Reactive & Functional Polymers 87 (2015) 37–45

Contents lists available at ScienceDirect

Reactive & Functional Polymers

journal homepage: www.elsevier.com/locate/react

Urease-carrying electrospun polyacrylonitrile mat for urea hydrolysis

Aref Daneshfar [a,b,*], Takeshi Matsuura [a,c], Daryoush Emadzadeh [a], Zahra Pahlevani [d], Ahmad Fauzi Ismail [a,*]

[a] Advanced Membrane Technology Research Centre (AMTEC), Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[b] Faculty of Chemical Engineering, Department of Polymer Technology, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[c] Industrial Membrane Research Laboratory, Department of Chemical and Biological Engineering, University of Ottawa, 161 Louis Pasteur St, Ottawa, ON K1N 6N5, Canada
[d] School of Engineering, Department of Materials Science and Engineering, Shiraz University, Shiraz, Iran

ARTICLE INFO

Article history:
Received 21 July 2014
Received in revised form 22 November 2014
Accepted 22 December 2014
Available online 6 January 2015

Keywords:
Electrospinning
Enzyme immobilization
Polyacrylonitrile
Urea hydrolysis
Urease

ABSTRACT

Electrospinning was used to fabricate beadless microfibrous polyacrylonitrile (ePAN) mats with an average fiber diameter of 1448 ± 380 nm from a 10 wt.% PAN in dimethylformamide (DMF) dope solution at applied voltage of 18 kV and 20 cm fiber collection distance. Urease (EC 3.5.1.5) was then covalently immobilized on dispersed microfibrous ePAN mats following the chemical treatment of fibers with ethylenediamine (EDA) and glutaraldehyde (GA). The optimal concentration of GA for immobilization was 5%. The amount of loaded urease reached 157 μg/mg mat, exhibiting 54% of the free urease activity. The surface chemistry of as-spun and chemically treated fibers was examined with Fourier transform infrared (FTIR) spectroscopy. Field emission scanning electron microscopy (FESEM) was used to study the morphology and diameter of the pristine, chemically treated, and urease-immobilized microfibrous mats. Immobilized urease showed increased temperature for maximum activity (from 37 to 50 °C for free and immobilized urease, respectively) and improved storage stability (20 days). The immobilized urease was also less sensitive to the changes in pH, especially in acid conditions. In addition, nearly 70% of initial activity of the immobilized urease was retained after 15 cycles of reuse, which proved the applicability of the electrospun fibers as successful enzyme carriers.

1. Introduction

In quest of a reliable way for daily removal of blood urea from patients suffering from permanent renal failure, artificial wearable or portable kidney machines have recently been developed [1,2]. In order to maintain the device within an acceptable working condition, the dialysate solution, which is mainly contaminated with urea, should be regenerated continuously. Urea is a remarkably stable molecule with degradation half-life of 3.6 years at 38 °C when dissolved in water. Due to its non-ionic polar nature, it can hardly be removed from water by ion-exchange media, activated carbon granules, or reverse osmosis membranes [3]. Thus, it seems that the most effective method for regeneration of the dialysate solution in the wearable kidney machine is the utilization of urease [4], a nickel-based enzyme that catalyzes urea hydrolysis to ammonia and carbon dioxide 10^{14} times faster than uncatalyzed urea hydrolysis.

To overcome the intrinsic vulnerability of free enzymes to the changes that intermittently happen in the reaction medium such as changes in pH and temperature, the immobilized form application of enzymes is preferable. More importantly, the immobilized enzyme provides a steady process, easy removal of the products, and convenient recovery of the catalyst from the stream. Three major methods are scrutinized for immobilization of different sorts of enzymes, i.e. adsorption, covalent binding, and encapsulation (entrapment) [5]. Among these techniques, covalent immobilization is widely studied as a durable technique for localization of enzymes on solid supports due to irreversibility of the bonds formed between the bioactive compartments and the support surface.

The choice of the support material and its conformation significantly affects the performance of the immobilized enzyme. Ideally, it should be inert, fairly inexpensive, and possess acceptable mechanical strength. In addition, its chemistry should allow easily incorporating of functional groups essential for direct or indirect covalent attachment of enzymes.

Polyacrylonitrile (PAN) is an important engineering polymer with good film and fiber forming characteristics which has widely been utilized for biomedical applications and enzyme

* Corresponding authors at: Advanced Membrane Technology Research Centre (AMTEC), Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia (A. Daneshfar). Tel.: +60 75535592; fax: +60 75581463.
E-mail addresses: darf2@live.utm.my (A. Daneshfar), afauzi@utm.my (A.F. Ismail).

http://dx.doi.org/10.1016/j.reactfunctpolym.2014.12.004
1381-5148/

immobilization. The presence of nitrile groups (C≡N) in the backbone of the polymer chains provides the possibility for incorporation of new reactive functional groups, macromolecules, or biofriendly species making PAN films or fibers hydrophilic and biocompatible [6]. Different PAN-based membranes has long been studied for direct or indirect (use of coupling agents) covalent immobilization of different sorts of enzymes. Poly (acrylonitrile-*co*-methylmethacrylate-*co*-sodium vinylsulfate) was activated with NaOH and hexamethylenediamine (HMDA) or hydroxylamine. Glucose oxidase (EC 1.1.3.4) was then covalently attached to the surface of membrane using glutaraldehyde (GA) as the coupling agent [7]. Similar procedure was also used for immobilization of urease (EC 3.5.1.5) on PAN hollow fibrous membranes [8].

The support conformation should pose minimum diffusional limitation and provide utmost surface area per unit mass for high enzyme loading [9]. Electrospun polymeric fibers are promising for enzyme immobilization [10–13] because the electrospun mats are more easily produced, dispersed in and recovered from the reaction media than other choices of nanoscaled geometries, e.g. nanoparticles and nanotubes [14]. Furthermore, electrospun fibers with high specific surface area could be prepared from a wide choice of polymers with disparate chemical characteristics, which render possibilities for different modes of enzyme immobilization [15–17].

Recently, enzyme immobilization on PAN-based nanofibrous membranes has become increasingly interesting because of the relatively easy procedures for introducing necessary functional groups on the fiber surface prior to immobilization and for fiber production. Lipase (EC 3.1.1.3) and cellulase (EC 3.2.1.4) have been successfully immobilized on electrospun PAN (ePAN) fibers through an amidination activation of nitrile groups and reaction with amine pendant groups of the enzymes [18–20]. Others have used PAN derivatives and tethered the electrospun fibers prior to immobilization with biomacromolecules such as collagen or protein hydrolysate from egg skin in order to circumvent the hydrophobic nature of PAN and to make it biocompatible [21,22].

