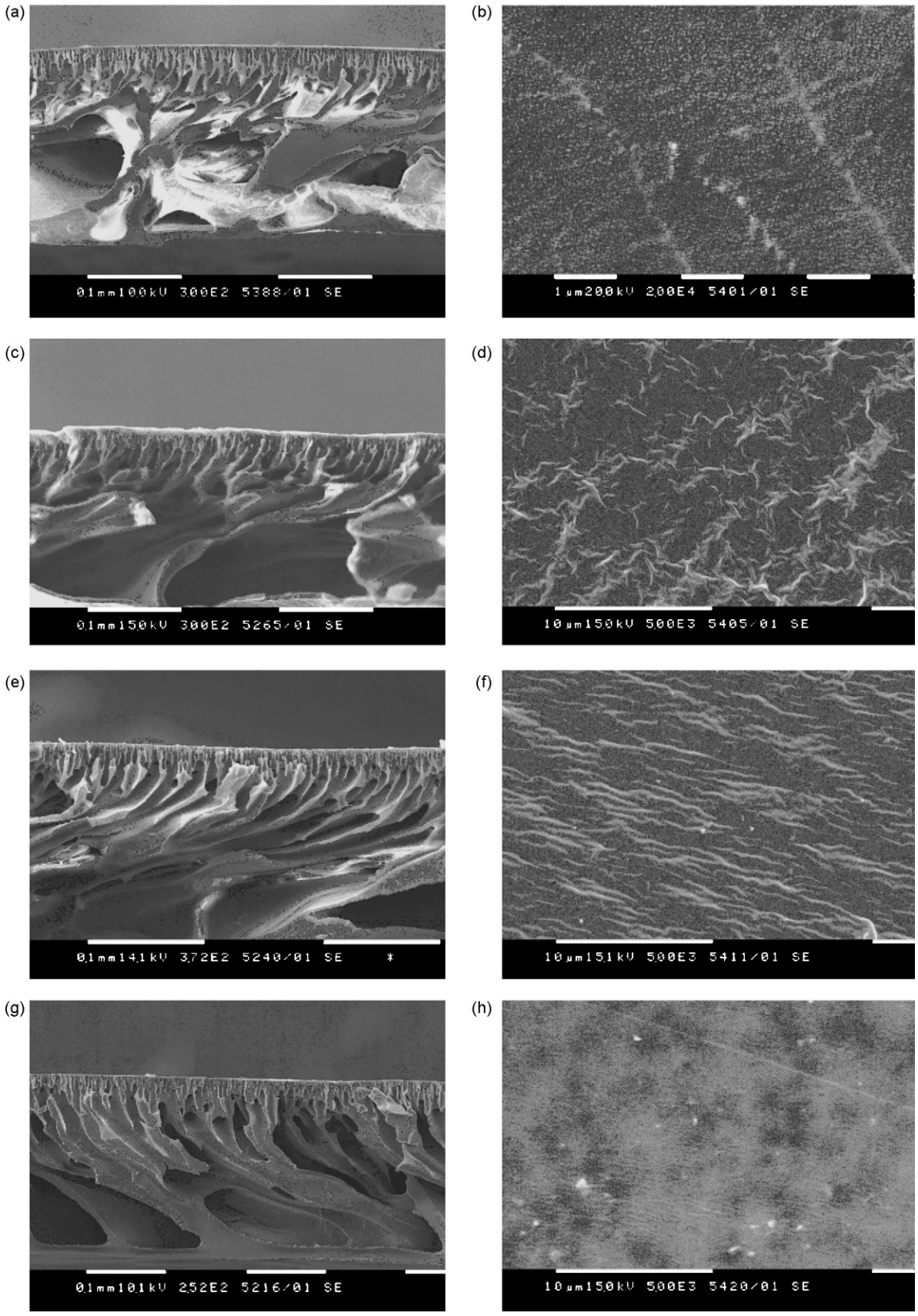


Fig. 3. SEM of the cross-section of PAN (a), PAN-NaOH + EDA (c), PANCHI-B (e) and PANCHI-C (g) and SEM of the surface of PAN (b), PAN-NaOH + EDA (d), PANCHI-B (f) and PANCHI-C (h).

