Behavior of Immobilized Glucose Oxidase on Membranes from Polyacrylonitrile and Copolymer of Methylmethacrylate-Dichlorophenylmaleimide

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Received 15 September 2005; accepted 2 February 2006
DOI 10.1002/app.24221
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Two new ultrafiltration membranes were obtained from a polymer mixture, containing 60% polyacrylonitrile (PAN) and 40% copolymer of methylmethacrylate-dichlorophenylmaleimide (MMA-DCPMI). Membrane 1 (MB1) contains 40% DCPMI of the copolymer, and membrane 2 (MB2) contains 15% of the copolymer. The pore size, the specific surface, the water content, the water flux, and the selectivity were determined for the two membranes. The presence of dichlorophenylmaleimide in the copolymer ensures the preparation of membranes suitable for direct covalent enzyme immobilization without further modifications. These membranes were used for immobilization of glucose oxidase (GOD). High amount of bound protein was found on each of the membranes. High relative activities of the immobilized GOD were achieved, 72% for MB1 and 68% for MB2. The properties of the immobilized enzyme (GOD) were determined: optimum pH and temperature and pH, thermal, and storage stability, and then compared with the properties of the native enzyme. The kinetic parameters of the enzyme reaction, Michaelis constant (K_m) and maximum reaction rate (V_{max}), were also investigated. The results obtained showed that the ultrafiltration membranes prepared from the mixture of PAN and the copolymer MMA-DCPMI were suitable for use as carriers for the immobilization of GOD. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 4334–4340, 2006

Key words: biological applications of polymers; membranes; structure; enzymes

INTRODUCTION

Immobilized catalyst, enzymes or whole cells, are successfully employed in an increasing number of industrial processes.[1,2] The success encountered by the employment of immobilized enzymes in biotechnological processes stimulated the interest of the researchers toward the basic research addressed to improve the performance of the biocatalytic membranes. To this aim, the study of the interactions between the enzyme and the support became of fundamental relevance. It is well known that the immobilization process affects the enzyme activity in respect to that of the soluble counterpart. The physicochemical natures of the carrier as well as the immobilization methods are the main causes of the observed differences. The carrier nature acts mainly through the "partitioning effect,"[3] which is responsible for the changes in the chemical composition of the microenvironment in which the immobilized enzyme is operating in respect to that of the bulk solution. The immobilization method acts through the nature of the binding forces or through the type and the position of the amino acidic residues involved in the enzyme attachment. Covalent coupling is the most convenient immobilization technique, since it allows high accessibility and reusability of the bound enzyme.[4] Covalent attachment in addition offers the advantage that no relevant leaking of enzyme takes place in repeated uses, since the binding forces are stronger than those occurring in the adsorption or ionic binding.

Polymer membranes are widely used as carriers for immobilization of enzymes.[5–9] They possess some specific characteristics, such as exact chemical composition and physical structure, fixed porosity, and hydrophilic–hydrophobic balance, which provide a possibility to carry out quantitatively-defined immobilization of enzymes. The membranes based on copolymers of acrylonitrile (AN) with other vinyl monomers (such as methylmethacrylate, ethylmethacrylate) are very useful as carriers for enzyme immobiliza-

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tion.[10–13] Unmodified membranes of AN copolymer are not suitable as carriers. An additional modification of these membranes is usually performed to increase their conveniences. A few studies have dealt with the chemical modification of AN copolymer membranes.[14–16]

There are no references concerning membrane preparation on the basis of a mixture of polyacrylonitrile (PAN) and the copolymer methylmethacrylate-dichlorophenylmaleimide (MMA-DCPMI). Matcjka and Bleha[17] described the membranes from copolymer of *N*-phenylmaleimide with styrene. Other authors have studied the application of methylmetacrylate-*N*-phenylmaleimide copolymer for polymer blend.[18] By adding PAN to the copolymer MMA-DCPMI, polymer mixture improves its film-casting properties. The presence of dichlorophenylmaleimide in the copolymer ensures the preparation of membranes, which are suitable for direct enzyme immobilization without further modifications.

