Covalent Immobilization of Penicillin G Acylase onto Chemically Activated Surface of Poly(vinyl chloride) Membranes for 6-Penicillic Acid Production from Penicillin Hydrolysis Process I. Optimization of Surface Modification and Its Characterization

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ABSTRACT: The covalent immobilization of penicillin G acylase (PGA) onto the surface of NH_2–poly(vinyl chloride) (PVC) membranes was studied. PGA was chosen because it plays a relevant role in the pharmaceutical industry, catalyzing the production of an important intermediate for the industrial production of semisynthetic penicillin and cephalosporine. Because PVC has no functional groups in its structure, in this work, we focused on the functionalization of PVC with primary amine functional groups for the covalent immobilization of PGA. This goal was achieved through an aminoalkylation process of the surface of the PVC membranes with ethylene diamine followed by activation with glutaraldehyde to finally immobilize the enzyme. Different factors affecting the modification and activation processes were studied, and their impacts on the catalytic activity of the immobilized PGA were followed. The functionalized membranes were characterized with Fourier transform infrared spectroscopy, thermogravimetric analysis, and scanning electron microscopy to verify the modification process. In addition, the changes resulting from the modification in physical characteristics, such as surface roughness, water uptake, and mechanical properties, were monitored. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: E27–E36, 2012

Key words: biological applications of polymers; biomaterials; enzymes; films; functionalization of polymers

INTRODUCTION

Enzyme immobilization is considered as to be one of the most used biotechnological applications of different synthetic polymers, including poly(vinyl chloride) (PVC).[1–9] Among the various methods available for enzyme immobilization, covalent binding is particularly important because it leads to the preparation of stable enzyme derivatives.[10–19] Suitable functional groups are essential for conducting such immobilization techniques. PVC has no functional groups in its structure, so chemical modification was carried out to introduce the proper functional groups. The grafting technique, with different types of polymers possessing different functional groups, has been presented extensively as the main solution, with either a chemical or radiation initiation system.[20–23] On the other hand, another simple technique using the aminoalkylation reaction with diamine was presented.[24] This technique depends on the reaction between the available chlorine atoms on the PVC surface and the amine groups of diamine. The introduced amine groups were further activated with a symmetric coupling agent, glutaraldehyde (GA), which covalently binds with the enzyme. The mechanism of PVC modification, activation, and immobilization with an enzyme is presented in Scheme 1. Even though such a technique has been investigated since almost 29 years ago,[9] still, no studies have been concerned with the optimization of the modification, and the activation processes have been published. Recently, the same

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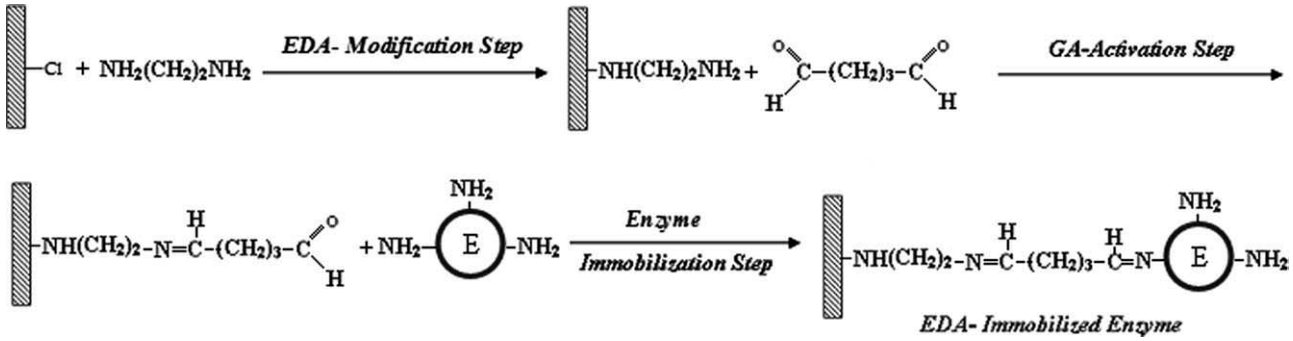

Scheme 1 Mechanism of PVC modification, activation, and enzyme immobilization.

technique was used by other authors in the immobilization of *Candida rugosa* lipase via GA coupling onto a functionalized hydrophobic polypropylene chloride membrane prepared by the amination of chlorinated polypropylene with hexamethylenediamine.[25] Other authors have immobilized invertase by covalent linking on the inner surface of a PVC tube. This was achieved through the introduction of an active functional group on the surface of an inert PVC tube through 1-fluoro-2-nitro-4-azidobenzene (FNAB), a precursor of highly reactive nitrene, which can be inserted into any C—H bond. CCl_4, lacking a C—H bond, is used as a solvent for loading the FNAB solution into the tube. The FNAB-loaded tube is then exposed to sunlight for 20 min, during which the azido group of FNAB generates nitrene and attaches itself to the PVC tube through an insertion reaction. Invertase is immobilized in the activated PVC tube at 50°C in 45 min. The invertase-embedded PVC tube is used as a flowthrough reactor to convert sucrose to invert sugar.[26]

In this work, penicillin G acylase (PGA) was covalently immobilized on the surface of GA-activated PVC membranes for the first time and evaluated in the penicillin hydrolysis process for the production of 6-penicillic acid (6-APA). Both the modification and activation processes were extensively investigated, and the optimum conditions were reached. Finally, the activated PVC membranes were characterized with Fourier transform infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA), and scanning electron microscopy (SEM) to monitor the resultant changes. PGA was selected according to its relevant role in the pharmaceutical industry, as it catalyzes the production of an important intermediate for the industrial production of semisynthetic penicillin and cephalosporine.