Direct amine functionalization of nanofibrous PAN mats have recently been studied for efficient heavy metal ion removal from aquatic environments [23–25]. Amine containing reagents such as ethanolamine, ethylenediamine [24,26], or hydroxylamine [25] react with PAN pendant nitrile groups through nucleophilic addition [24]. After the amine functionalization, bioactive compartments containing —NH_2 groups can be immobilized on the surface of aminated carrier by use of glutaraldehyde (GA) through the formation of stable amino-aldehyde bonds [27]. However, there are no references concerning the application of directly aminated PAN membranes or fibers for immobilization of enzymes.

In this work, the surface aminated electrospun polyacrylonitrile (NH_2-ePAN) mats were prepared by electrospinning of PAN dope solution followed by amination with ethylenediamine (EDA). Urease was then covalently immobilized on NH_2-ePAN mats using glutaraldehyde (GA). Properties of the immobilized urease were studied and compared to those of free enzyme. Results will possibly lead to the use of urease-immobilized ePAN mats (Urs-ePAN) in miniaturized wearable kidneys to regenerate the valuable dialysate fluid by preserving the catalytic activity for increasing the number of reuses.

2. Experimental

2.1. Materials

Jack bean urease type III (EC 3.5.1.5), PAN (MW = 150,000), and GA (25 wt.% aqueous solution) were purchased from Sigma (USA) and used as received. Dimethyl formamide (DMF) (analytical grade), ethylenediamine (EDA) (analytical grade), potassium dihydrogen phosphate (monobasic) (analytical grade), potassium monohydrogen phosphate (dibasic) (analytical grade), and urea (biology grade) were purchased from Merck (Germany). The activity of the native or immobilized urease was measured by Atlas Medical Berthelot urea kit. All other chemicals were of analytical grade. Deionized water (DI) (>18 MΩ cm) was used for washing or preparation of all aqueous solutions unless otherwise stated. The urea and urease solutions were prepared in 22 mM phosphate buffers (PBS) containing 1 mM ethylenediamine tetra acetic acid (EDTA) as an ion chelating compartment of the PBS.

2.2. Preparation of NH_2-ePAN mats

The 10 wt.% PAN dope solution was prepared by slowly adding of PAN powder to DMF followed by mechanical stirring for 24 h at ambient temperature. The solution was then loaded to a 10 ml glass syringe and was upheld for 2 h to remove the dispersed air bubbles. A metal needle spinneret (D = 0.7 mm) was attached to the syringe tip. Electrospinning was performed for 7 h to collect a detachable thickness of the fibers. A syringe pump (Cole-Parmer® 100 Touch Screen) was used to deliver the polymer solution to the tip of the needle at a constant flow rate of 1 ml/h. High voltage power supply (ES30P-5W-Gamma High Voltage Research, Florida, USA) with low current output (166 μA) was used as the electricity power source. Positive charge (18 kV) was applied to the needle tip and the produced electrospun fibers were collected on a grounded aluminum sheet. The distance between the needle tip and the collector was kept constant at 20 cm. Afterwards, the nonwoven sheets were cut into 2 × 2 cm^2 sheets which were dried overnight in a vacuum oven and weighed carefully after drying. The sheets were then soaked in 70% ethanol aqueous solution in small glass bottles, followed by shaking on a rocking shaker at 250 rpm for 24 h to disentangle the fibers by increasing their wettability. Further surface functionalization was carried out on the dispersed spun fibrous mats. The dispersed fibers were washed successively and kept immersed in 3 M aqueous ethylenediamine (EDA) solution at 99 ± 1 °C for 4 h. Those pieces were further washed to remove residual EDA. Aminated electrospun ePAN mats, called NH_2-ePAN, were stored in DI for later use.

2.3. Immobilization of the urease using glutaraldehyde

Aminated electrospun mats, NH_2-ePAN, were soaked in aqueous GA solutions at different concentrations, 0–10 wt.%, for 2 h at ambient temperature, followed by washing with a copious amount of water. GA treated mats (GA-ePAN) were further loaded in glass bottles containing 5 ml of urease solution in PBS (1 mg/ml) at pH 7. Then, the bottles were shaken for 1 h at ambient temperature. Afterwards, the bottles were transferred to a refrigerator for further urease covalent immobilization at 4 °C ± 1 °C for 24 h.

2.4. Characterization

The attenuated total reflection-FTIR (ATR-FTIR) (Thermo Nicolet Instrument Corporation, Madison, WI) was used to characterize the surface chemistry before and after the surface modification of the pristine electrospun PAN mats (ePAN).

The morphology and diameter of the pristine, the chemically treated, and the urease immobilized ePAN mats were investigated by field emission scanning electron microscope FESEM (JEOL JSM-6701F, Japan). The samples were prepared by placing 0.5 × 0.5 cm^2 pieces over FESEM specimen stabs, followed by sputtering a thin

layer of gold. Surface imaging was carried out using 10 kV potentials for 1000–25,000 times magnification.

2.5. Measurement of the activity and immobilized protein

Urease catalyzes the hydrolysis of urea according to the following reaction (1):

$$CO(NH_2)_2 + 2H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2 \quad (1)$$

The numbers of moles of ammonia liberated from the urease-catalyzed hydrolysis of urea in different time sequences were measured and the specific catalytic activity of the enzyme was expressed in μmol NH_3 min^{-1} mg^{-1} dissolved enzyme and μmol NH_3 min^{-1} mg^{-1} immobilized enzyme, for free and immobilized urease, respectively.

For free urease, 2 ml of 0.25 mg/ml of enzyme solution was mixed with 2 ml of 10 g/l of urea solution. For immobilized urease, the urease-immobilized electrospun mat was immersed in 2 ml of urea solution (10 g/l) and 2 ml of PBS. These two solution mixtures are called working solutions. The pH of the working solutions was 7 and the experiments were conducted at ambient temperature unless otherwise stated. The concentration of liberated ammonia was measured by the Berthelot method using Atlas Medical urea kit reagents. As a function of time, 10 μl of the sample was withdrawn and mixed with 1 ml of reagent R_2 followed by addition of 1 ml of R_1 of the kit. A blue–green indophenol color was developed after 30 min as the result of the reaction between the liberated ammonia, sodium hypochlorite (R_2), and sodium salicylate in the presence of sodium nitroprusside (R_1). Absorbance of the colored solution was measured at 580 nm and the concentration (μmol/ml) of evolved ammonia was determined using the calibration curve. The ammonia concentrations were plotted versus time and the slope of the linear portion was used to calculate the specific activity.

The protein content of the urease-immobilized ePAN mats was determined by subtraction of the amount of unbound protein in filtrate and washing liquids from the content of the initial solution using the Bradford method.

2.6. pH and thermal stability

The effect of pH on the activity of both free and immobilized urease was determined through changing the pH of the working solution in a range from 5.5 to 8.5 by adjusting the amount of PBS. The relative enzyme activity at different pH was reported as percent maximum activity.

To study the effect of temperature on the free and immobilized urease, the temperature of the working solution (pH = 7) was changed in a range from 4 to 90 °C by keeping the working solution either in a laboratory fridge or in an oven for 75 min. The activity measurement was made at room temperature.