The present work describes the preparation of new type of membranes out of a mixture of 60% PAN and 40% copolymer MMA-DCPMI, which are used as carriers for a direct immobilization of glucose oxidase (GOD). The aim of this work is to study the characteristics of the ultrafiltration polymer membranes as well as the properties of the enzyme covalently immobilized onto them. GOD is selected for the experiments because of its widespread use and its importance as a reagent in medical diagnostics for determination of glucose concentration in biological liquids.[19–21]

EXPERIMENTAL

Materials

The following materials were used for the preparation of the ultrafiltration membranes polyacrylonitrile (PAN) (commercial product of Lukoil Neftochim Bourgas, Bulgaria), copolymer of methylmethacrylate-dichlorophenylmaleimide (synthesized through radical copolymerization of methylmethacrylate (MMA) and dichlorophenylmaleimide (DCPMI; "Konstantin Preslavsky" University, Shoumen, Bulgaria), in dimethylformamide, lithium nitrate, and glycerol (analytical reagent grade, Fluka Chemie AG, Switzerland). The immobilization was carried out with glucose oxidase (GOD) with a specific activity of 21 U/mg (Fluka Chemie AG, Switzerland).

Preparation of polymer membrane

About 18 g mixture of PAN (60%) and copolymer of MMK-DCPMI (40%) was dissolved in 100 cm^3 dimethylformamide. Then, 1 g lithium nitrate and 3 g glycerol were added to the solution. The polymer solution was stirred continuously in water bath at 60°C until the mixture was fully dissolved. Membranes were cast from the homogeneous solution by the phase-inversion method.[22] Distilled water at room temperature (20°C) was used as coagulating solution.

Immobilization of GOD

The membrane (100 cm^2) was immersed in a 25 cm^3 solution of GOD (with preliminary determined optimum concentration of 3 mg/cm^3), which was prepared in 0.1*M* phosphate buffer (pH 7). The immobilization was conducted at 4°C for 18 h. After that, the membrane was washed with distilled water, 1*M* NaCl, and 0.1*M* phosphate buffer (pH 7). The membrane thus prepared was stored in a 0.02 wt % water solution of NaN_3 at 4°C.

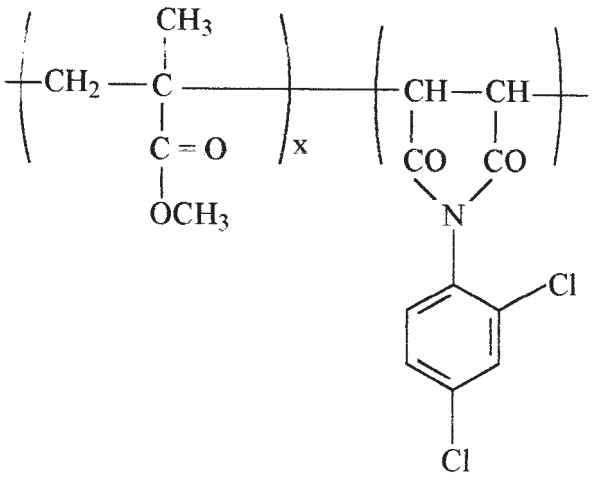
Analyses

The structure of PAN-copolymer composition was proved by infrared spectroscopy (IR-20, Carl Zeiss, Jena, Germany). The porosity of the membranes was studied by means of mercury porosimetry (Porometer 1500, Carlo Erba, Italy). The water content (per gram membrane) of membranes was calculated as being the weight difference between the water-swollen and dry membrane.[23] The water flux of the membranes was determined by using an ultrafiltration cell (Sartorius, Germany) at 25°C under nitrogen pressure of 2×10^5 Pa. The coefficient of permeability of the membranes was calculated from the water flux.[22] Membrane selectivity was determined with a Sartorius ultrafiltration cell using calibrants.[22] The determination of the amount of enzyme bound to the membrane was carried out by the method of Lowry et al.,[24] and absorption was registered by spectrophotometer Specol 11 (Carl Zeiss Jena, Germany) at wave length $\lambda = 750$ nm. The free and immobilized GOD specific activities were determined at static condition spectrophotometrically (Specol 11, Carl Zeiss Jena, Germany). The membrane (1 cm^2) with bound enzyme was immersed in 18 wt % solution of glucose in phosphate buffer with optimum pH. The enzyme GOD catalyzed the oxidation of β-D-glucose to β-D-gluconic acid and hydrogen peroxide. The latter easily diffuses into the solution and can be measured quantitatively (spectrophotometrically at 460 nm) by its interaction with 0.1 wt % *o*-dianizidine in the presence of 0.02 wt % peroxidase.[25]

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.