EXPERIMENTAL

Materials

PVC, ethylenediamine (EDA), and PGA (E.C.3.5.1.11) were obtained from Sigma Chemical Co. (St. Louis, MO), GA was obtained from Fluka (packed in Switzerland), and penicillin G was obtained from El-Nasr Pharmaceutical Co for Chemicals (Egypt). All other chemicals used were analytical-reagent grade. The buffer solutions were prepared with distilled water.

Methods

Each experimental point represents the average of three measurements.

Preparation of the PVC membranes

The PVC membranes were prepared by the dissolution of 0.125 g of PVC in 10 mL of tetrahydrofuran. The polymer solution was poured into a Petri dish of (diameter = 9 cm). It was rotated on a horizontal, flat surface to get an even distribution of the polymer over the glass. Then, the membrane was washed many times with distilled water to remove an excess amount of tetrahydrofuran solvent.[27]

Membrane surface modification

The PVC membranes were functionalized by treatment with a large excess of aqueous solution of EDA with a preselected concentration. Thus, a small piece of PVC membrane (2×1 cm^2) was added to 20 mL of the amine solution in distilled water and shaken in a shaking water bath maintained at 80°C for 60 min unless other conditions are stated. After completion of the reaction, the membranes washed with distilled water to remove unreacted EDA and dried in an air dryer.[28]

Enzyme immobilization

The modified PVC membranes were activated normally with 20 mL of GA (1%) of pH 8.0 at 40°C for 60 min unless other conditions are mentioned. After completion of the activation process, the activated PVC membranes were washed with distilled water to remove excess unreacted GA. The activated PVC membranes were then transferred to an

enzyme–buffer solution with a definite concentration for 16 h at 4°C to complete the immobilization process.

Determination of the immobilized enzyme activity

The catalytic activity of the immobilized enzyme was measured with a potassium penicillin G (PGK) buffer solution. The hydrolysis of PGK by PGA yielded 6-APA. The produced 6-APA was determined by means of a spectrophotometric method with *para*-dimethylaminobenzaldehyde as a colorimetric substrate.[29] One enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 μmol of 6-APA per min in a 4% (w/v) solution of PGK at pH 7.8 and 37°C.

TGA

The thermal degradation behaviors of the PVC membranes were studied with a thermogravimetric analyzer (Shimadzu TGA-50, Japan) in the temperature range from 20 to 600°C under nitrogen at a flow rate of 20 mL/min and at a heating rate of 10°C/min.

Morphological characterization (SEM)

The surface morphology of PVC and the modified PVC membranes was observed with the help of a scanning electron microscope (JEOL JSM 6360LA, Japan) at an accelerated voltage of 20 kV. The fracture surfaces were vacuum-coated with gold for SEM.

FTIR spectroscopic analysis

The structures of the PVC membranes and modified PVC membranes were analyzed by FTIR spectroscopy. The samples were mixed with KBr to make pellets. FTIR spectra in the absorbance mode were recorded with an FTIR spectrometer (Shimadzu FTIR-8400 S, Japan) and connected to a PC, and the data was analyzed by IR Solution software, version 1.21.

Surface roughness measurements

The surface roughnesses of the PVC and modified PVC were observed with the help of a surface roughness tester (Mitutoyo, SJ-201P, Japan),with cut-off length displayed change in the range of 0.25 mm.

Determination of the water uptake (*W*%)

For a membrane previously immersed in distilled water at room temperature for 24 h, the surface was dried by wiping with a filter paper and weighing. The obtained results were the average of three samples.[30]

$$W\% = \frac{\text{Weight of the wet membrane(g)} - \text{Weight of the dry membrane(g)}}{\text{Weight of the dry membrane(g)}} \times 100$$

Mechanical properties

The tensile strength (TS) and percentage elongation (%*E*) at break were measured on rectangular strips 3×1 cm^2 and about 0.02 mm thick. The TS indicates the maximum stress developed in a film during a tensile test, whereas %*E* indicates the capacity of the film to stretch.[31]

RESULTS AND DISCUSSION

Few publications concerning the covalent immobilization of enzymes onto a chemically modified, nongrafted, PVC surface matrix were recognized in the literature.[32–34] Here, the modification process of the PVC membrane surface followed by activation were studied. The modification process with EDA was characterized with different characterization techniques, including FTIR spectroscopy, SEM, and TGA. Different factors affecting the modification and activation processes were studied, and their effects on the activity of the immobilized PGA were examined. The processes of membrane modification, activation, and enzyme immobilization are illustrated in Scheme 1. The obtained results are discussed in detail accordingly.

Modification process

The surface modification of the PVC membrane was carried out with EDA to be functionalized with primary amine groups. Factors affecting the immobilization process of PGA, namely, EDA concentration, reaction time, reaction temperature, and PVC amount were studied. The obtained results are discussed in detail in the following text.

Effect of the EDA concentration

The effect of the variation of EDA concentration on the catalytic activity of immobilized PGA is shown in Figure 1(a). It is obvious that there was a linear decrease of activity with increasing of EDA concentration.