2.7. Kinetic study of free and immobilized urease

The Eq. (2) relates the rate of enzyme reaction, V, represented here by specific activity of urease (μmol NH_3 min^{-1} mg $urease^{-1}$), and the substrate (urea) concentration S (mol/l), according to the Michaelis–Menten kinetics. V_m is the maximum reaction rate and K_m is the Michaelis constant (mol/l).

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m} \times \frac{1}{S} \quad (2)$$

Thus, Eq. (2) allows calculating V_m and K_m from the intercept and the slope of $(1/V)$ versus $(1/S)$ plot, which is also called the Lineweaver–Burk plot.

2.8. Reuse cycles and storage durability

The process of separating products of a typical enzymatic reaction that mostly takes place in solution, even if the free enzyme remains active at the end of the reaction, will inexorably lead to deactivation of the enzyme. Enzyme immobilization on the surface of the insoluble supports enables recovery of the active catalyst from the reaction mixture and facilitating the separation of the products from the reaction mixture. However, a big question remains; is it viable to reuse the immobilized enzyme-support system repeatedly?

In order to study the reusability of the immobilized urease, its specific activity was measured at pH 7 and ambient temperature 15 times within 75 min. After each time of reuse, the urease-ePAN mats were washed with PBS at pH 7 three times.

Free and immobilized ureases were stored for 20 days at 4 °C or 25 °C and the samples were taken from time to time for the activity measurement. The activities relative to the first day activity is reported as % maximum activity.

3. Results and discussions

3.1. Preparation and characterization of chemically treated ePAN mats

An as-spun ePAN sheet and several pieces of 2×2 cm^2 square mats are shown in Fig. 1(a). An efficient enzyme immobilization requires the maximum surface area available per unit mass of the support. This implies that a non-woven electrospun mat should be disentangled to get access to inner fibers. In order to increase the wettability of the fibers and the surface area available for immobilization, the square mats were soaked either in DI or 70% ethanol aqueous solution. The as-spun ePAN mat remained undispersed even though it was immersed in DI and shaken for 24 h (Fig. 1(b)). In contrast, the mat was almost completely disentangled after immersion in 70% ethanol solution followed by washing and vigorous shaking for 5–7 times with DI (Fig. 1(c)). Undispersed and dispersed as-spun ePAN mats in the flask are shown in Fig. 1(d) for comparison. As shown in Fig. 1(c), immersion the fiber mats in aqueous alcohol solution liberated the small amount of air trapped within the mat and enabled the complete wetting of the surfaces of the inner fibers. This happens due to the surfactant-like action of ethanol. Alkyl group of ethanol preferentially were absorbed to the surface of ePAN fibers while its hydroxyl group interacted with water. Thus, the hydrophobic interaction between neighboring fibers would be reduced so that further washing and shaking would weaken agglomeration of the fibers and make them dispersed in water well [28].

During amine activation in EDA solution, the color of fibers changed gradually from white to mild yellow, as shown in Fig 2(b), and amine content of the fibers, determined through acid–base titration, reached to 2.3 ± 0.4 μmol/mg mat after 4 h. Extended duration of amination resulted in only negligible change in amine content of the fibers. Following the reaction with GA, the color was changed to orange or mild pink (Fig. 2(c)). The changes in the color of the fibers during the activation of the ePAN fibers prior to immobilization proved the occurrence of stepwise chemical reactions between functional groups after each step.

The FTIR spectra of the pristine, NH_2-ePAN and GA-ePAN mats are shown in Fig. 3. For pristine ePAN, the characteristic peaks are 2939 cm^{-1} (C—H stretching band related to the backbone of PAN), 2245 cm^{-1} (C≡N stretching), and 1450 cm^{-1} (C—H scissoring of methylene groups). The peak at 3633 cm^{-1} is related to the OH stretching possibly due to the absorbed water on the surface of the fibers [29]. The peaks at 1736 cm^{-1}, 1250 cm^{-1}, and 1173 cm^{-1} are assigned to the stretching vibration of (C=O),

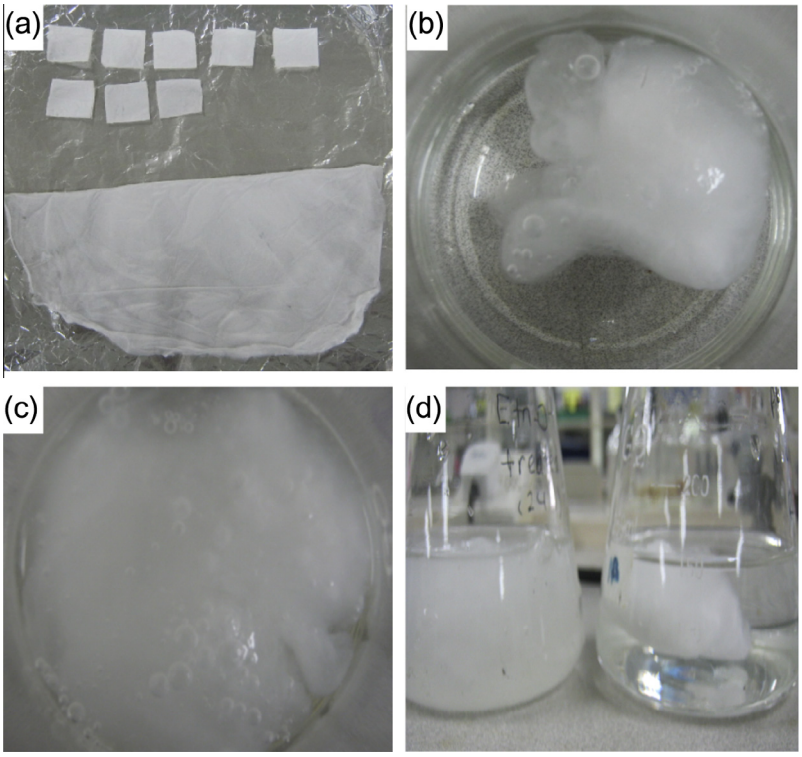


Fig. 1. (a) As-spun PAN (ePAN) mat and $2 \times 2\ cm^2$ pieces, (b) undispersed fibrous mats after shaking in and washing with deionized water, (c) dispersed ePAN fibers after immersion in 70% aqueous ethanol solution and washing with DI, and (d) front view of dispersed and undispersed fibers.