RESULTS AND DISCUSSION

The membranes prepared from a mixture of 60% PAN and 40% copolymer MMC-DCPMI featured functional



Scheme 1

groups, which were suitable for immobilization of biologically active substances. The copolymer was synthesized in "Konstantin Preslavski " University (Shumen), and its structure is as in Scheme 1.

Two types of copolymers were used being differentiated by the amount of dichlorophenylmaleimide: CP1 (40% of DCPMI) and CP2 (15% of DCPMI). The amount of active groups (Cl-ions) measured by mercurometric titration[26] was 12.25% for CP1 and 8.43% for CP2. Each of the two copolymers was further mixed with PAN (60% PAN + 40% copolymer) to prepare two membranes: MB1 (PAN-CP1) and MB2 (PAN-CP2). It was preliminarily found out that the optimum concentration of copolymer was 40%, because above this concentration, it is difficult to prepare a good polymer film. The thickness of both membranes was 150 μm and the specific surface area for MB1 was 55 m^2/g and for MB2 was 53 m^2/g. The pore size radius of both membrane selective layers was from 0.01 to 0.02 μm.

The water content of the two membranes was investigated, and the results are presented in Table I. It can be seen that MB1 is characterized by lower water content. The most probable reason for that is the greater amount of DCPMI in CP1, which imparts hydrophobic properties of the membranes.

The water flux and the coefficient of permeability were determined for the two membranes (Table II). Both membranes have similar values of the indicated

TABLE I
Water Content of Polymer Membranes

Membrane number	Polymer	Water content (%)
MB1	PAN-CP1	85.7 ± 0.4
MB2	PAN-CP2	88.8 ± 0.3

TABLE II
Characteristics of Polymer Membranes

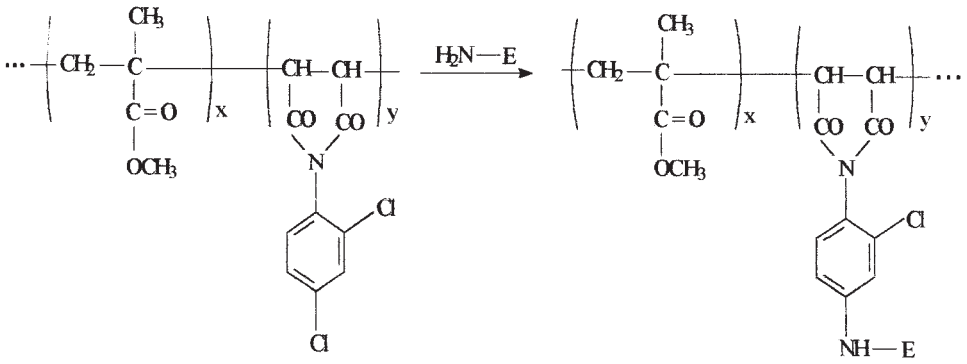
Membrane number	Water flux[a] ($m^3/(m^2\ h)$)	Coefficient of permeability ($m^3/(ms\ Pa)$)	Rejection[b] (%)
MB1	0.08 ± 0.04	0.50×10^{-10}	75 ± 0.9
MB2	0.11 ± 0.04	0.65×10^{-10}	80 ± 0.8
PAN	0.13 ± 0.07	0.68×10^{-10}	80 ± 0.8

[a]Measured at a pressure of 2×10^5 Pa and temperature of 25°C.
[b]For 0.1 % solution of albumin.

parameters. The selectivity of membranes towards 0.1% solution of albumin was also determined (Table II). These values correspond well to the values of industrially manufactured PAN membranes (cut off 60,000; commercial product of Ecofilter, Bourgas, Bulgaria), which indicates that the prepared membranes MB1 and MB2 are ultrafiltration ones.[27] The two membranes were used for immobilization of the enzyme GOD. A covalent immobilization of GOD to the DCPMI units was conducted according to Scheme 2.

