Studies of Adsorption of Alkaline Trypsin by Poly(methacrylic acid) Brushes on Chitosan Membranes

Gülay Bayramoğlu, Hakan Erdogan, M. Yakup Arica

Biochemical Processing and Biomaterial Research Laboratory, Faculty of Science, Kırıkkale University, 71450 Yahşihan, Kırıkkale, Turkey

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ABSTRACT: Poly(methacrylic acid)-grafted chitosan membranes (chitosan-*g*-poly(MAA)) were prepared in two sequential steps: in the first step, chitosan membranes were prepared by phase-inversion technique and then epichlorohydrin was used as crosslinking agent to increase its chemical stability in acidic media; in the second step, the graft copolymerization of methacrylic acid onto the chitosan membranes was initiated by ammonium persulfate (APS) under nitrogen atmosphere. The chitosan-*g*-poly(MAA) membranes were first used as an ion-exchange support for adsorption of trypsin from aqueous solution. The influence of pH, equilibrium time, ionic strength, and initial trypsin concentration on the adsorption capacity of the chitosan-*g*-poly(MAA) membranes have been investigated in a batch system. Maximum trypsin adsorption onto chitosan-*g*-poly(MAA) membrane was found to be 92.86 mg mL^{-1} at pH 7.0. The experimental equilibrium data obtained for trypsin adsorption onto chitosan-*g*-poly(MAA) membranes fitted well to the Langmuir isotherm model. The adsorption data was analyzed using the first- and second-order kinetic models, and the experimental data was well described by the second-order equation. More than 97% of the adsorbed trypsin was desorbed using glutamic acid solution (0.5*M*, pH 4.0). In addition, the chitosan-*g*-poly(MAA) membrane prepared in this work showed promising potential for various biotechnological applications. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 108: 456–465, 2008

Key words: chitosan-*g*-poly(MAA) membrane; ion-exchange membrane; polymer brushes; adsorption; trypsin; thermodynamic parameters

INTRODUCTION

Ion-exchange separation by membrane chromatography plays an important role in isolation and purification of proteins from biological fluids.[1–5] Particularly, ion-exchange chromatography is based on differential adsorption of charged biological macromolecules at oppositely charged surfaces of chromatographic solid supports.[6–8] In bioseparation, the modification of membrane surface is widely used to change the character of the base membrane surface from hydrophobic to hydrophilic to create selective absorptive surface for adsorption of protein.[8–10]

Several natural polymeric support materials belonging to the class of polysaccharides have some inherent disadvantages such as poor mechanical strength, susceptibility to microbial degradation, uncontrolled water uptake, low-binding capacity to proteins.[11–16] Among them, chitosan is the N-deacytylated form of chitin that is obtained by alkaline treatment of chitin. Chitosan is abundant, available, renewable sources for valuable polymeric starting blocks, and that the development of new routes to produce valuable products in important research task.[17–20] Among the methods of modification of polymers, grafting is one of the promising methods, in principle graft copolymerization is an attractive method to impart a variety of functional groups to a polymer. For example, a grafting of functional pendant group carrying acrylate monomer such as methacrylic acid onto chitosan backbone could introduce a novel absorptive function.[17,18] The grafted brush side chain could increase the adsorption capacity of the support by allowing multilayer protein binding to this hybrid support.[21–24] Fibrous materials are among the most suitable matrices for isolation of protein from biological fluids because of their intrinsically high-specific surfaces, providing the quantity and accessibility of the binding sites necessary for high-adsorption capacity.[18–24]

Trypsin (EC 3.4.21.4) is a serine protease found in the digestive system, where it breaks down proteins. Trypsin specifically hydrolyzes peptide bonds at the carboxyl side of lysine and arginine residues. It is used for numerous biotechnological processes such as protein primary structure analysis, to breakdown casein in milk for baby food, and to resuspend cells adherent to the cell culture dish wall during the process of harvesting cells.[25–27]

Correspondence to: M. Y. Arica (yakuparica@tnn.net or yakuparica@kku.edu.tr).