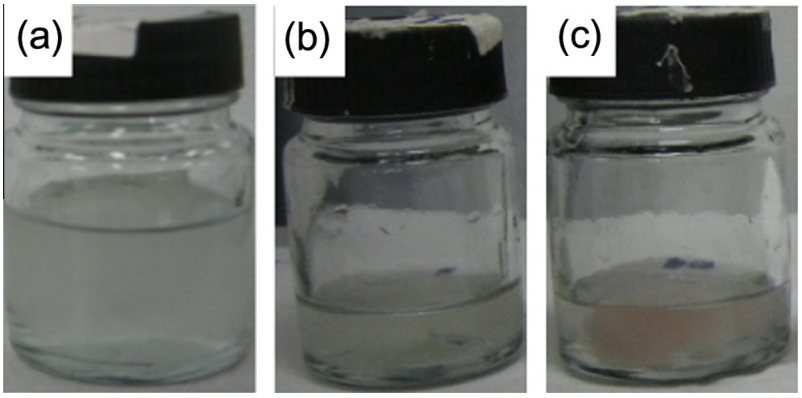


Fig. 2. The changes in color of ePAN mats following the stepwise chemical treatment (a) pristine ePAN mat (white), (b) NH_2-ePAN mat (mild yellow), and (c) GA-ePAN mat (pink to mild orange). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

C—C(=O)—C, and —OCH_3 in the methyl methacrylate constituent groups, respectively [30,31], suggesting that the precursor material was the co-polymer of acrylonitrile and methyl methacrylate.

Amination with ethylenediamine (EDA) resulted in the formation of a new broad peak at 3371 cm^{-1} (NH stretching) that indicates the existence of primary amine groups in NH_2-ePAN. Characteristic bands related to the acetate groups of methyl methacrylate co-monomer show a considerable decrease in their intensity whereas the peak related to the nitrile group of the PAN does not reveal a dramatic change. Of particular importance, this is interpreted as the preferential reaction of EDA with acetate groups forming amide II groups [32]. The strong peak of NH_2-ePAN observed at 1635 cm^{-1} can be assigned to either amide II groups [29] or the CN bending in C(=NH)—NH— (amidine group) [33]. However, because the nitrile groups remained almost intact, the peak is largely contributed by the amide groups. The peak at 1566 cm^{-1} is related to the bending vibration of NH in primary amine.

Following the treatment of NH_2-ePAN mat with 10 wt.% GA solution, the peak at 1566 cm^{-1} has nearly vanished. This might be attributable to the condensation reaction between the aldehyde groups of GA and the amine end groups, to form imine groups (Schiff base imine) and intensification of the peak at 1635 cm^{-1} [34]. In addition, the characteristic peak of aldehyde carbonyl group at 1720 cm^{-1} has overlapped with the peak of methacrylate carbonyl at 1735 cm^{-1} [35]. The two other characteristic peaks related to —CH_2— groups of the GA [36] are combined with those of PAN and resulted in intensification of the peak at 2940 cm^{-1}. Scheme 1 shows formation of possible functional groups on the surface of ePAN fibers following the reaction with EDA and GA. The reaction between GA and primary amine end groups present on the surface of the NH_2-ePAN fibers proves the feasibility of

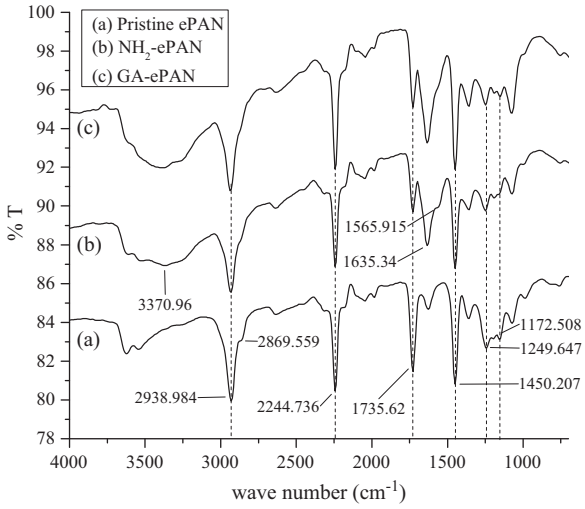


Fig. 3. ATR-FTIR spectral bands related to the (a) pristine ePAN mats, (b) EDA treated ePAN resulted in NH_2-ePAN, and (c) GA treated NH_2-ePAN resulted in GA-ePAN.

the covalent bonding of enzymes via the reaction between the surface amine groups of the enzyme and the unreacted end group of the GA-ePAN fibers [37].

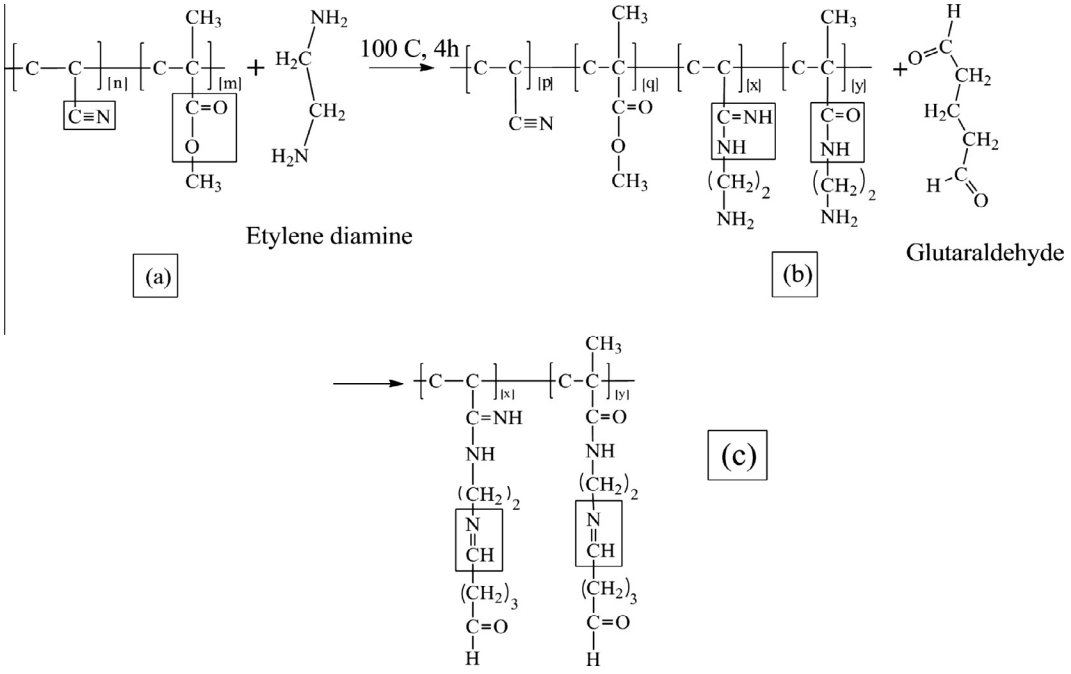
FESEM images of pristine and chemically treated ePAN fibers, presented in Fig. 4(a)–(c), revealed changes occurred during the stepwise chemical treatments. For each micrograph, the diameters of 20 arbitrarily chosen fibers were measured and the average fiber diameter was reported. Fig. 4(a) shows smooth nonwoven as-spun ePAN ultrafine microfibers with an average fiber diameter of 1448 ± 380 nm. During the amination, as can be seen in Fig. 4(b), fiber surfaces were corroded and rugged due to EDA. However, fibers preserved their integrity and no flaws were observed. The average diameter of NH_2-ePAN fibers slightly increased (1503 ± 300 nm). Fig. 4(c) depicts the morphology of cross-linked NH_2-ePAN mats when treated with 10 wt.% GA solution. Possibly, cross-linking took place as a result of Schiff base reaction, as indicated earlier, between free unreacted amines and aldehyde end group of the neighboring fibers at their touching points. Schiffman and Schauer [38] reported the similar observations when chitosan nanofibers were cross-linked using GA.