This behavior could be explained in correlation with a decrease in the amount of immobilized

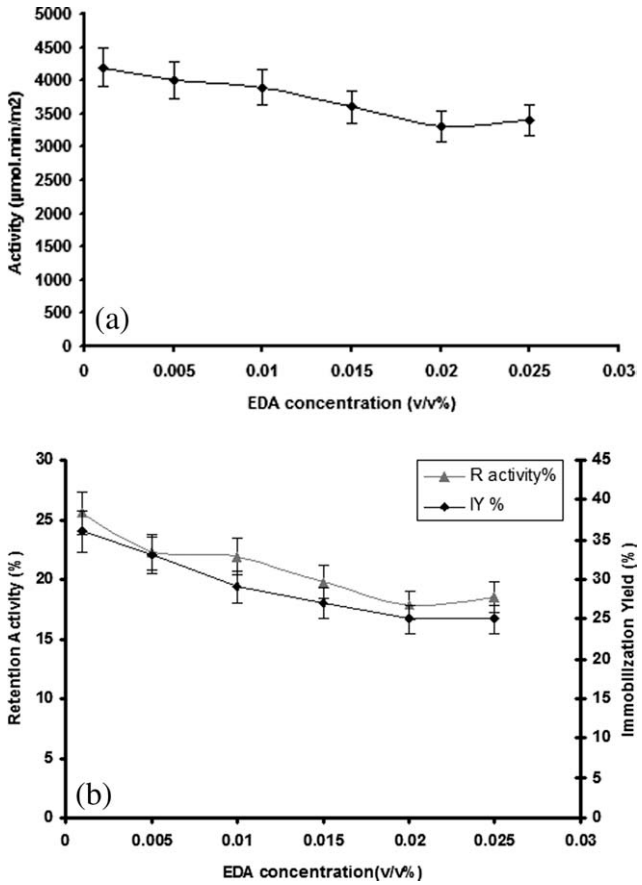

Figure 1 Effect of EDA concentration on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

enzyme with variation of EDA concentration according to two causes. The first one was the consumption of the two amine groups of EDA in some kind of binding between two Cl atoms on the surface of PVC; this led to reductions in the left NH_2 end groups available to react with GA and, mutually, the amount of binding enzyme. The second cause was the incorporation of the two end aldehyde groups in some kind of crosslinking with the terminal amine groups, which led to a reduction in the available end aldehyde groups for binding the enzyme molecules. The individual effect of each cause or the synergetic effect of both causes could be present an explanation of the obtained behavior. This assumption may explain the retention activity behavior [Fig. 1(b)].

Effect of the reaction temperature

The variation of the reaction temperature with EDA was found to have an obvious determining effect on the catalytic activity of the immobilized PGA [Fig. 2(a)]. From inspection the figure, we found that the activity decreased sharply with increasing temperature from 40 to 50°C. The membranes continued to lose their activity but with lower rate with increasing reaction temperature up to 60°C, which was the minimum value.

Beyond 60°C, the activity started to recover again. However, the activity at 80°C was still lower than its counterpart at 40°C, at which maximum value was obtained. On the other hand, the amount of immobilized enzyme, immobilization yield (IY%), showed the reverse behavior [Fig. 2(b)]. The obtained data indicated an increase of IY% with temperature elevation up to 50°C, and then, it leveled off. Joining together this behavior with the behavior of catalytic activity led directly to a sharp decrease of enzyme retention activity [Fig. 2(b)], from 32% to around 15%.

In general, an increase the activation temperature over 40°C decreased the activity in the same manner as the retention activity. This behavior could be have been due to the formation of protein–protein interaction [Fig. 2(b)].

Effect of the reaction time

The effect of the variation in reaction time on the catalytic activity of the immobilized PGA was investigated [Fig. 3(a)]. The obtained results confirmed the given explanation concerning the reaction between Cl on the surface of the PVC membranes with EDA. In general, an increase in the activation time with EDA

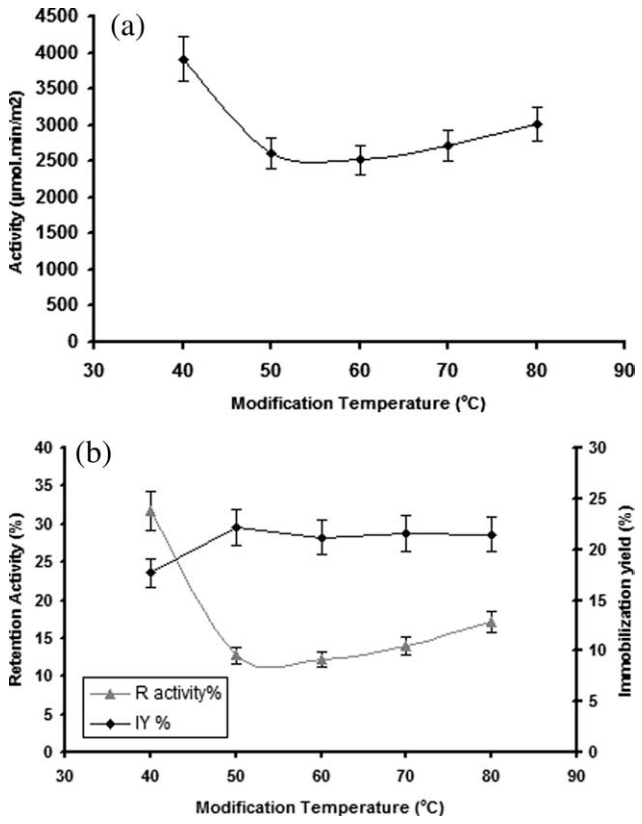

Figure 2 Effect of the reaction temperature with EDA on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

