Chitosan-Grafted Poly(hydroxyethyl methacrylate-*co*-glycidyl methacrylate) Membranes for Reversible Enzyme Immobilization

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ABSTRACT: Epoxy group-containing poly(hydroxyethyl methacrylate/glycidyl methacrylate), p(HEMA/GMA), membrane was prepared by UV initiated photopolymerization. The membrane was grafted with chitosan (CH) and some of them were chelated with Fe(III) ions. The CH grafted, p(HEMA/GMA), and Fe(III) ions incorporated p(HEMA/GMA)-CH-Fe(III) membranes were used for glucose oxidase (GOD) immobilization via adsorption. The maximum enzyme immobilization capacity of the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes were 0.89 and 1.36 mg/mL, respectively. The optimal pH value for the immobilized GOD preparations is found to have shifted 0.5 units to more acidic pH 5.0. Optimum temperature for both immobilized preparations was 10°C higher than that of the free enzyme and was significantly broader at higher temperatures. The apparent K_m values were found to be 6.9 and 5.8 m*M* for the adsorbed GOD on p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes, respectively. In addition, all the membranes surfaces were characterized by contact angle measurements. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 3084–3093, 2007

Key words: membrane; contact angle; immobilized enzyme; adsorption; glucose oxidase

INTRODUCTION

Polymer membranes are widely used in the fields of medicine and biotechnology for clinical applications, affinity separation of a target protein from biological fluids, or the construction of enzyme reactors and enzyme electrode.[1,2] The performance of immobilized enzyme depends greatly on the characteristic structure of the carrier materials.[3,4] Many efforts have been concentrated on modifying the support to obtain high immobilization efficiency and high retained enzyme activity.[5–8]

Among the various immobilization techniques available, adsorption may have a higher commercial potential than other methods because the adsorption process is simpler, less expensive, retains a high catalytic activity, and most importantly the support could be repeatedly reused after inactivation of the immobilized enzyme.[9–11] A number of methods for reversible immobilization of enzymes have been reported in the literatures.[11–13] However, the adsorption is generally not very strong and some of the adsorbed enzyme is desorbed during washing and operation. Thus, reversible enzyme immobilization via adsorption requires a strong hydrophobic or ionic interaction between the enzyme and support. Natural and synthetic ionic polymers grafted supports were used for the reversible immobilization of various enzymes.[12,13] Recently, various transition metal ions such as Zn(II), Ni(II), Cu(II), Al(III), and Fe(III) were immobilized onto suitable metal chelating polymers and have been used as new selective adsorbents for protein immobilization. Metal chelating adsorbent can be prepared from natural and synthetic polymers with metal chelating groups.[14,15] Coating a hydrophilic and functional natural polymer on synthetic support may provide an increase in the enzyme immobilization capacity and in retaining enzyme activity.[3,16,17] On the other hand, desorption of enzymes from the ionic polymer-grafted support was found to require the use of denaturing conditions (under low pH and high ionic strength), but this desorption would be necessary after inactivation of the enzyme upon use.

In this study, a reactive epoxy group carrying copolymer was prepared in the membrane form by copolymerization of 2-hydroxyethylmethacrylate (HEMA) and glycidyl methacrylate (GMA). The copolymer provides reactive epoxy groups for covalent attachment of macromolecules (epoxy groups react with sulfhydryl-, amino-, carboxyl-, or hydroxyl groups). By this way, the epoxy group could be readily introduced into the copolymer backbone at a desired density by adjusting

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the concentration of epoxy group carrying comonomer in the polymerization mixture and thus, the support activation procedure is eliminated for surface modification. The copolymer membrane was then grafted with chitosan (CH) and the polycationic amino groups of the CH are used for the reversible immobilization of an enzyme (i.e., glucose oxidase). Glucose oxidase is an acidic protein and it contains large quantity of carboxylic groups on the surface. In addition, GOD contains tightly bound two flavin adenine dinucleotides (FAD) and, each of them has Fe(III) ions chelated through the phosphate group of the FAD molecules. Therefore, the enzyme was selected as a model enzyme to evaluate its immobilization on the membrane grafted with an oppositively charged natural polymer ''chitosan'' and/or chelated Fe(III) ions. The immobilization of GOD via adsorption onto the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes from aqueous solutions containing different amounts of enzyme at different pH was investigated in a batch system. In addition, the membranes were characterized by FTIR and contact angle studies.

EXPERIMENTAL

Materials

Glucose oxidase (GOD, EC 1.1.3.4. Type X-S from *Aspergillus niger*), peroxidase (POD, EC 1.11.1.7. Type II from horseradish), chitosan, and glucose were obtained from the Sigma Chemical (St. Louis) and used as received. 2-Hydroxyethylmethacrylate (HEMA), glycidyl methacrylate (GMA, methacrylic acid 2,3 epoxypropyl isopropyl ether), and α-α′-azoisobisbutyronitrile (AIBN) were obtained from Fluka Chemie, AG (Buchs, Switzerland), and the monomers distilled under reduced pressure before use. All the other analytical grade chemicals were purchased from Merck AG (Darmstadt, Germany).