It is well known that the Cl-ion is very active and the investigated copolymer allows direct immobilization of the enzyme.[4] Considering the copolymer structure, an interaction could be expected between Cl-ions in *p*-position with respect to a primary amino group of the enzyme molecule (lysine ε-amino group preferably). Since the nonmodified PAN had no functional groups suitable for enzyme immobilization, it is not presented in reaction for the covalent immobilization (Scheme 2). The direct immobilization of GOD was proven by the comparison of the IR spectra of the membrane MB2 with the one with immobilized GOD (Fig. 1). The absorption band of C—Cl noticed at 700 cm^{-1} almost disappears, when comparing the two IR spectra. The change in the second spectrum was due to the immobilized enzyme molecules.

The amount of bound protein and the activity of the immobilized GOD were determined (Table III). The enzyme activity was expressed as micromoles of glucose converted per minute per millgram of bound enzyme. As it can be seen, the immobilization yield was quite high for both membranes. The higher amount of DCPMI in CP1 explains the higher amount of protein bound to MB1. On the other hand, the activity of GOD immobilized on MB2 was higher. The relative enzyme activities for both membranes are as follows: 68% for MB1 and 72% for MB2. We assume that the local accumulation of protein on the surface of MB1 hinders the penetration of the substrate molecules into the active center of the enzyme molecules, which in turn determines the lower activity of GOD immobilized on MB1. These results clearly show the suitability of the membranes for being used as carriers for immobilization, since the specific activities of the



Scheme 2

bound enzymes are relatively high with respect to the activity of the free enzyme.

The properties of the immobilized GOD such as optimum pH and temperature, pH and thermal stability, and storage stability were investigated. As it is known from references, pH_{opt} of the native GOD does not depend significantly on the enzyme producer and it is between 5.6 and 5.8.[28,29] The used native GOD in this work was characterized by pH_{opt} 5.8 (Fig. 2, Curve 0), which complies with the references.

The pH optimum of GOD for both membranes was determined. Usually, pH_{opt} depends on a wide range of factors such as the type of carrier, activation mechanism of carrier, type of modifying agent, immobilization method, etc. Since the nonuniform proton distribution in the microenvironment of the bound enzyme and in the bulk solution (due to diffusion limitations and surface electric charges of the membrane), changes in the value of pH_{opt} for the bound enzyme was observed. The results obtained clearly show that pH_{opt} shifted to a greater value for both membranes: 6.2 (MB1, Curve 1) and 6.4 (MB2, Curve 2). The broader curve profiles of the immobilized GOD (on both membranes) clearly indicate the greater stability of the immobilized enzyme toward pH changes in the bulk solution as compared with the free enzyme. As it can be seen, the pH tolerance of GOD is much greater for MB2 (Curve 2).

Figure 3 represents the dependence of the enzyme activity (of both free and immobilized GOD on MB1 and MB2) on temperature. The conducted experiments showed 28°C as the optimum temperature for the free enzyme, whereas the optimum temperature for the immobilized enzyme was above 30°C (30°C for MB2, Curve 2; and 32°C for MB1, Curve 1). The curve profile of the immobilized enzyme is broader than that of the free enzyme. As it can be seen, the temperature tolerance of GOD is much greater for MB1. It is most likely that the greater amount of bound protein stabilizing the enzyme molecules shifts the optimum temperature to a higher value.