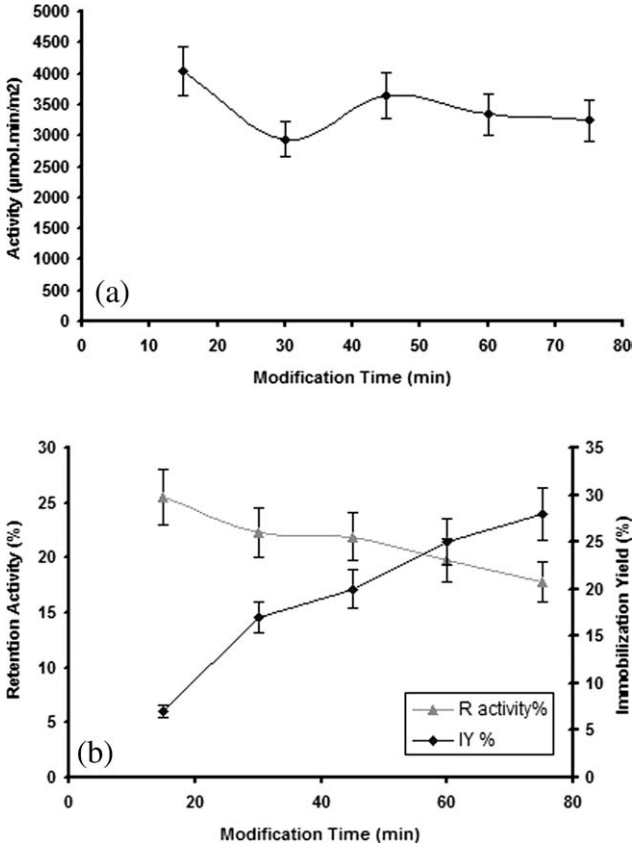

Figure 3 Effect of the reaction time with EDA on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

beyond 15 min decreased the activity. The activity decreased sharply with the reaction time with EDA until a minimum value was reached after 30 min.

The effect of the variation in reaction time on IY% and the retention activity is also illustrated in Figure 3(b). From the figure, it is clear that a decline of activity was a combined with an increase in the amount of immobilized enzyme. This behavior could be explained by the formation of protein–protein interaction due to an increase in the protein surface density, which reduced the diffusion of substrate to the enzyme active sites. This explanation was confirmed by the obtained results from the investigation of the retention activity [Fig. 3(b)].

Effect of the PVC amount

It is clear from Figure 4(a) that an increase in the PVC concentration used in the preparation of the membranes had a linear positive effect on the activity up to a concentration of 0.03%. With a further increase of the PVC concentration up to 0.05%, the rate of activity increase became lower and tended to level off with a continuous increase of PVC concentration up to 0.3%. The amount of immobilized enzyme was measured and was related to the retention activity measurement [Fig. 4(b)]. The illustrated data revealed that the maximum retention activity was obtained with samples prepared with 0.12% PVC. This behavior was a combined with mirror image behavior in IY%. The obtained data could be explained by the consumption of the two amine groups of EDA in some kind of binding between the two Cl atoms on the surface of PVC; this led to reductions in the left NH_2 end groups available to react with GA and, mutually, the amount of binding enzyme.

Although the amount of immobilized enzyme decreased with increasing PVC concentration, the activity was increased. This could be explained by the existence of protein–protein interactions at high IY%, which led to a reduction of activity. This effect reached its minimum value at 0.12% PVC, in which the maximum activity and retention activity (%) were obtained.

Activation process

PVC membranes with primary amino functional groups were activated with GA. Factors affecting the activation step, namely, GA concentration, reaction

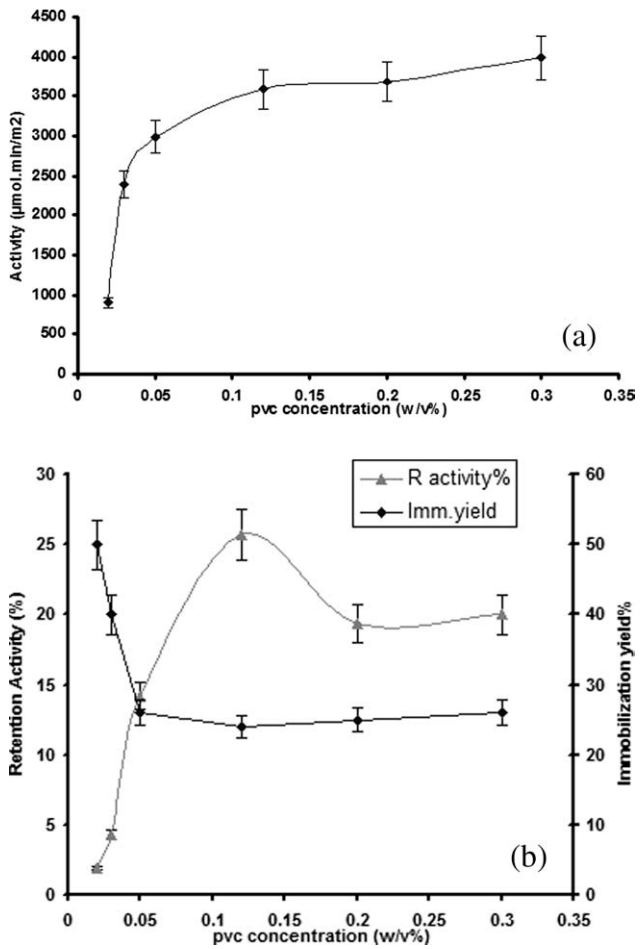

Figure 4 Effect of the PVC–EDA ratio on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