Table 1
Basic characteristics of modified membranes

Type of membrane	Bound chitosan ($g\,g^{-1}$)	Amino groups ($mgequiv.\,g^{-1}$)	Degree of hydrophilicity (%)	Water flow, J ($m^3\,m^{-2}\,h^{-1}$)
PAN-NaOH + EDA	–	0.35	76	0.39
PANCHI-A	0.15	0.79	78	0.44
PANCHI-B	0.19	0.85	79	0.32
PANCHI-C	0.13	0.76	77	0.12
PAN	–	–	67	0.52

Table 2
Basic characteristics of enzyme membranes

Type of membrane	Relative activity (%)	Bound protein ($\mu g\,cm^{-2}$)	V_{max} ($\mu mol\,mg^{-1}\,min^{-1}$)	K_m (mM)
PAN-NaOH + EDA	50	30	0.75	9.55
PANCHI-A	82.5	80	0.46	1.17
PANCHI-B	84.2	84	4.40	3.03
PANCHI-C	83.2	79	2.73	2.48
Free enzyme	100	–	23.10	0.9

covalent coupling with protein. It should be pointed out that the difference between these immobilization methods had influenced the amount of bound protein and the relative activity of the immobilized enzyme.

The effect of the substrate concentration on the kinetics of the reaction catalyzed by free and immobilized AChE was studied using acetylthiocholine iodide as substrate at constant temperature and pH. Michaelis constant (K_m) and the maximum reaction rate (V_{max}) of the immobilized enzymes were calculated from Lineweaver–Burk plot. They were compared to the values, characteristic of free AChE (Table 2). It is obvious that V_{max} of free AChE is approximately 10 times higher than V_{max} of the immobilized AChE. The immobilized enzyme on the dual-layer biomimetic membranes exhibited higher V_{max} with comparison to that on the PAN-NaOH + EDA, membrane. A comparison of the V_{max} results for immobilized AChE on different dual-layer membranes has shown, that the highest V_{max} was observed for the immobilized AChE on PANCHI-B and PANCHI-C membranes.

A comparison of the values of K_m for a given enzyme in both immobilized and free states could provide information about the interaction between this enzyme and its support. An increase in K_m once an enzyme has been immobilized indicates that the immobilized enzyme has an apparent lower affinity for its substrate than the free enzyme does. The calculated values of K_m showed that the immobilized AChE on PANCHI membranes had lower apparent affinity to its substrate in comparison with free AChE. When comparing K_m values for the different modified membranes it is clear that they are lower for the chitosan-treated membranes.

The pH is one of the important parameters capable of altering enzyme activities in aqueous solution. The effect of pH on activity of the free and immobilized enzyme on all kinds of modified membranes was investigated within pH interval 6.0 and 8.6. Relative activity as function of pH is depicted in Fig. 4.

As shown in Fig. 4, optimum pH for free AChE was found to be 8.0. It should be noted that pH optimum of immobilized enzyme changed depending on the type of the modified membrane [23]. In our case the membranes were charged positively and pH_{opt} was shifted toward low pH value.

The pH stability of the free and immobilized AChE on all kind of modified membrane was determined by incubating in different buffers (pH 3.0–9.0) for 20 min at 35 °C (Fig. 5). The activity was then assayed under optimal conditions. The immobilized AChE showed improved stability retaining a considerable amount of activity at lower and higher pH values when compared to the free enzyme [24].

The temperature optima curves for the free and immobilized AChE onto modified membranes in the temperature range 20–40 °C are shown in Fig. 6. The optimum temperature was found to be 30 °C for free enzyme, whereas the temperature optimum of the immobilized AChE was shifted to 35 °C. This indicates that the immobilized AChE resisted denaturation due to temperature rise. Similar results have been reported for the immobilized AChE on different supports and an immobilization method applied [25,26].

Thermal stability experiments were carried out with the free and the immobilized enzymes, witch were incubated in the absence of substrate at 60 °C. Relative activity as a function of temperature is illustrated in Fig. 7. The bound enzymes on PANCHI-B and PANCHI-