Stability studies are an important part of the whole set of analyses of immobilized systems. The results obtained from these studies are always compared with the results for free enzymes. In most of the cases, immobilized enzymes are more tolerant of exterior factors (temperature, pH, etc.) than the free ones. Therefore, it is widely accepted that enzyme immobilization retains the enzyme activity and enhances its conformational stability.[28] The pH stability of both free and immobilized GOD are represented in Figure 4. The experiment was conducted in buffer solutions

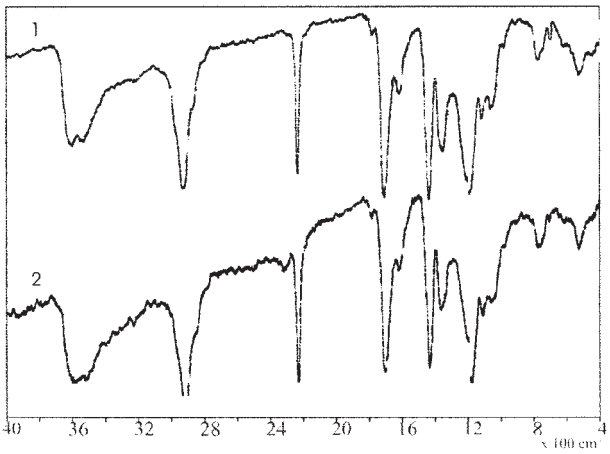


Figure 1 Infrared spectra of (1) MB2 (containing 15% DCPMI) and (2) MB2 with immobilized GOD.

TABLE III
Amount of Bound Protein and Enzyme Activity of GOD Immobilized on Polymer Membranes

Membrane	Bound protein (mg/cm^2)	Relative activity (%)
MB1	0.089	68.0
MB2	0.054	72.0

The enzyme activity was measured at static condition, pH 6.2 and 32°C (for GOD immobilized on MB1) and pH 6.4 and 30°C (for GOD immobilized on MB2), using 18% glucose in 0.1*M* phosphate buffer, for 5 min.

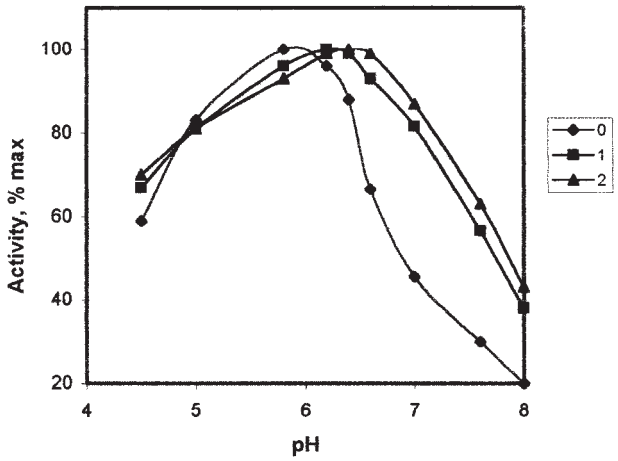


Figure 2 Effect of pH on the activity of free (0) and immobilized GOD on the membranes containing PAN and copolymer of MMA-DCPMI: MB1 (1) and MB2 (2). The activities were measured with 18% glucose solution in 0.1*M* buffer, at optimum temperature (28°C for free enzyme and 32 and 30°C for enzyme immobilized on MB1 and MB2, respectively) for 5 min.

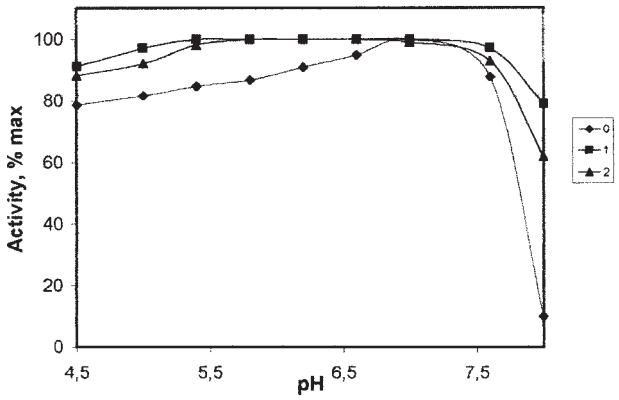


Figure 4 pH stability of free (0) and immobilized GOD on the membranes containing PAN and copolymer of MMA-DCPMI: MB1 (1) and MB2 (2). The enzyme was incubated for 30 min in 0.1*M* buffer (pH 4.5–8.0) at optimum temperature (28°C for free enzyme and 32 and 30°C for enzyme immobilized on MB1 and MB2, respectively). The activities were measured using 18% glucose solution in 0.1*M* phosphate buffer, pH 5.8 and 28°C (for free GOD) and pH 6.2 and 32°C (for GOD immobilized on MB1) and pH 6.4 and 30°C (for GOD immobilized on MB2), for 5 min.