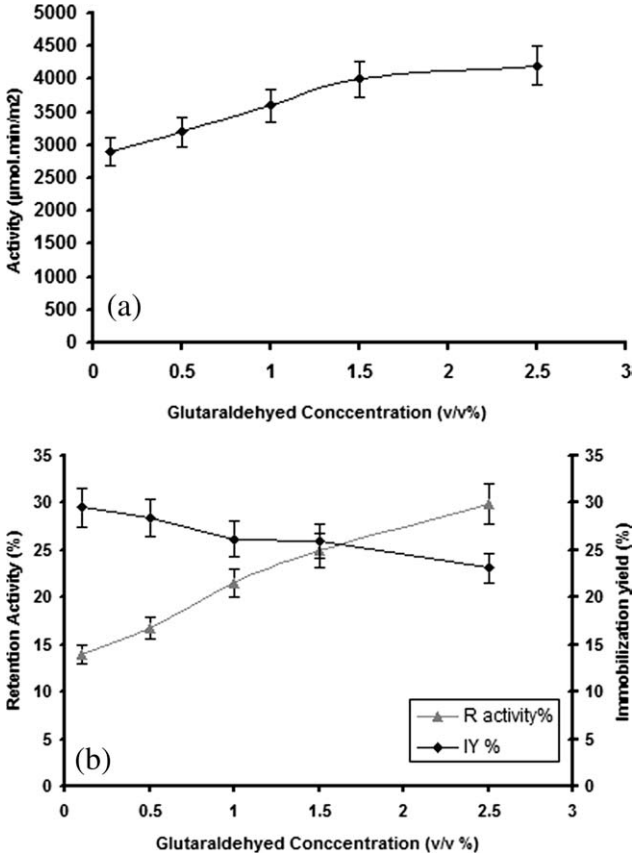

Figure 5 Effect of the GA concentration on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

time, reaction temperature, and reaction pH were investigated, and the obtained results are discussed in the following.

Effect of the GA concentration

The effect of the variation of GA concentration on the catalytic activity of the immobilized enzyme was studied [Fig. 5(a)]. A linear increment of the activity was observed with increasing GA concentration up to 1.5%. A further increase of the GA concentration to 2.5% increased the activity slightly.

Increasing the GA concentration was expected to increase the amount of immobilized enzyme; this caused an activity increment. Contrary to our expectations, the amount of immobilized enzyme decreased with increasing GA concentration [Fig. 5(b)]. This behavior may be explained by the consumption of both aldehyde end groups of GA in the crosslinking of the left terminal NH_2 groups on the surface of the aminated PVC. This led to a reduction of the terminal aldehyde groups available for enzyme immobilization. A decrease in the amount of immobilized enzyme reduced the occurrence of the protein–protein interaction process; this finally increased both the activity and the retention activity of immobilized enzyme.

Effect of the reaction temperature

When we studied the dependence of immobilized enzyme activity on the temperature of the activation process with GA solution, the maximum enzyme activity was obtained at 40°C. The activity decreased exponentially with increasing temperature of the reaction medium up to 50°C, where it lost 60% of its initial activity at 40°C. Further increases in the reaction temperature to 80°C continuously decreased the activity but at a lower rate to a loss of 80% of its activity at 40°C [Fig. 6(a)].

The retention activity was found to be almost constant, where IY% increased linearly [Fig. 6(b)]. The decline of activity in such a manner could have been due to the presence of a high enzyme density immobilized on the surface of the activated PVC membrane in the range causing protein–protein interaction.

Effect of the reaction time

Figure 7(a) illustrates the effect of the variation of the reaction time with GA on the catalytic activity of the immobilized PGA. The obtained data indicated that the activity of the immobilized enzyme decreased with increasing reaction time with GA. It

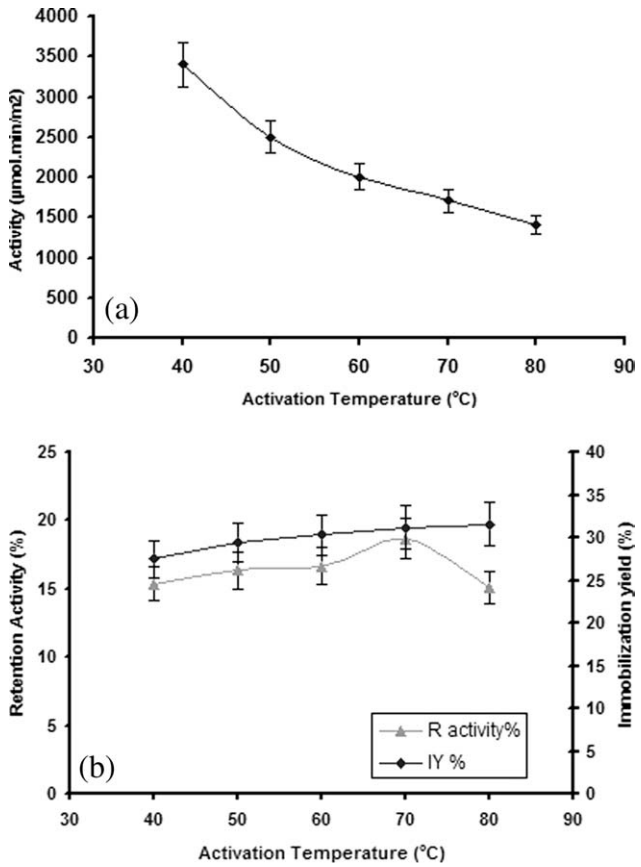

Figure 6 Effect of the reaction temperature with GA on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