Preparation of p(HEMA/GMA) membrane

The poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate), p(HEMA-GMA), were prepared via UV initiated photopolymerization. The polymerization was carried out in a round glass mold (diameter: 9.0 cm) at 25°C under a nitrogen atmosphere for 1 h. HEMA (1.4 mL), GMA (0.4 mL), AIBN (10 mg) as polymerization initiator and isopropyl alcohol (2.0 mL) as a monomer diluent were mixed with phosphate buffer (2.0 mL, 50 m*M*, pH 7.0). The resulting mixture was mixed and equilibrated at 25°C for 15 min in a thermostated water-bath. The mixture was then poured into the mold and exposed to long-wave ultraviolet radiation for 20 min. After polymerization, the membrane was washed with distilled water and cut into circular pieces (diameter: 1.0 cm) with a perforator.

Coating of p(HEMA/GMA) membrane with chitosan

Functional epoxy group carrying p(HEMA/GMA) membrane disks (diameter 1.0 cm; about 10 g) were equilibrated in acetate buffer (50 m*M*, pH 4.5) for 2 h, and transferred to the medium containing chitosan (1.0% (v/v)). Coating of chitosan on the p(HEMA/GMA) membrane surface was carried out at 65°C for 12 h, while continuously stirring the reaction medium. After this period, the p(HEMA/GMA)-CH membranes were removed from the reaction medium and washed with 1.0*M* NaCl and then acetate buffer (50 m*M*, pH 4.5).

To determine the leakage of covalently grafted chitosan from the p(HEMA/GMA) membranes, the chitosan-grafted p(HEMA/GMA) membranes were magnetically stirred at 150 rpm in various buffer solutions (10 mL, pH between 4.0 and 8.0) at room temperature for 2 days. At the end of this period, the chitosan content of the membrane samples were determined by potentiometric titration. Then, this value was subtracted from the initial value to calculate remaining covalently bound chitosan on the membrane surface.

Incorporation of Fe(III) ions onto p(HEMA/GMA)-CH membrane

To prepare p(HEMA/GMA)-CH-Fe(III) complexed membrane, a 50 ppm solution of Fe(III) ions was prepared from $FeCl_3$ in distilled water at pH 4.5. The p(HEMA/GMA)-CH membrane was placed in the Fe(III) ions solution while stirring 100 rpm at 25°C for 1.0 h. The concentration of the Fe(III) ions in the resulting solution was determined with an atomic absorption spectrophotometer [(AAS), Shimadzu AA 6800, Japan].

Fe(III) leakage from the p(HEMA/GMA)-CH-Fe(III) membrane was investigated as a function of medium pH (4.0–8.0). The membrane disks were stirred for 18 h at room temperature. The Fe(III) ion concentration was then determined in the medium as described above and, the Fe(III) leakage was not observed from the metal incorporated membrane disks in the studied pH ranges.

Immobilization of GOD onto chitosan-grafted and Fe(III) ion-chelated membranes

Immobilization of GOD on the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) via adsorption was studied at various pH values, in either acetate (7.5 mL, 50 m*M*, pH 4.0–5.5) or phosphate buffer (7.5 mL, 50 m*M*, pH 6.0–8.0). The initial concentration of GOD was 1.0 mg/mL in buffer solution. The immobilization