with varying pH values (4.5–8.0) for 30 min at optimum temperature for each form of GOD. The free GOD showed maximum relative activity in a narrow pH interval (6.6–7.0), whereas the immobilized GOD featured much broader interval of pH stability (5.4–7.6). It is evident that the immobilization increases pH stability of the enzyme in both alkali and acidic range of pH interval. For example, at pH 8, the immobilized GOD activity was 80% (on MB1) and 60% (on MB2). The free enzyme activity decreased to 8% of the initial one at the same pH value.

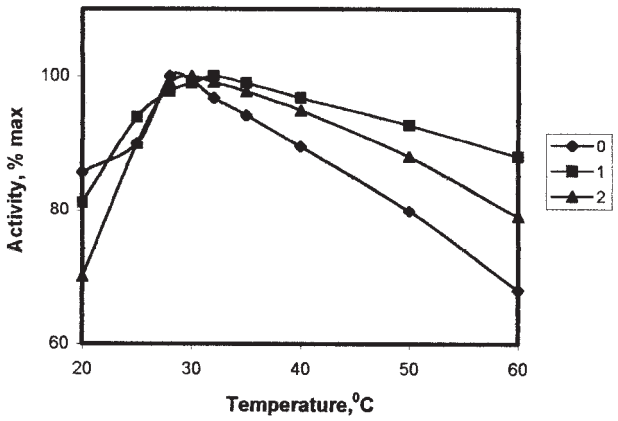


Figure 3 Effect of temperature on the activity of free (0) and immobilized GOD on the membranes containing PAN and copolymer of MMA-DCPMI: MB1 (1) and MB2 (2). The activities were measured with 18% glucose solution in 0.1*M* phosphate buffer with optimum pH (5.8 for free enzyme and 6.2 and 6.4 for enzyme immobilized on MB1 and MB2, respectively) for 5 min.

The investigation of the thermal stability for both free and immobilized GOD was conducted by incubating each enzyme form in 0.1*M* sodium phosphate buffer with optimum pH, at 30 and 50°C for 7 h (Fig. 5). During the indicated period and temperatures, the

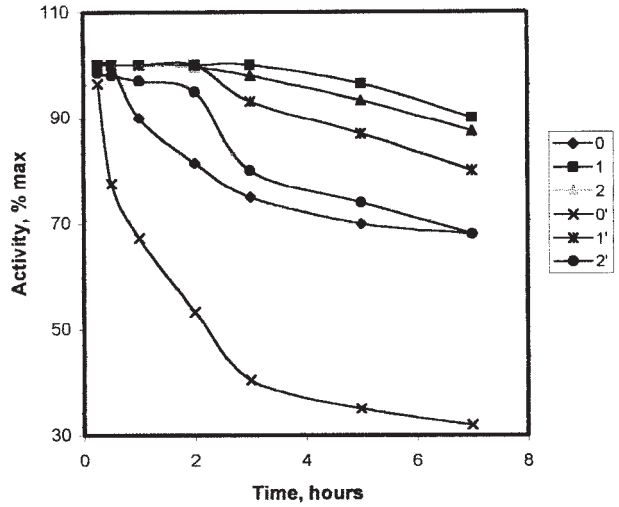


Figure 5 Thermal stability at 30°C (0, 1, 2) and 50°C (0′, 1′, 2′) of free (0, 0′) and immobilized GOD on the membranes containing PAN and copolymer of MMA-DCPMI MB1 (1, 1′) and MB2 (2, 2′). The enzyme was incubated for 7 h in 0.1*M* phosphate buffer with optimum pH (5.8 for free enzyme and 6.2 and 6.4 for enzyme immobilized on MB1 and MB2, respectively). The activities were measured using 18% glucose solution in 0.1*M* phosphate buffer, pH 5.8 and 28°C (for free GOD) and pH 6.2 and 32°C (for GOD immobilized on MB1) and pH 6.4 and 30°C (for GOD immobilized on MB2), for 5 min.