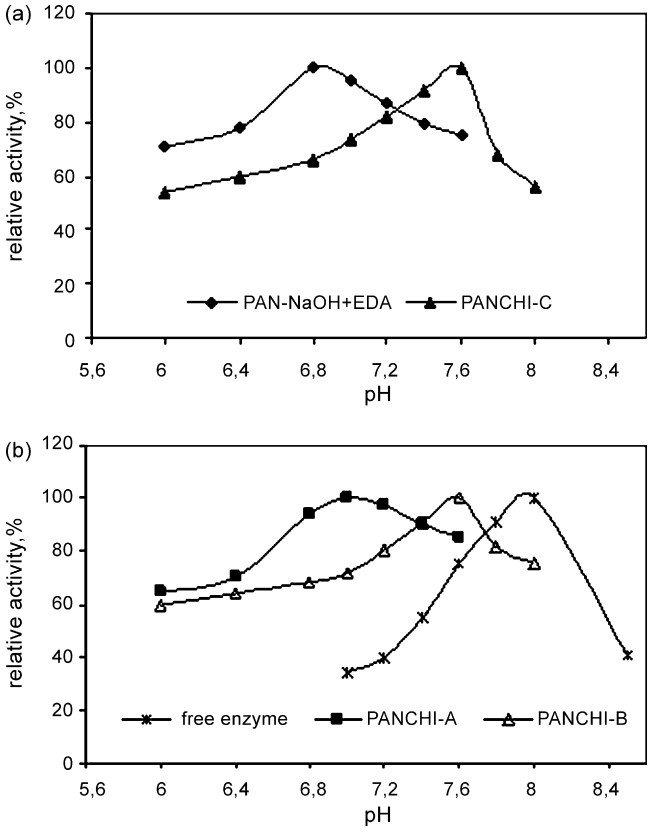


Fig. 4. Optimum pH of free and immobilized AChE.

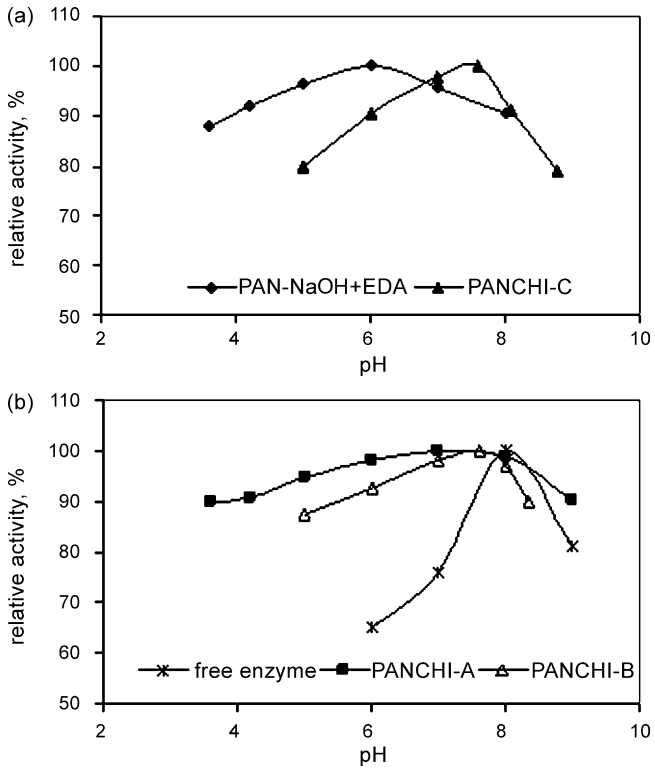


Fig. 5. pH stability of free and immobilized AChE.

C were more stable with time (20% of the enzyme activity was lost for 100 min) in comparison with AChE on membrane modified with PAN-NaOH + EDA and PANCHI-A. For this time the free AChE was almost inactivated.

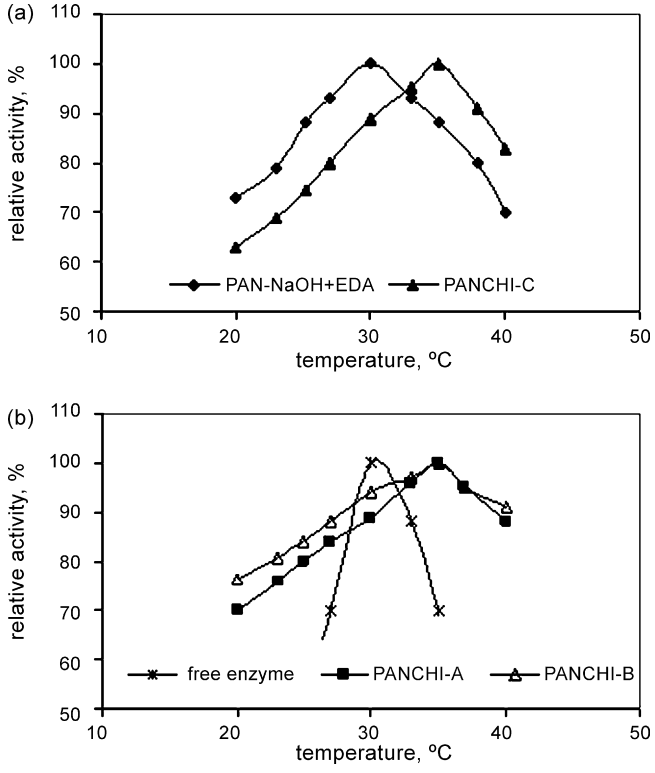


Fig. 6. Optimum temperature of free and immobilized AChE.