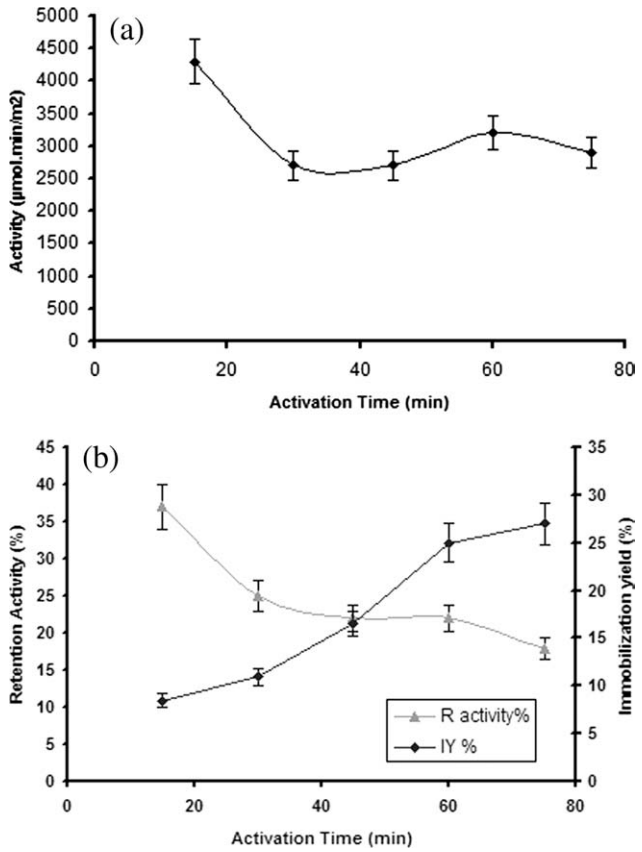

Figure 7 Effect of the reaction time with GA on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

was expected that with increasing reaction time, the amount of immobilized enzyme would increase and so would the activity. Over a certain protein concentration immobilized on the PVC membrane surface, protein–protein interaction tended to appear and lead to reductions in both the activity and the retention activity of the immobilized enzyme [Fig. 7(b)]. This limit was reached after 15 min of reaction time.

Effect of the reaction pH

It is well known that the pH of the medium greatly affected the reaction rate between aldehyde and primary amine groups [Fig. 8(a)]. From the figure, it is clear that there was a sharp increase in the immobilized enzyme activity with increasing reaction pH from 2.0 up to 8.0, when it reached its maximum value. A further increase of pH slightly affected the activity.

The retention activity of the immobilized enzyme followed the same trend as the activity. We could explain this trend by taking into consideration the amount of immobilized enzyme, which was found to increase linearly with the increasing rate of aldehyde group reaction with amine groups on the surface of the aminated PVC membranes because of the deprotonation of the amine groups in moving to an alkaline medium [Fig. 8(b)].

Membrane characterization

The verification of the surface modification process was obtained through FTIR spectroscopy and TGA of the modified PVC membranes. In addition, the morphological changes resulting from surface modification were monitored through SEM micrographs and surface roughness measurements of the membranes. *W*% and TS reflected the impact of the modification process on the physical and chemical prosperities, respectively. The obtained results are discussed in the following sections.

FTIR analysis

The FTIR spectroscopic analyses of the PVC membrane, activated membrane, and immobilized membrane were carried out from 400 to 4000 cm^{-1} (Fig. 9). The IR spectrum of the PVC membrane showed characteristic peaks (curve A).The spectra for aminated PVC showed a new broad peak at 3357 cm^{-1}; this corresponded to NH_2 groups, which indicated the presence of amine groups on the surface of the

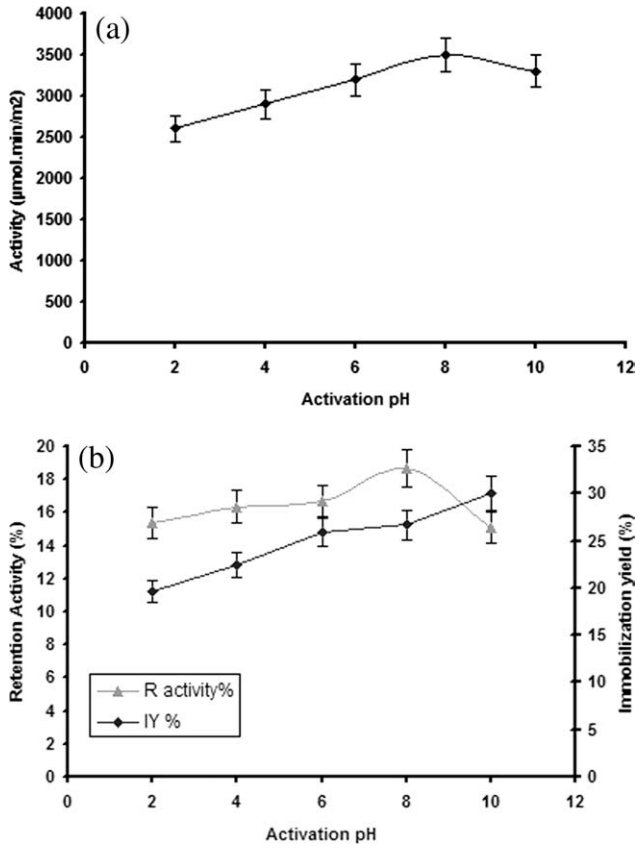

Figure 8 Effect of the pH of GA solution on the (a) catalytic activity of the PVC membranes and (b) retention (R) activity and IY% of immobilized PGA.