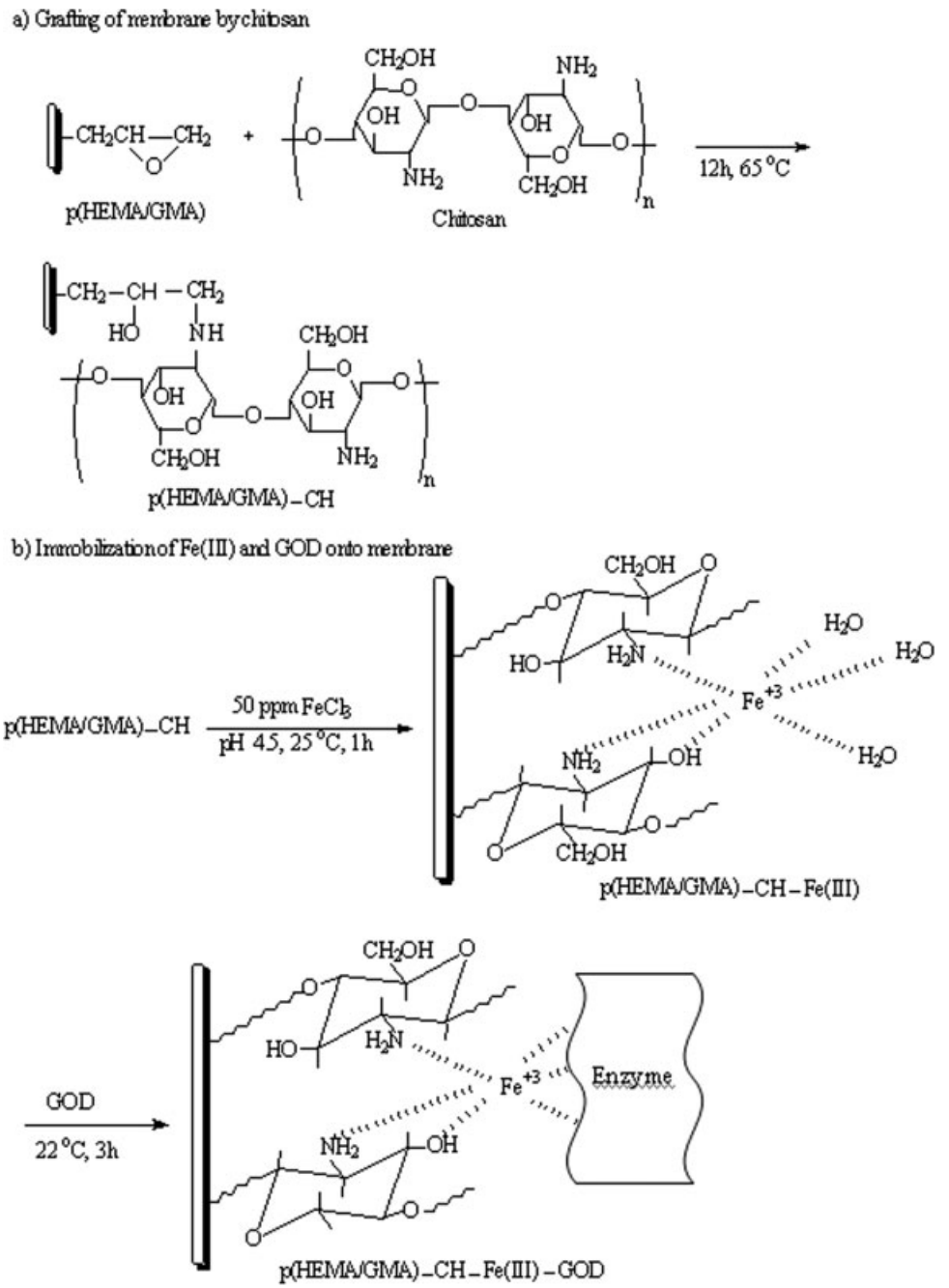

Figure 1 Schematic representation coating of the p(HEMA/GMA) membranes with chitosan.

experiment was conducted for 3 h at 22°C while continuous stirring. Effect of initial GOD concentration on the immobilization efficiency was studied at different GOD concentrations. In a typical immobilization experiment, GOD was dissolved between 0.25 and 2.0 mg/mL in acetate buffer (25 mL, 50 m*M*, pH 5.0) and 10 membrane disks were added. The membrane radius and thickness were 1.0 and 0.06 cm, respectively.

The adsorption experiments were carried out for 3 h at 22°C at a stirring rate of 100 rpm. The amounts of immobilized GOD on the membranes were determined by measuring the initial and final concentrations of protein within the immobilization medium using a (Jasco, Model FP 750, Tokyo Japan) spectrofluorimeter (excitation at 280 nm and emission at 340 nm). A calibration curve was prepared from crystalline GOD as a standard (0.02–0.2 mg/mL) and was used in the calculation of enzyme concentration.

Activity assays of free and adsorbed glucose oxidase

The activities of both free and immobilized GOD preparations were determined by measuring the accumula-

tion of hydrogen peroxide, a side product of glucose conversion, in the medium as previously described.[1]

$$\text{D-glucose} + O_2 + H_2O \xrightarrow[\text{POD}]{\text{GOD}} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 + o\text{-dianisidine} \longrightarrow o\text{-dianisidine} + H_2O$$
$$\text{(Colorless)} \qquad \text{(Red Colored)} \quad (2)$$

The absorbance of the reaction mixture was determined at 530 nm using a UV–vis spectrophotometer (Shimadzu, Model 1601, Tokyo, Japan). One unit of GOD activity was defined as the amount of enzyme which oxidases 1.0 μmol of glucose to gluconic acid and hydrogen peroxide per minute at 30°C and at pH 5.5. The relationship between the initial rate (V) of the enzymatic reaction and glucose concentration (S) (between 2.5 and 50 mM) in acetate buffer (50 mM, pH 5.5) at 30°C was measured, from which the Michaelis constants K_m and $V_{\max}$ of the free and immobilized GOD were determined.

For each set of data, the arithmetic mean values and standard deviations were calculated and the margin of the error for each data set was determined according to a confidence interval of 95% using statistical package under Excel for Windows.

Thermal and pH stability

The thermal stabilities of the free and immobilized GOD were determined by measuring the residual activity of the GOD exposed to 50 and 60°C for 180 min in acetate buffer at pH 5.5. After a predetermined incubation time interval, the remaining activities of the free or immobilized enzyme preparations were determined under standard conditions. The pH stability of the free and immobilized enzyme was determined after preincubating of enzyme in substrate free solutions at 30°C and at different pH's for 24 h.

Characterization of p(HEMA/GMA) membranes

The available epoxy groups content of the p(HEMA/GMA) membrane was determined by pyridine–HCl method as described previously.[18] The free amino group content of the p(HEMA/GMA)-CH membrane was determined by potentiometric titration. Briefly, the p(HEMA/GMA)-CH membrane about 1.0 g was transferred in HCl solution (0.1M, 20 mL) and it was then incubated in a shaking water-bath at 35°C for 6 h. After this reaction period, the final HCl concentration in the solution was determined by a potentiometric titration with 0.05M NaOH solution.

FTIR spectra of the p(HEMA/GMA) and p(HEMA/GMA)-CH membranes were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry membrane (about 0.1 g) was mixed with KBr (0.1 g) and pressed into a tablet form. The FTIR spectrum was then recorded.

Scanning electron micrographs (SEM) of the membranes were obtained using a JEOL, JMS 5600 scanning electron microscope, after coating with gold under reduced pressure.