The FESEM images presented in Fig. 4(d) and (e) shows urease aggregates immobilized on or within ePAN fibers when the concentration of GA changed from 5 wt.% to 10 wt.%. As shown in Fig. 4(d), urease was mainly immobilized on individual separate fibers, whereas, in Fig. 4(e) some enzyme aggregates were entrapped between the cross-linked fibers.

3.2. Effect of GA concentration on the amount of immobilized urease and activity

It has been demonstrated that the ligand mobility is the governing factor affecting the activity of an immobilized enzyme [39]. Therefore, rather than the total number of immobilized biomolecules, the number of active immobilized enzymes and their freedom of movement in the surrounding microenvironment determine the activity retention. In Fig. 5, the specific activity and the amount of immobilized urease are plotted versus GA concentrations. As it can be seen, the amount of immobilized enzyme increased steadily with the increase in GA concentration. However, the specific activity shows a drastic decline when the GA concentration was >5 wt.%. It was because GA reacts preferentially with —NH_2 and —SH groups [40] of urease molecule, which are essential for catalytic activity [41].

Zhu and Sun [11] proved that upon increment in GA concentration, the number of aldehyde groups on the surface of polyvinyl alcohol fibers and the overall content of immobilized lipase increased. The decrease in activity, however, was ascribed to more multi-point chemical bondings that reduced the ligand mobility. Another explainable scenario was the limited mass diffusion of the substrate (feed) to the enzyme active site as a result of the intermolecular steric hindrance of crowded enzyme molecules immobilized on the fibers.



Scheme 1. (a) Polyacrylonitrile-co-methyl methacrylate precursor of the fibers and reaction with EDA, (b) possible amidine and amide II groups formed during the amination reaction leaving unreacted amine end groups, and (c) reaction of aldehyde groups with amine end groups.

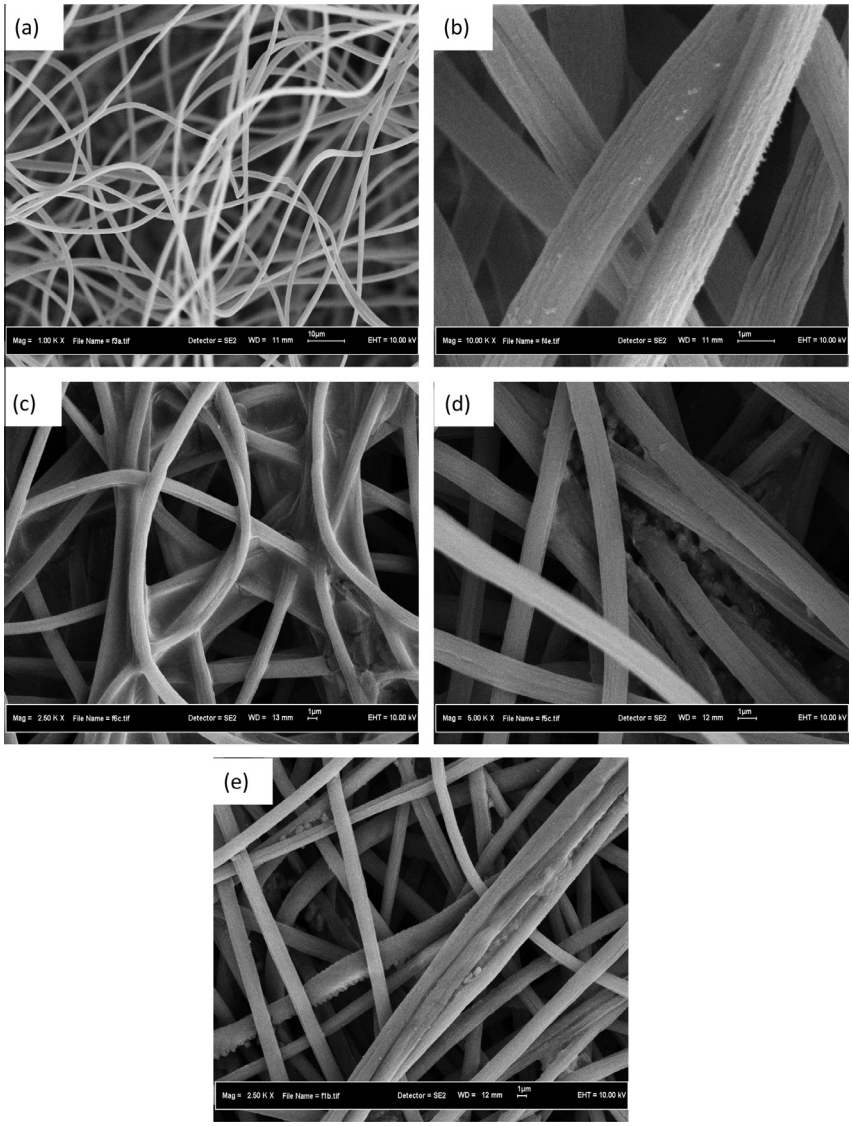


Fig. 4. The FESEM images of (a) as spun ePAN, (b) NH_2-ePAN, (c) GA-ePAN when NH_2-ePAN mat was treated by 10 wt.% GA and crosslinking of the neighboring fibers at their touching points, (d) urease immobilized on 5 wt.% GA-ePAN, and (e) urease immobilized on 10 wt.% GA-ePAN.

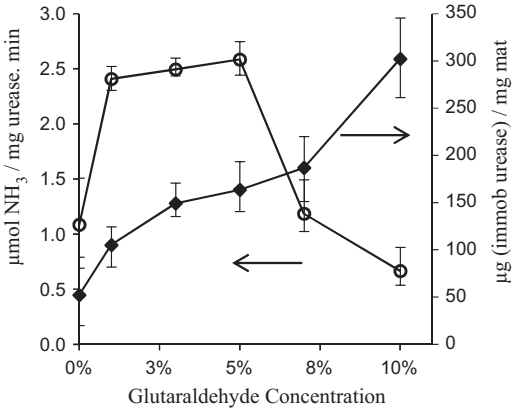


Fig. 5. Effect of GA concentration on the specific activity and the amount (μg) of free urease immobilized on mg ePAN mat at 25 °C and pH 7.