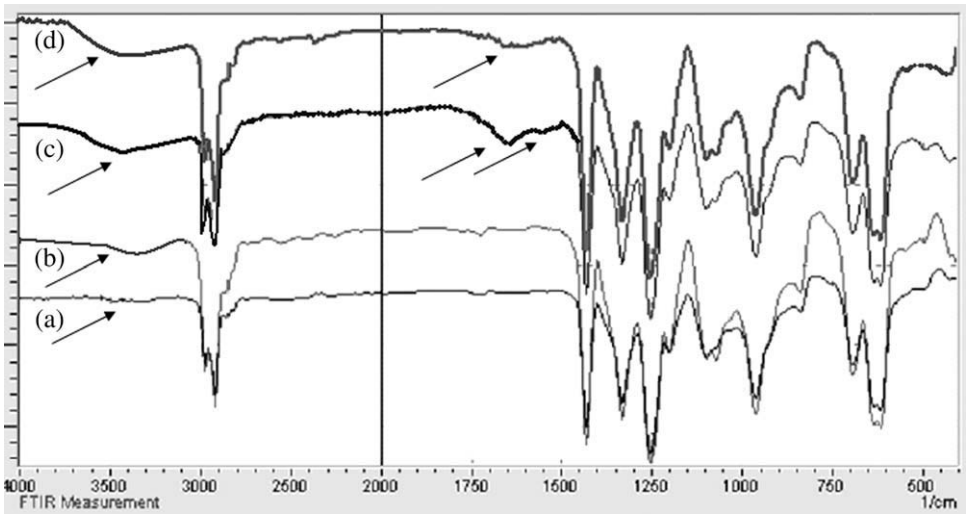

Figure 9 FTIR spectra of (a) PVC membrane, (b) aminated membrane, (c) activated membrane, and (d) immobilized one.

membrane (curve B). The GA-activated membranes showed new two peaks. The first of them at 1720 cm^{-1} referred to the carbonyl groups (C=O) of the free aldehyde end of GA, and the other peak at 1670 cm^{-1} referred to C=N— bonds, which resulted from the reaction of NH_2 end groups with GA (curve C). Finally, the spectra of the catalytic membranes gave a broader peak at 3392 cm^{-1}; this indicated an increase in the concentration of NH_2 groups found naturally in the enzyme and a reduction of the intensity for the peaks at 1720 cm^{-1} as a result of the reaction with enzyme surface amine groups. From all of these results, we could be sure that the processes of amination, activation, and immobilization took place successfully. This result was in agreement with other published results.[35]

TGA

TGA is a simple and accurate method for studying the decomposition pattern and thermal stability of polymers. As shown in Figure 10(a), the PVC membrane, we found that there were two characteristic peaks. The fist one at 374.4°C resulted from the breaking of the C—Cl bond. The second one at 591°C referred to the decomposition of the ethylene bond. In the second curve [Fig. 10(b)], the aminated PVC, a new band appeared at 135°C, which resulted from the evaporation of water molecules from an increase in the hydrophilicity after the reaction with EDA. The C—Cl band shifted to 378.2°C, which indicated some stability of the membrane. In the third curve [Fig. 10(c)], the activated PVC membranes, there was no effect of humidity, and the mean band shifted back to 360°C. In the last curve [Fig. 10(d)], the catalytic PVC membranes, the band of humidity appeared again because of the NH_2 groups present in the enzyme. On the other hand, the mean band shifted to 386.6°C; this referred to more stability for the membrane immobilized with enzyme.

SEM morphological examination

Figure 11 displays SEM results for the PVC, aminated PVC, activated PVC, and immobilized one. From these curves [Fig. 11(a–d)], we noticed changes after each step, and this was confirmed by surface roughness measurements.

Surface roughness measurements

The surface roughness values of the PVC membrane, aminated membrane, and immobilized one are shown in Table I. It was clear that the roughness of the membranes increased with amination, activation, and finally immobilization with enzyme. This

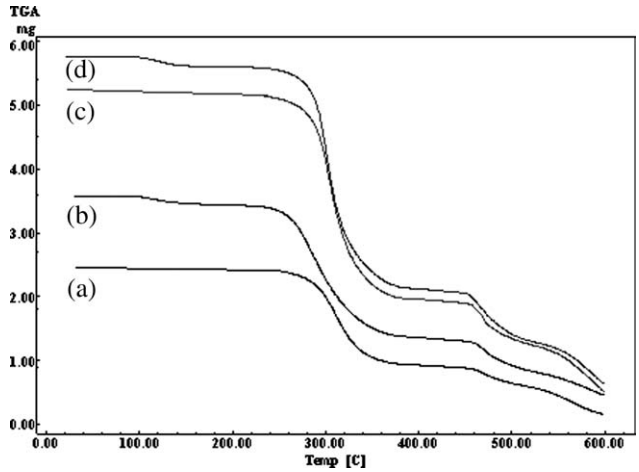

Figure 10 TGA thermographs of the (a) PVC membrane, (b) aminated membrane, (c) activated membrane, and (d) immobilized one.