The specific surface area of the p(HEMA/GMA) and p(HEMA/GMA)-CH membrane was measured by a surface area apparatus and calculated using the BET (Brunauer, Emmett, and Teller) method.[19]

Contact angles to water, glycerol, and diiodomethane of the p(HEMA/GMA), p(HEMA/GMA)-CH, p(HEMA/GMA)-CH-Fe(III), p(HEMA/GMA)-CH-GOD, and p(HEMA/GMA)-CH-Fe(III)-GOD membranes samples were measured by sessile drop method at ambient temperature using a digital optical contact angle meter CAM 200 (KSV Instruments, Helsinki, Finland). The surface free energy parameters of the membranes were calculated using the contact angle data of the probe liquids. The results are analyzed according to acid–base method of van Oss' et al.[20] In this method, the contact angles against at least three liquids with known values of γ^{LW}, γ^{+}, and γ^{-} are measured and the superscripts (LW), (+) and (−) refers to dispersive, Lewis acid and base components, respectively. The values for each experiment are put into the following equation:

$$(1 + \cos\theta)\gamma_1 = 2[(\gamma_s^{LW}\gamma_1^{LW})^{1/2} + (\gamma_s^{+}\gamma_1^{-})^{1/2} + (\gamma_s^{-}\gamma_1^{+})^{1/2} \quad (3)$$

The total surface energy γ^{TOT} is regarded as the sum of Lifschitz–van der Waals and the Lewis acid and base components.

$$\gamma^{TOT} = \gamma^{LW} + \gamma^{AB} \quad (4)$$

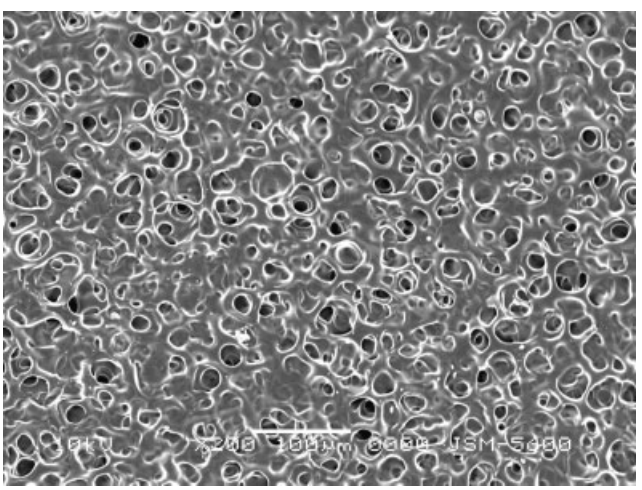

Figure 2 SEM micrograph of the p(HEMA/GMA) membrane.

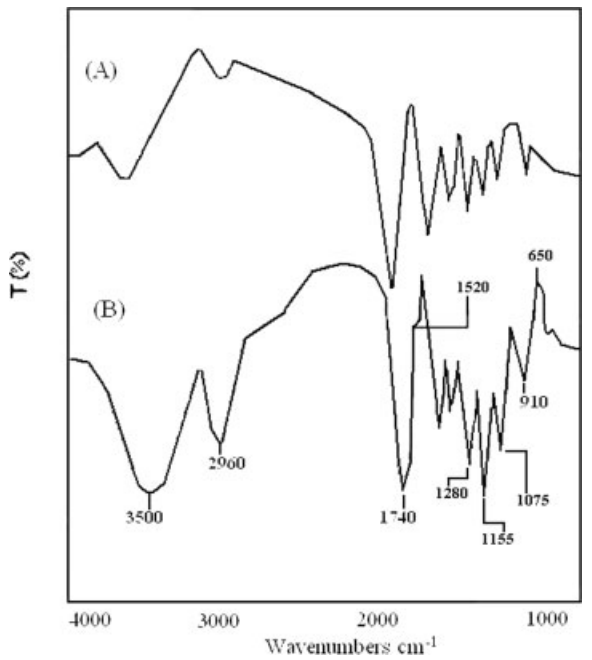

Figure 3 FTIR spectra. (A) p(HEMA/GMA) membrane; (B) p(HEMA/GMA)-CH membrane.

where γ^{LW} designated Lifschitz–van der Waals interaction was calculated from the measured diiodomethane contact angle, and γ^{AB} designated such acid–base interactions as hydrogen bonding, and γ^{+} and γ^{-} refer to proton and electron donating character, respectively. The method equations were solved using CAM 200 software packages operated under Windows 98 (KSV Instruments).

RESULTS AND DISCUSSION

Immobilization and comparison of recovered enzyme activity

The main physical and morphological properties of the chitosan-grafted p(HEMA/GMA) membrane were as follows: the thickness in the wet state of the membrane was 0.06 cm; the density of the membrane in the dry state was 1.23 g/cm^3; the plain surface area (smooth surface of the membranes without pores) of the 1.0 mL wet membrane was 34.6 cm^2. The specific surface area of the p(HEMA/GMA) and p(HEMA/GMA)-CH membranes was measured by BET method and were found to be 1.98 and 2.56 m^2/g membrane, respectively. After chitosan grafting, the specific surface area of the membrane was increased about 1.3 folds. The chitosan polymer was linked to the acrylic backbone (i.e., p(HEMA/GMA)) by the reaction between the —NH_2 groups chitosan and the epoxy group of copolymer (Fig. 1). The amounts of available epoxy groups were determined by titration of pyridine–HCl solution with 0.1*M* NaOH and were found to be as 2.18 mmol/g membrane. The free amino group content of the p(HEMA/GMA)-CH membrane was determined by potentiometric titration and the amount of free amino group on the chitosan-grafted membrane was found to be as 5.6×10^{-3} mmol/g membrane. The leakage studies show that a maximum 6% of the grafted chitosan was leaked from the membrane surface after 2 days of incubation period in different buffer solutions. It should be noted that the covalently-grafted chitosan on the membrane surface was stable under given experimental conditions. The presence of cationic charges on the membrane surface can provide an interaction site with the opposite charges of the protein and this leads to immobilization of protein via adsorption on the resultant membrane surface. The amount of immobilized Fe(III) ions on the membrane was found to be 2.17×10^{-3} mmol/g membrane, respectively.