Based on FESEM micrographs (Fig. 4(d) and (e)), it was probable that enzyme molecules developed multiple bonds with two or more adjacent fibers or were entrapped between them. This could partially or completely deactivate urease molecules or ban substrate to reach the active site of enzyme, respectively.

With the specific activity of free urease at pH 7 and 25 °C equal to 5.26 (μmol NH_3/min mg urease), urease immobilized on 5% GA-ePAN microfibers retained 54% of initial activity of free enzyme and immobilized urease content reached 157 μg/mg ePAN mat. This concentration was then used in the following studies of urease activity.

3.3. Effect of pH and temperature on urease activity

The pH of the solution must be set in order to maintain the ionic groups on the active sites in a suitable form to function. Rather than changes in the ionic groups of the active site, variations in pH alter the three-dimensional shape of the enzyme and, thus, the enzyme activity and reaction rate. The pH-activity profiles for both free and immobilized urease are shown in Fig. 6. The maximum activity slightly shifted from pH 7 for free urease to 6.5 for immobilized urease. Also, immobilized urease retained relatively high values in a broader range of acidic pH than free urease did.

Immobilization through multi-point attachment can preserve the structure of enzyme molecule when pH changes. However,

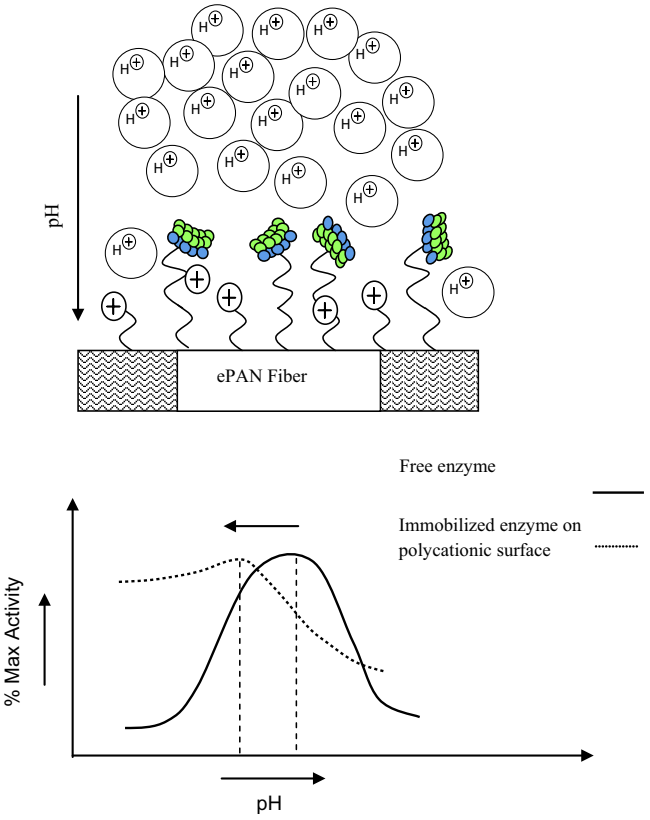
the charge of the surface affects the microenvironment of the immobilized enzyme substantially. Generally, immobilization on polycationic surfaces-such as chitosan or polyethyleneimine shifts the optimum pH toward acidic pH while immobilization on polyanionic surfaces such as polylactic acid tends to shift it towards more alkaline pH [42]. In this work, PAN microfibers were tethered with amine groups, which changed the microenvironment around the immobilized urease; i.e., the positively charged surface of NH_2-ePAN microfibers repulsed the H^+ ions and preserved the activity of the immobilized urease at low pH. Schematic representation of the effect is shown in Scheme 2.

Effect of temperature on the activity of free and immobilized urease is shown in Fig. 7. The maximum activity for free and immobilized urease was found to be at 37 and 50 °C, respectively. In addition, thermal stability of urease was enhanced in a broad range of temperature by immobilization. This might be due to the enzyme multi-point attachment to the surface of the fibers through covalent bonding, which would lead to reduce enzyme conformational changes at higher temperatures.

3.4. Kinetic studies

The changes in the rate of the enzymatic reaction as the function of substrate concentration provide information about the kinetic parameters i.e., V_m (maximum reaction rate) and K_m (the Michaelis constant). K_m is an approximate representation of binding tightness of the substrate to the enzyme. The lower the K_m, the higher the affinity between enzyme and substrates will be. V_m, on the other hand, demonstrates the maximum velocity that a given enzymatic reaction can theoretically reach. The Lineweaver–Burk plots for both free and immobilized urease are shown in Fig. 8. It was found that K_m values for the free and immobilized urease were 40.35 and 57.6 mM, respectively. Whereas the V_m value for the immobilized urease (10.1 μmol NH_3 min^{-1} mg^{-1} immobilized urease) was lower than that of free urease (17.54 μmol NH_3 min^{-1} mg^{-1} free urease). The linear nature of these plots shows that both the free and immobilized urease follows Michaelis–Menten kinetics.

Immobilization of urease on different support materials and conformations has been widely studied and comprehensively reviewed by Krajewska [43]. It was found that a similar trend for an increase in K_m and a decrease in V_m, more or less, almost always happens regardless of the carrier material chemistry, its conformation, and covalent immobilization technique. The increase in K_m is the result of inefficient substrate access to the immobilized enzyme active site which results in lower rate of the reaction and V_m. Mass transfer limitations within the support structure, conformational changes of enzyme during the immobilization and washing steps, restricted freedom of movement due to multi-point attachment, and non biospecific orientation of the enzyme active site through hydrophobic interaction of the enzyme with support are the most important reasons that explain the difficulty of the substrate to reach the enzyme active site afterwards the immobilization on a solid support [42]. However, in comparison with the results of other works, the urease immobilized on the fibers of the present study showed much lower increase in K_m (57.6 (immobilized)/40.35 (free) = 1.42). This result demonstrates that the affinity of the urease for the substrate (urea) has not changed significantly and that the binding sites in the enzyme is not significantly affected due to its immobilization on the ePAN microfibers. One possible reason for the difference seen is that there would be a reduced diffusion path for the substrate around the dispersed fibers.



Scheme 2. The effect of polycationic surface charge on microenvironment pH and immobilized enzyme optimum activity.

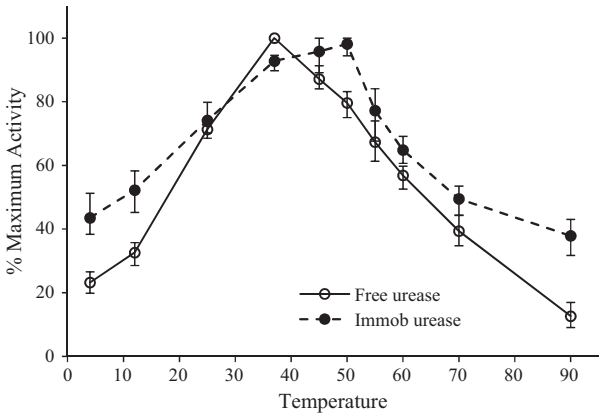


Fig. 7. Effect of temperature on the activity of free and immobilized urease.