TABLE IV
Kinetic Parameters of Free and Immobilized GOD

Enzyme kind	Membrane	V_{max} (mol/(min mg))	K_m (mol/L)
Free	–	9.38×10^{-6}	1.9×10^{-2}
Immobilized	MB1	9.20×10^{-8}	2.4×10^{-2}
Immobilized	MB2	1.36×10^{-7}	2.0×10^{-2}

activity of the free enzyme decreased significantly with respect to the activity of the immobilized enzyme (68% from the initial enzyme activity at 30°C and 32% at 50°C). At 30°C, the immobilized enzyme activity was 90% from the initial enzyme activity (for MB1) and 87% (for MB2) for the same period of time, whereas at 50°C the obtained results were as follows: 80% (for MB1) and 68% (for MB2). Considering the indicated values in Figure 5, a conclusion can be drawn that the MB1 immobilized system is the most tolerant to temperature incubation, which could be explained by the greater amount of bound protein on the membrane surface.

Another characteristic of the immobilized enzyme is storage stability. It was estimated by measuring the enzyme activity at certain intervals for an appropriate period of time. The membranes with immobilized GOD were immersed in 0.02 wt % aqueous solution of sodium azide and stored for 90 days at 4°C. After that period of time, the immobilized enzyme activity decreased only by 4% (MB1) and 6% (MB2). The activity of the free enzyme decreased with 15% for the same period.

The kinetics of the enzyme reactions catalyzed by both enzyme forms was also investigated. Lineweaver–Burk method was used. A series of measurements (6–7) of the enzyme activity were conducted under optimum conditions (optimum temperature and pH for each enzyme form), varying the substrate concentration within the range of 5×10^{-3}–1×10^{-1} mol/L. Michaelis–Menten constants and the maximum reaction rates were calculated on the basis of the function $1/v = f(1/S)$ (Table IV).

The similar values of K_m for the free and immobilized GOD undoubtedly indicate that the enzyme bonding to the membrane surface does not influence significantly the enzyme affinity to glucose. During the covalent immobilization, the affinity to the substrate molecule is usually reduced. In our case, the values of K_m of the immobilized GOD and the free enzyme are almost equal. These results confirm the advantages of this immobilization method and this kind of support. The maximum reaction rate of the immobilized GOD lessened about 100 and 69 fold for MB1 and MB2 respectively, with respect to V_{max} of the enzyme reaction catalyzed by the free GOD. The decline of the V_{max} values after the immobilization is fully expected, since it is well known that covalent immobilization features greater diffusion limitations with respect to the substrate. If we compare these results with similar ones from other references,[5,30] we will find out that they are almost identical. Considering Table IV, it is easy to distinguish the immobilized system based on MB2 as the one with better kinetic parameters ($K_m = 2 \times 10^{-2}$ mol/L; $V_{max} = 1.36 \times 10^{-7}$ mol/(min mg) enzyme). The main reason for this involves the lower amount of bound protein to the membrane surface, which in turn leads to smaller diffusion limitations with respect to the substrate and better kinetic parameters of the immobilized GOD.

CONCLUSIONS

The new type of ultrafiltration membranes was prepared out of a mixture of 60% PAN and 40% copolymer MMA-DCPMI. Two types of copolymers differentiated by the amount of DCPMI were used: CP1 (40% of DCPMI) and CP2 (15% of DCPMI). The suitability of the membranes for direct covalent immobilization of GOD was demonstrated as well. A high immobilization yield was observed for both membranes. High relative activities of the immobilized GOD were achieved: 72% (MB2) and 68% (MB1). The results obtained proved MB2 (with 15% DCPMI in the copolymer) to be a better carrier for a covalent immobilization in comparison with MB1.

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