Scanning electron microscopy (SEM) micrographs presented in Figure 2 shows the porous surfaces structure of the membrane. The membrane has a rough surface due to the pores, which formed during the polymerization process. The porous surface structure should be considered as a factor providing an increase surface area and thus, which will provide a high immobilization capacity.

FTIR spectra of plain and chitosan-grafted p(HEMA/GMA) membranes are presented in Figure 3. The broad band in the 3300–3500 cm^{-1} range indicates —OH stretching vibrations in the structure of the modified membranes. Among the characteristic vibration of GMA is the methyl vibration at 2960 cm^{-1}. The vibration at 1740 cm^{-1} represents the ester configurations of both HEMA and GMA. The epoxide group

TABLE I
Contact Angles of Water, Glycerol, and Diiodomethane on the Membrane Samples

	Water (θ°) ($\gamma_l = 71.3$)[a]	Glycerol (θ°) ($\gamma_l = 64.0$)[a]	Diiodomethane (θ°) ($\gamma_l = 50.8$)[a]
p(HEMA/GMA)	57.3 ± 1.3	49.4 ± 1.7	21.7 ± 0.8
p(HEMA/GMA)-CH	69.9 ± 1.9	68.9 ± 1.6	43.3 ± 2.1
p(HEMA/GMA)-CH-Fe(III)	83.8 ± 2.4	81.2 ± 1.1	44.9 ± 0.6
p(HEMA/GMA)-CH-GOD	43.3 ± 1.1	70.4 ± 0.3	42.2 ± 2.3
p(HEMA/GMA)-CH-Fe(III)-GOD	81.3 ± 0.5	80.9 ± 1.2	42.2 ± 1.7

[a] Test liquids and their surface tension, (γ_l).

TABLE II
Surface Free Energy Parameters (mN/m^2) of the Membrane Sample According to the van Oss et al.[20] Method

Membrane samples	γ^{LW} (mN/m^2)	γ^+ (mN/m^2)	γ^- (mN/m^2)	γ^{AB} (mN/m^2)	γ^{Total} (mN/m^2)
p(HEMA/GMA)	47.48	0.48	4.23	4.09	51.57
p(HEMA/GMA)-CH	38.13	0.07	3.86	0.56	38.69
p(HEMA/GMA)-CH-Fe(III)	37.15	0.54	2.75	2.99	40.14
p(HEMA/GMA)-CH-GOD	43.95	0.15	5.95	1.77	45.72
p(HEMA/GMA)-CH-Fe(III)-GOD	43.82	0.06	1.82	0.21	44.03

gives the band at 910 cm^{-1}. The FTIR spectra of the chitosan-grafted membranes have some adsorption bands different from those of the plain membranes. The most important adsorption band at 1520 cm^{-1} representing N—H bending, is due to chitosan bonded to the p(HEMA/GMA) membrane.

We had expected changes in surface properties of the membranes after chitosan coating, Fe(III) ions chelation, and GOD immobilization. The change in the surface properties of a material can be characterized by contact angle measurements. Considering that low contact angle to water represent hydrophilic surfaces and high contact angle represent hydrophobic surfaces, the p(HEMA/GMA) membranes can be defined as relatively hydrophilic. Chitosan coating caused an increase in the water contact angle value of the membrane (from 57.3° ± 1.3° to 69.9° ± 1.9°) compared to the plain membrane (Table I). The decreased hydrophilicity of the membrane surface caused by the incorporated of CH chains on the membrane surfaces and that was used as an active component for enzyme capture by charge interactions. The contact angle value of the membrane surface was further increased (83.8° ± 2.4°) after Fe(III) ions incorporation. On the other hand, the contact angle values of both the membranes were decreased to some extent after GOD adsorption.

The information about surface properties of a material can be obtained after the calculation of surface free energy using contact angle value of different test liquids.[21,22] It should be noted that the surface property of a support is the most important factor when enzyme immobilization is contemplated via adsorption. In the present work, the surface free energy of the membrane was determined using the acid–base method of van Oss' et al., consisting of the sum of the Lifschitz–van der Waals (γ^{LW}) and the acid–base components (γ^{AB}) applies for all investigated membranes at different values (Table II). As can be seen in table, chitosan-grafted membrane seems to exhibit only basic character (γ^-). This is an important observation because GOD is an acidic protein. From this point of view, the main interaction between CH-grafted support and GOD should be ion exchange. As expected, the investigated membranes (i.e., chitosan-grafted, Fe(III) ions incorporated, and GOD immobilized) exhibit different acid–base components (γ^{AB}) of the surface free energy because of different interactive functional groups of the membrane samples, and all the investigated membrane sample, the basic parameter (γ^-) is significantly larger when compared with the acidic parameter (γ^+). It should be noted that the Lifschitz–van der Waals component of the surface free energy of the p(HEMA/GMA)-CH membrane was also significantly different compared to Fe(III) ions incorporated counterpart. It is interesting to observe that the acid parameter of Fe(III) ions-incorporated membrane increased when compared with chitosan-grafted membrane (i.e., p(HEMA/GMA)-CH). Chitosan coating, Fe(III) ions incorporation, and GOD immobilization follow all the variations of both the dispersive and polar components. The comparison between before and after GOD immobilization, the surface free energy components of the CH-grafted and Fe(III) ions-incorporated membrane show that there is clear correlation with these parameters. It found that the incorporation of metal ions enhanced GOD immobilization capacity most significantly, and there is a large difference of surface energy component between the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes. These results indicated that GOD immobilization capacity of the membrane strongly depend on