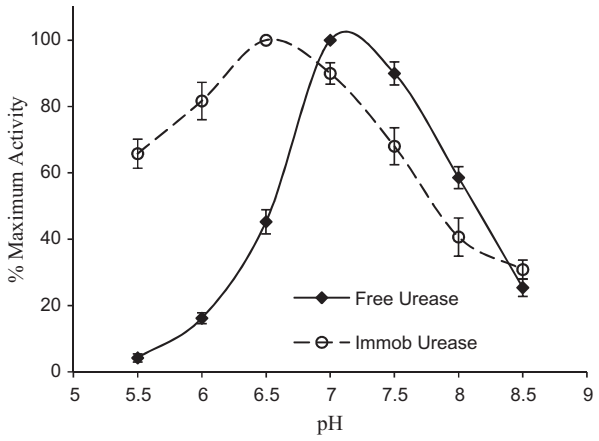


Fig. 6. Effect of pH on activity of free and immobilized urease.

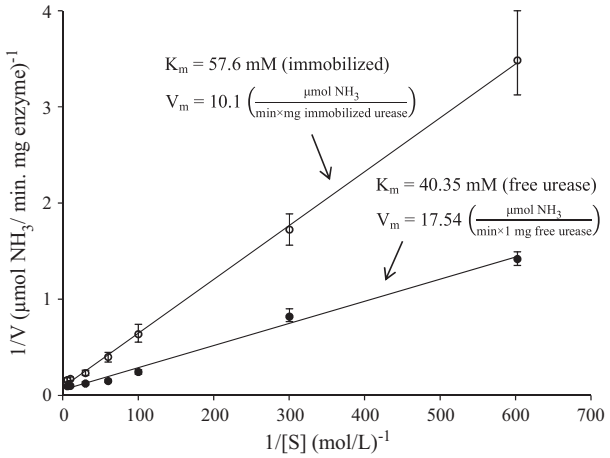


Fig. 8. Lineweaver–Burk plots for free and immobilized urease at pH 7 and 25 °C.

3.5. Reusability and storage stability

The activity of immobilized urease was measured repeatedly and, as shown in Fig. 9, the activity decreased only slowly. After 10 times of reuse cycles the enzyme retained over 85% of its initial activity. Even after 15 cycles, this system preserved around 70% of its initial activity. Generally, immobilization prevents enzyme to lose its 3-dimensional conformation, its active site and its activity during repeated number of reuse. Moreover, in this study the considerable urease loading (157 μg/mg mat) implies strong enzyme aggregates which prevents the enzyme from leaching into solution and denaturing during reuse cycles [44].

Another important challenge that faces real application of enzymes is their activity retention during the storage period. In this work, we studied the effect of storage on the activity of both free and immobilized urease by measuring the activity retention for 20 days storage duration at 4 °C and 25 °C. The results are shown in Fig. 10.

As can be seen in Fig. 10, immobilization has improved enzyme storage stability. Immobilized urease stored at 4 °C has retained almost 80% of its initial activity while the activity of free enzyme was below 40% of the initial value. Also, immobilized enzyme retained near 40% of its initial activity, while free enzyme lost almost all activity at 25 °C. These results which demonstrate the preservation of enzyme structure after immobilization suggest the applicability of ePAN mats as successful biocatalyst carriers.

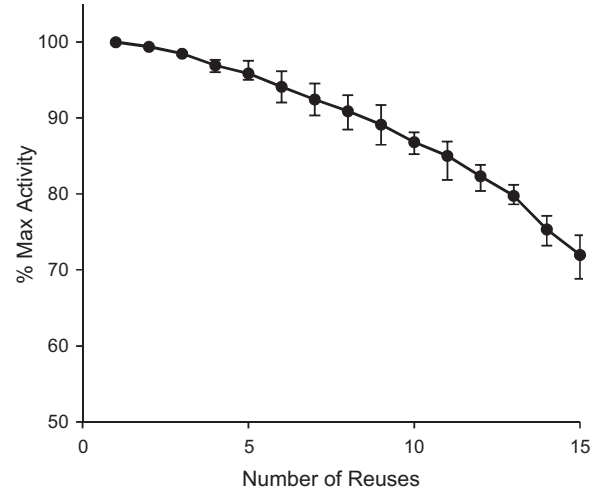


Fig. 9. Reusability of the urease immobilized-ePAN microfibrous mats.

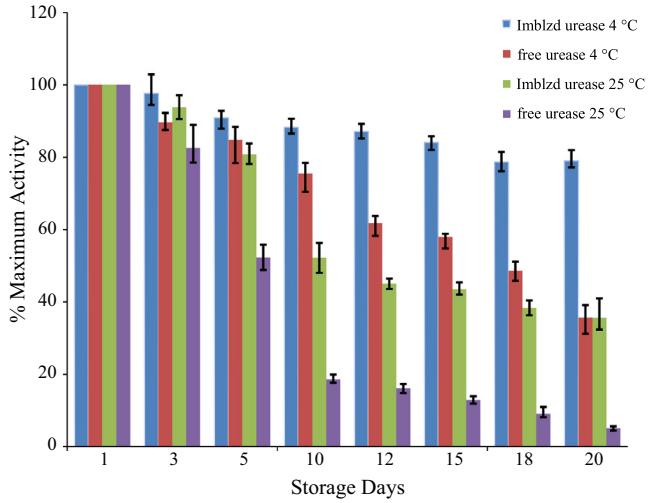


Fig. 10. Storage stability for free and immobilized urease at 4 °C and 25 °C.

4. Conclusion

This study revealed the successful covalent immobilization of urease for the first time on electrospun polyacrylonitrile (ePAN) microfibrous mats. At first, electrospinning of a 10 wt.% PAN (MW = 1,500,000)/DMF dope solution at 18 kV and 20 cm resulted in microfibrous ePAN mats with an average fiber diameter of 1448 ± 380 nm. Then, urease was immobilized on disentangled fibers following the treatment of ePAN fibers with EDA and GA solutions. At 5% GA concentration urease loading reached 157 μg/mg mat while preserving 54% of the free urease initial activity. The FESEM images revealed enzyme aggregates on the surface of the fibers and they confirmed the success of immobilization. Immobilized urease tolerated the changes in pH (5.5–7) and temperature (10–90 °C) significantly better than the free urease did. Furthermore, it also showed improved storage stability (20 days) and could retain nearly 70% of initial activity after 15 cycles of reuse. This study provided an inexpensive, simple, efficient and reliable technique for immobilization of urease on ePAN fibers which is prognosticated to find application in dialysate liquid regeneration system in artificial kidney machines.