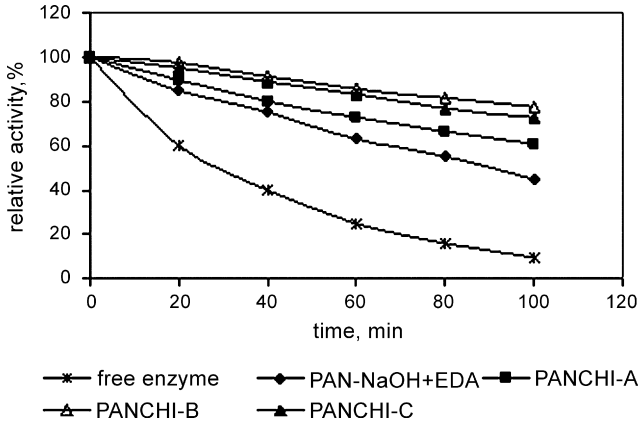


Fig. 7. Thermal stability at temperature 60 °C for free and immobilized AChE.

3.3. Industrial applicability

In order to investigate the industrial practicability of an immobilized enzyme, the loss of enzyme activity, known as storage stability, is an important parameter to be taken into account. The immobilized and free AChE was stored in phosphate buffer solution at 4 °C and activities were measured periodically over duration of 60 days. Upon 60 days of storage, the catalytic activity of free enzyme was retained 3% of its original activity (Fig. 8). In the same time immobilized AChE on all kinds of modified membranes retained above 45% of its activity, while AChE immobilized on PANCHI-B membrane retained about 75%. The covalent immobilization definitely held the enzyme in a stable position in comparison to the free counterpart [27]. It should be noted that the biocompatibility of the support could also play an important role on the stabilization of enzyme conformation [28].

Reusability of immobilized enzymes was important for their practical application. Reusability was carried out by measuring the activity of the immobilized enzyme (1 cm^2 enzyme membrane PANCHI-B) successive times. The maximum activity in the range of 100% was obtained at the beginning of reusability experiments. As shown in Fig. 9 the activity of bound AChE on PANCHI-B membranes showed good operation stability, and the enzyme activity loss was less than 50% after 10 times. This indicated the resultant bound AChE on modified PAN membranes with chemically bound chitosan had excellent reusability, which was desirable for applications in biotechnology.

These results demonstrated that the dual-layer biomimetic membranes were a potential support in the enzyme immobiliza-

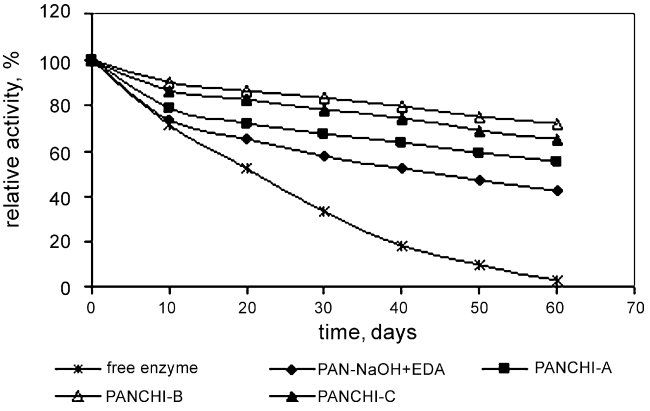


Fig. 8. Storage stability of free and immobilized AChE.

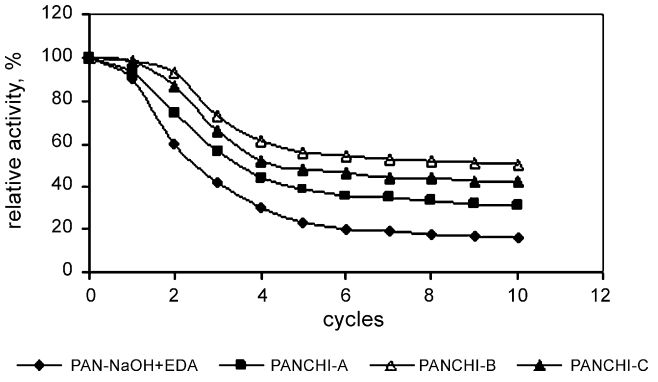


Fig. 9. Operation stability of immobilized AChE.

tion technology for analytical applications such as the detection of pesticide. The obtained results show that the membrane modified with low molecular weight chitosan (PANCHI-B) is most suitable carries for AChE immobilization.

Acknowledgements

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