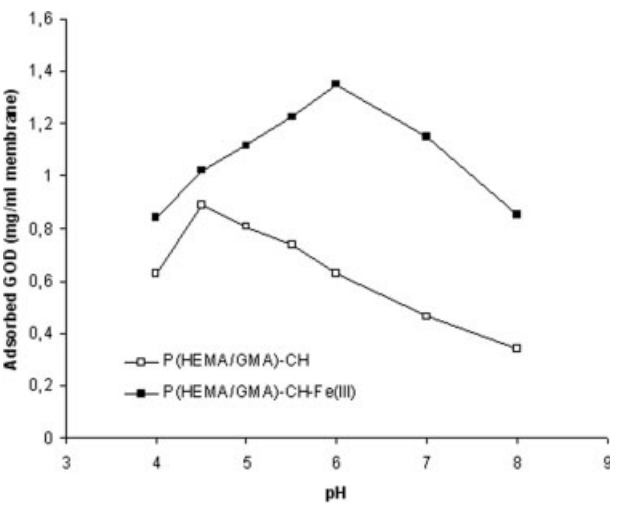

Figure 4 Effect of pH on GOD adsorption on the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes: the immobilization of GOD on the membranes at various pH values was studied under the following conditions; initial concentration of enzyme 1.0 mg/mL, temperature 22°C.

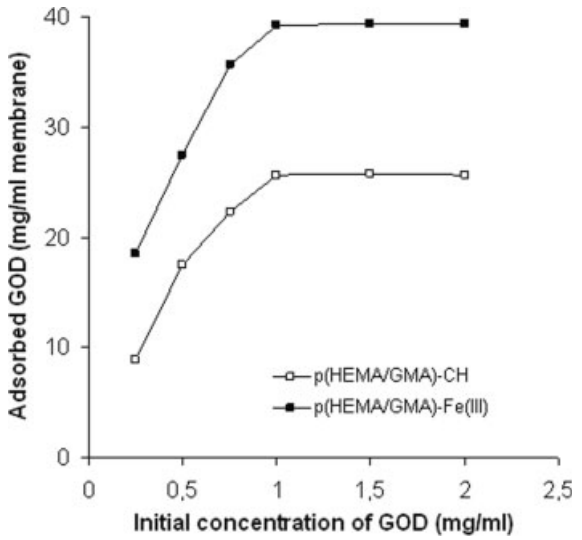

Figure 5 Effect of initial GOD concentration on the enzyme loading on the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes: the experimental conditions are pH 6.0, initial concentration of enzyme varied between 0.25 and 2.0 mg/mL, temperature 22°C.

the dispersive and polar components (i.e., functional hydrophobic, ionic, and Lewis acid–base groups and metal ions) on the surface of the support. Thus, all these parameters should be effective in determining the interactions of GOD with the ionic polymer chitosan and the chelated Fe(III) ions on the membranes.

Immobilization of glucose oxidase onto membranes

To maximize the amount of immobilized GOD onto p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes, the medium pH and initial enzyme concentration were changed for each individual set of batch immobilization experiment. The amount of immobilized GOD on the modified membrane is expressed as the weight of immobilized GOD per milliliter of the membrane (mg bound protein/mL membrane). The optimal pH values for the immobilization of GOD via adsorption onto the modified membranes were studied in the pH range of 4.0–8.0. The maximum GOD immobilization was obtained at pH 4.5 for p(HEMA/GMA)-CH (about 0.89 mg/mL) and at pH 6.0 for p(HEMA/GMA)-CH-Fe(III) (about 1.36 mg/mL) membranes (Fig. 4).

Glucose oxidase with a pI value of 4.0 contains large number of carboxyl groups. In the present study, the maximum adsorption was not observed at pI value of GOD, but shifted 0.5 unit to less acidic pH. This could be resulted from preferential interaction between anionic GOD and cationic CH molecules at pH 4.5. On the other hand, the maximum GOD adsorption was obtained at pH 6.0 with p(HEMA/GMA)-CH-Fe(III) membrane. The trivalent Fe(III) ions are considered hard Lewis acids and interact with hard Lewis base sides of the enzyme such as phosphate, carboxylic, and phenolic oxygen. It should be noted that GOD is an iron-containing flavoprotein, and the covalently linked flavin nucleotide contains phosphate groups, which provides additional binding sites for the enzyme to immobilization on the Fe(III) ions-incorporated membrane. As seen in Figure 4, the hard Lewis metal (Fe(III)) ions-incorporated membrane showed a different adsorption pH profile to enzyme when compared with Fe(III) ions free membrane.

The dependence of enzyme concentration on the immobilization efficiency was evaluated using six different concentration of enzyme in the range of 0.25–2.0 mg/mL (Fig. 5). An increase in GOD concentration in the medium led to an increase in immobilization efficiency for both CH-grafted and Fe(III)-incorporated membranes, but this levelled off at GOD concentration of 1.0 mg/mL for both membranes. As presented in Figure 5, with increasing enzyme concentration in solution, the amount of GOD adsorbed per unit area by p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes increases almost linearly at low concentrations. This could be explained by saturation of interacting groups of the grafted CH molecules and Fe(III) ions with the adsorbed GOD molecules, as a result of which achieve maximum immobilization capacity.