Acknowledgements

We acknowledge the Ministry of Higher Education (Malaysia) and Universiti Teknologi Malaysia for the financial support through Fundamental Research Grant Scheme Vot. R. J130000.7809.4F157.

References

[1] V. Gura, C. Ronco, F. Nalesso, A. Brendolan, M. Beizai, C. Ezon, A. Davenport, E. Rambod, Kidney Int. 73 (2007) 497–502.
[2] V. Gura, A.S. Macy, M. Beizai, C. Ezon, T.A. Golper, Clin. J. Am. Soc. Nephro. 4 (2009) 1441–1448.
[3] J.P. Chen, S.H. Chiu, Enzyme Microb. Technol. 26 (2000) 359–367.
[4] D.H. Chen, J.C. Leu, T.C. Huang, J. Chem. Technol. Biotechnol. 61 (1994) 351–357.
[5] R.A. Sheldon, Adv. Synth. Catal. 349 (2007) 1289–1307.
[6] Z.G. Wang, L.S. Wan, Z.K. Xu, J. Membr. Sci. 304 (2007) 8–23.
[7] T.S. Godjevargova, A.R. Dimov, N. Vasileva, J. Appl. Polym. Sci. 54 (1994) 355–359.
[8] M.C. Yang, C.C. Lin, Biomaterials 22 (2001) 891–896.
[9] J. Kim, J.W. Grate, P. Wang, Chem. Eng. Sci. 61 (2006) 1017–1026.
[10] T. Srisook, T. Vongsetskul, J. Sucharitakul, P. Chaiyen, P. Tangboriboonrat, React. Funct. Polym. (2014)
[11] J. Zhu, G. Sun, React. Funct. Polym. 72 (2012) 839–845.
[12] X.-J. Huang, D. Ge, Z.-K. Xu, Eur. Polym. J. 43 (2007) 3710–3718.
[13] Z.G. Wang, J.Q. Wang, Z.K. Xu, J. Mol. Catal. B-Enzym. 42 (2006) 45–51.

[14] K. Rege, N.R. Raravikar, D.-Y. Kim, L.S. Schadler, P.M. Ajayan, J.S. Dordick, Nano Lett. 3 (2003) 829–832.
[15] T.E. Herricks, S.H. Kim, J. Kim, D. Li, J.H. Kwak, J.W. Grate, S.H. Kim, Y. Xia, J. Mater. Chem. 15 (2005) 3241–3245.
[16] Z.G. Wang, L.S. Wan, Z.M. Liu, X.J. Huang, Z.K. Xu, J. Mol. Catal. B-Enzym. 56 (2009) 189–195.
[17] T. Vongsetskul, P. Kongjumnean, P. Sunintaboon, R. Rangkupan, P. Tangboriboonrat, Polym. Bull. 69 (2012) 1115–1123.
[18] S.F. Li, J.P. Chen, W.T. Wu, J. Mol. Catal. B-Enzym. 47 (2007) 117–124.
[19] T.C. Hung, C.C. Fu, C.H. Su, J.Y. Chen, W.T. Wu, Y.S. Lin, Enzyme Microb. Technol. 49 (2011) 30–37.
[20] S.F. Li, W.T. Wu, Biochem. Eng. J. 45 (2009) 48–53.
[21] P. Ye, Z.K. Xu, J. Wu, C. Innocent, P. Seta, Biomaterials 27 (2006) 4169–4176.
[22] X.-J. Huang, A.-G. Yu, J. Jiang, C. Pan, J.-W. Qian, Z.-K. Xu, J. Mol. Catal. B-Enzym. 57 (2009) 250–256.
[23] B. Bagheri, M. Abdouss, M.M. Aslzadeh, A.M. Shoushtari, Iran. Polym. J. 19 (2010) 911–925.
[24] N. Arsalani, R. Rakh, E. Ghasemi, A.A. Entezami, Iran. Polym. J. 18 (2009) 623–632.
[25] L.C. de Santa Maria, M.C.V. Amorim, M.R.M.P. Aguiar, P.I.C. Guimarães, M.A.S. Costa, A.P. de Aguiar, P.R. Rezende, M.S. de Carvalho, F.G. Barbosa, J.M. Andrade, R.C.C. Ribeiro, React. Funct. Polym. 49 (2001) 133–143.
[26] N. Arsalani, M. Hosseinzadeh, Iran. Polym. J. 14 (2004) 345–352.
[27] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G.D.-O.C. Mateo, R. Fernández-Lafuente, J.M. Guisán, Enzyme Microb. Technol. 39 (2006) 877–882.
[28] N. Wu, J. Parris, Colloid Surf. A 167 (2000) 179–187.
[29] S. Deng, R. Bai, J.P. Chen, Langmuir 19 (2003) 5058–5064.
[30] A.M. Shoushtari, M. Zargaran, M. Abdouss, J. Appl. Polym. Sci. 101 (2006) 2202–2209.
[31] P. Tahaei, M. Abdouss, M. Edrissi, A. Shoushtari, M. Zargaran, Materialwiss. Werkst. 39 (2008) 839–844.
[32] C. Xu, J. Du, L. Ma, G. Li, M. Tao, W. Zhang, Tetrahedron (2013).
[33] Y.G. Ko, Y.J. Chun, C.H. Kim, U.S. Choi, J. Hazard. Mater. 194 (2011) 92–99.
[34] L. Marin, B. Simionescu, M. Barboiu, Chem. Commun. 48 (2012) 8778–8780.
[35] S. Jain, S. Chattopadhyay, R. Jackeray, H. Singh, Anal. Chim. Acta 654 (2009) 103–110.
[36] H.C. Wu, T.W. Wang, P.L. Kang, Y.H. Tsuang, J.S. Sun, F.H. Lin, Biomaterials 28 (2007) 1385–1392.
[37] S. Nair, J. Kim, B. Crawford, S.H. Kim, Biomacromolecules 8 (2007) 1266–1270.
[38] J.D. Schiffman, C.L. Schauer, Biomacromolecules 8 (2006) 594–601.
[39] V. Bulmuş, K. Kesenci, E. Pişkin, React. Funct. Polym. 38 (1998) 1–9.
[40] S. Di Martino, H. El Sheriff, N. Diano, A. De Maio, V. Grano, S. Rossi, U. Bencivenga, A. Mattei, D. Mita, Appl. Catal. B: Environ. 46 (2003) 613–629.
[41] K.H. Yeon, R.M. Lueptow, J. Chem. Technol. Biotechnol. 81 (2006) 940–950.
[42] J.N. Talbert, J.M. Goddard, Colloids Surf., B 93 (2012) 8–19.
[43] B. Krajewska, J. Mol. Catal. B-Enzym. 59 (2009) 22–40.
[44] B.C. Kim, S. Nair, J. Kim, J.H. Kwak, J.W. Grate, S.H. Kim, M.B. Gu, Nanotechnology 16 (2005) S382.