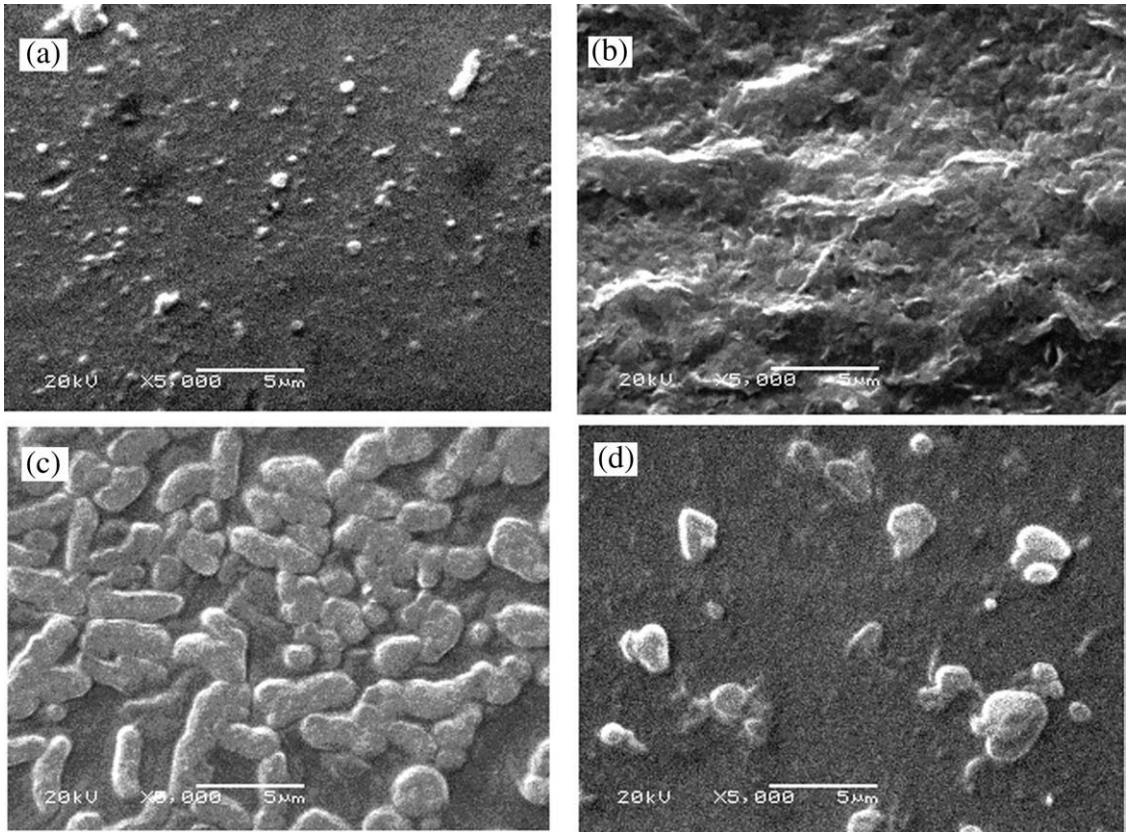

Figure 11 SEM of the (a) PVC membrane, (b) aminated membrane, (c) activated membrane, and (d) immobilized membrane.

finding was confirmed the occurrence of the amination, activation, and immobilization processes.

Water uptake (W%)

The *W*% values of the PVC membrane, aminated membrane, and immobilized membrane are displayed in Table I. From this table, the increase of water sorption of the aminated membrane over PVC itself is clear. This was due to the increase in the hydrophilic groups on the membrane surface, which were represented by NH_2 groups. On the other hand, after treatment of the aminated membrane with the GA, the water sorption also increased. This was due to the increase in the hydrophilic groups (H—C=O). Also, the immobilized one showed more water sorption, and this was confirmed by the greater increase in hydrophilic groups. From all of the previous results, we noticed that *W*% improved slightly. This was due to the fact that the amination, reaction with GA, and immobilization processes occurred on the surface of the membranes. This was confirmed by SEM examination.

TS measurements

The tensile properties of the PVC, activated, and immobilized membranes were measured and are shown in Table I. These were determined from the critical breaking point of the stretching test pieces. The effect of force and the elongation of the membranes were observed as a positive results as the elongation of the membranes increased with each step; this meant that after activation, the membrane became more elastic than the native one, except the one immobilized with enzyme. On the other hand, the activation of the membrane and further immobilization of enzyme led to a noticeable improvement of its mechanical properties and TS. In general, an improvement in the mechanical properties of the PVC modified, activated, and immobilized membranes with enzyme was obtained.

TABLE I
W%, Maximum Force, and Elongation and Surface Roughness Values of the PVC Membranes, Aminated Membranes, Activated Membranes, and Immobilized Membranes

Sample	*W*%	Max force (N)	Elongation (mm)	Surface roughness (μm)
PVC	0.67	2.960	0.888	1.40
PVC + EDA	3.6	3.125	1.151	1.75
PVC + EDA + GA	6.3	3.750	1.801	2.27
PVC + EDA + GA + enzyme	8.5	4.032	0.683	2.33

CONCLUSIONS

PVC membranes were successfully modified with EDA, and as a result, their surface was functionalized with amine groups. Minimum EDA concentration and minimum reaction temperature with EDA gave the best results of catalytic activity of the immobilized enzyme: 0.020% EDA and 40°C, respectively. The reaction time with EDA for 15 min was found as the optimum time needed to have the best catalytic activity. Aminated PVC membranes were activated with GA. The conditions of the activation process, including GA concentration, pH, reaction time, and reaction temperature, were investigated. The optimum conditions within the studied range were GA = 1.5%, pH = 8.0, 15 min, and 40°C, respectively. The modified PVC membranes were characterized with FTIR spectroscopy and TGA to prove the occurrence of functionalization with amine groups. A characteristic band at 3357 cm^{-1} for amine groups was recognized. A new thermogram stage at 25–150°C appeared in the TGA of the aminated PVC membranes. This range referred to the loss of water, which confirmed the hydrophilicity improvement of the surface due to the grafting of amine groups. The monitoring of the surface morphology changes was performed with SEM and surface roughness measurements. In conclusion, this technique proved to be effective in the immobilization of PGA enzyme on PVC membranes with high catalytic activity, which reached 4000 μmol min m^2.

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