Kinetics parameters K_m and V_{max}

When a biocatalyst is immobilized, kinetic parameters K_m and V_{max} undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change for the substrate. The kinetic parameters K_m and V_{max} for the free and the immobilized enzyme preparations were determined using

TABLE III
Properties of the Adsorbed GOD on the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) Membranes

Enzyme preparations	V_{max} (U/mg enzyme)	K_m (mM)	Recovered activity (%)
Free GOD	238 ± 8	5.4 ± 0.2	–
p(HEMA/GMA)-CH-GOD	143 ± 6	6.9 ± 0.3	60 ± 1.3
p(HEMA/GMA)-CH-Fe(III)-GOD	182 ± 8	5.8 ± 0.3	76 ± 1.9

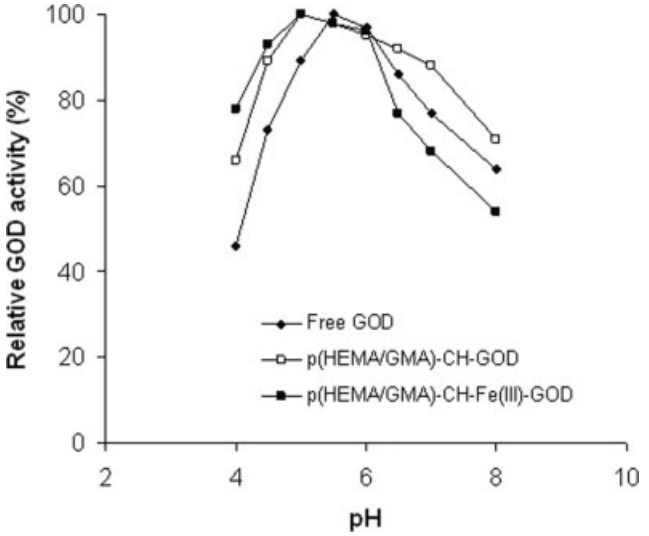

Figure 6 pH profiles of the free and immobilized glucose oxidase preparations.

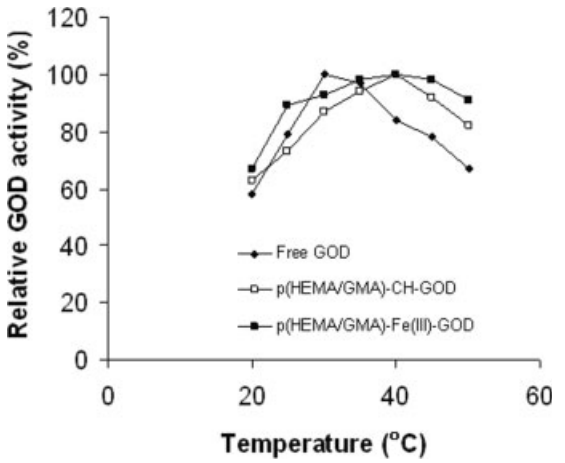

Figure 7 Temperature profiles of the free and immobilized glucose oxidase preparations.

Line weaver-Burk plot. For the free enzyme, K_m was found to be 5.4 m*M*, whereas V_{max} value was calculated as 238 U/mg proteins. The apparent K_m values were found to be 6.9 and 5.8 m*M* for the adsorbed GOD on p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes, respectively. As expected, the K_m and V_{max} values were significantly affected after immobilization of GOD on to the CH-grafted and/or Fe(III) ions-incorporated membranes (Table III). The decrease in the V_{max} values of both immobilized GOD preparations can be related with the increase in the corresponding K_m values, because an increase in the K_m value leads to a decrease in the affinity of the enzyme for its substrate.[23–25]

Effect of pH and temperature on the catalytic activity

The pH value for optimum activity for the free GOD was found to be at 5.5, which was similar to that reported previously.[1] The optimal pH value for the immobilized GOD preparations is found to have shifted to more acidic pH 5.0. In general, immobilized enzyme on the cationic supports can result in an acidic shift in the pH optimum. As seen in Figure 6, a shift to more acidic pH region for both immobilized GOD preparations is observed. Polyionic supports cause the partitioning of protons between the bulk phase and the enzyme microenvironment causing a shift in the optimum pH value. Furthermore, the pH profiles of the immobilized GOD preparations display significantly improved stability on both sides of the optimum pH value, in comparison to that of the free form, which means that the immobilization methods preserved the enzyme activity in a wider pH range. These results could probably be attributed to the stabilization of GOD molecules resulting from multipoint ionic interaction and/or coordination complex formation with the grafted CH molecules and Fe(III) ions.[1,5,11]

The activities obtained in a temperature range of 20–50°C were expressed as percentage of the maximum activity (Fig. 7). The activity of the free GOD is strongly dependent on temperature, with the optimum temperature being observed at about 30°C. The optimum reaction temperature for both immobilized GOD preparations was at 40°C, and the temperature profiles of the immobilized enzymes were broader than that of the free one. The main reason for shift toward higher temperatures, with adsorbed enzyme could be multipoint ionic interaction, which consequently leads to an increase in the activation energy for the enzyme to reorganize to proper conformation for binding to substrate.[5,26,27]

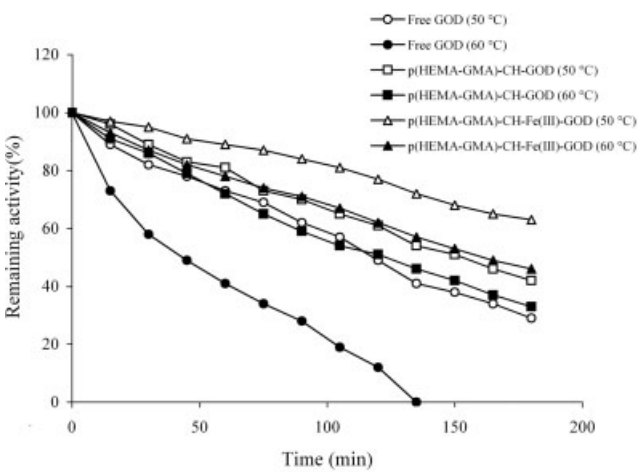

Figure 8 Temperature stabilities of the free and immobilized GOD preparations.

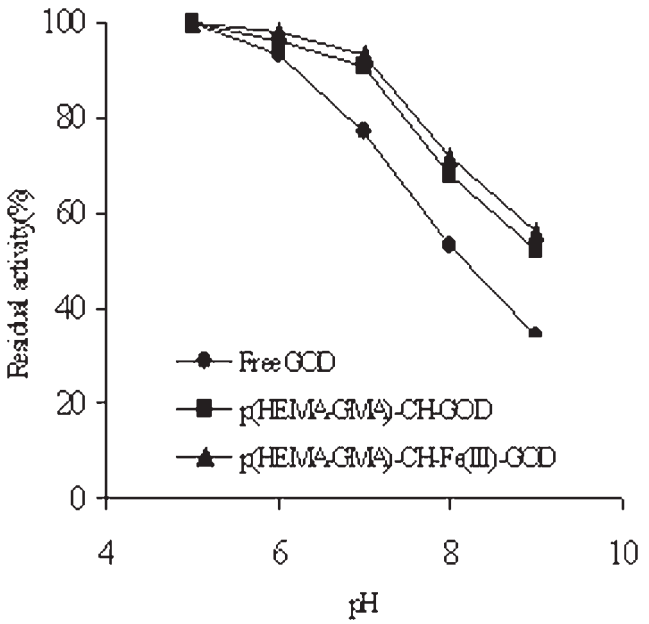

Figure 9 pH stabilities of the free and immobilized GOD preparations.

Thermal and pH stabilities of free and immobilized GOD preparations

Thermal stability experiments were carried out with the free and the immobilized enzymes, which were incubated in the absence of substrate at various temperatures. Figure 8 shows the heat inactivation curves between 50 and 60°C for the free and the immobilized GOD preparations. As seen from the figure, at 50 and 60°C, both immobilized enzyme preparations were inactivated at a much slower rate than those of the free enzyme. At 60°C, the free GOD lost all its initial activity within about 135 min. At 60°C, GOD adsorbed on both the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes preserve their initial activity about (33 ± 3.7)% and (46 ± 3.1)% in 180 min, respectively. It may be contributed to the stabilizing action on GOD conformation by chitosan and/or chitosan-Fe(III) ions complex on the membranes. It should be noted that the biocompatibility of the support could also be playing an important role in the stabilization of enzyme conformation and the resistance of enzyme to thermal denaturation. These observations suggest that the thermostability of immobilized GOD increased considerably as a result of immobilization onto natural polymer-grafted (i.e., chitosan) membrane.[28–31]

The relationship between pH and remaining activity of the free and the immobilized GOD preparations is presented in Figure 9. The enzyme preparations were incubated in the absence of substrate at different pH (between 5.0 and 9.0) for 24 h. There was no significant activity loss for the free GOD in the pH range 5.0–6.0, while it was found to be stable up to a pH value of 7.0 for both immobilized GOD preparations. As seen in the figure, the immobilized GOD preparations were less sensitive to pH than their free counterpart. These data indicated that the pH stability of GOD was significantly improved after immobilization via adsorption.

CONCLUSIONS

The p(HEMA/GMA) membrane was prepared form HEMA and GMA monomer in the presence of an initiator AIBN via UV-initiated photopolymerization. The chitosan polymer was grafted on the membrane as a cationic polymer ligand. The desired amount of enzyme can be loaded on the membranes by changing the initial concentration of enzyme in the immobilization medium. A high immobilization yield was obtained (1.36 mg/mL membrane) with the p(HEMA/GMA)-CH-Fe(III) membranes with respect to p(HEMA/GMA)-CH (0.89 mg/mL membrane). The thermal and pH stabilities of the immobilized glucose oxidase preparations were increased when compared with free GOD. At 60°C, immobilized GOD on the p(HEMA/GMA)-CH and p(HEMA/GMA)-CH-Fe(III) membranes retained activities after 120 min about (51 ± 3.7)% and (62 ± 3.1)%, respectively; whereas the activity retained by the free enzyme was only (12 ± 2.9)%. The chitosan-grafted and Fe(III)-incorporated p(HEMA/GMA) membranes have numerous advantages such as (i) the reactive membrane can be prepared without any activation steps and no need of any toxic substance for surface modifications; (ii) it can be easily used for enzyme immobilization under mild experimental conditions; the amount of immobilized enzyme on the membrane surface can be adjusted by changing initial enzyme concentration in the immobilization medium; iii) the presence of chitosan on the membrane surface could also provide resistant to microbial attach and high biocompatibility of CH molecules could also offer a natural micro-environment to the guest biomolecule; iv) finally, the reusability of the membrane supports may provide economic advantage for large scale biotechnological application.

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