In this study, chitosan membranes were prepared by phase-inversion technique and then crosslinked with epichlorohydrin under alkaline condition; secondly, the graft copolymerization of acrylic acid onto the chitosan membranes was initiated by ammonium persulfate (APS) under nitrogen atmosphere. After crosslinking process, the membranes were grafted using methacrylic acid in the presence of an initiator. The degree of grafting was measured gravimetrically. The reversible ion-exchange binding of model protein (tyrpsin; isoelectric point about: pI 10.5) was studied to evaluate the binding properties of the trypsin on the functional polymer brush layers. The adsorption parameters such as adsorption conditions (e.g., initial trypsin concentration, pH, ionic strength, and temperature) were varied to evaluate the nature of binding mechanisms of trypsin on the chitosan-*g*-poly(MAA) ion-exchange membranes.

EXPERIMENTAL

Materials

Chitosan powder, APS, and methacrylic acid monomer (MAA) were obtained from Fluka AG (Buchs, Switzerland), the monomer was distilled under reduced pressure and stored at 4°C until use. Trypsin (EC. 3.4.21.4; Type 1) and epichlorohydrin were supplied from Sigma Chemical (St Louis, MO) and used as received. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany). The water used in these experiments was purified using a Barnstead water purification system (Dubuque, IA).

Preparation of ion-exchange membranes

The preparation of chitosan membranes was carried out via phase-inversion techniques. Chitosan (5.0 g) was dissolved in acetic acid solution (4.0%, 100 mL). Chitosan solution (10 mL) was transferred into a flat glass dish and the water was allowed to evaporate for 18 h at 35°C. The dried membrane was then transferred in sodium hydroxide solution (2.5*M*, 5 mL). The formed chitosan membranes were washed twice with 50-mL purified water. The chitosan membranes were crosslinked by reaction with epichlorohydrin. The crosslinking reaction was carried out by immersing the chitosan membranes into epichlorohydrin solution in the flat glass dish (pH 12, 20 mL) at 50°C for 2.0 h at 100 rpm. The resulting crosslinked membrane was washed with acetone for 30 min to remove any possible impurities. The membrane was then washed several times with distilled water and phosphate buffer (0.1*M*, pH 7.0) in a sonicated water bath, and it was cut into disks (diameter: 0.5 cm) with a perforator.

The graft copolymerization of methacrylic acid on the crosslinked membrane was carried out using APS as initiator in a two naked round-bottomed glass reactor. In a typical grafting, crosslinked chitosan membrane disks (about 2.0 g) and APS aqueous solution ($6.58 \times 10^{-3}M$, 18 mL) were transferred into the reaction vessel and stirred magnetically at 100 rpm for 10.0 min. The monomer (i.e., methacrylic acid, 1.6 mL) was added drop wise to this reaction mixture. The reactor was allowed to warm to 60°C and stirring was continued for 3.0 h under nitrogen atmosphere. After the grafting reaction, the methacrylic acid-grafted chitosan membrane disks were filtered and washed several times with purified water. Finally, the methacrylic acid-grafted chitosan membranes were soaked in purified water for about 48 h and then dried under reduced pressure at 60°C. The grafting percentage was determined from the mass of dried membrane before and after grafting by using the following equation:

$$\%\ \text{of poly(MAA) grafting} = (W_2 - W_1)/W_1 \times 100 \quad (1)$$

where W_1 and W_2 are the mass of crosslinked chitosan membrane disks and poly(MAA) grafted chitosan membrane disks, respectively.

Characterization studies

The amount of available surface-functional carboxyl groups of the chitosan-*g*-poly(MAA) membrane was determined by potentiometric titration. The chitosan-*g*-poly(MAA) membrane (0.2 g) was allowed at room temperature for 24.0 h in purified water (10 mL). Then, 2*M* NaOH (10.0 mL) was added to the mixture and shaken for 1.0 h. At the end of this period, the membranes were filtered and assayed by titration with 0.1*M* HCl solution. The free amino groups' content of the crosslinked chitosan membrane was determined by potentiometric titration before and after grafting reaction. Briefly, the membrane (about 1.0 g) was transferred in HCl solution (0.1*M*, 20.0 mL) and the medium was incubated in a shaking water bath at 35°C for 6.0 h. After this period, the final HCl concentration in the solution was determined by a potentiometric titration with 0.05*M* NaOH solution.

The dried chitosan-*g*-poly(MAA) membranes were coated with gold under vacuum and their scanning electron micrographs (SEM) were obtained using a JEOL (JSM 5600) SEM. FTIR spectra of the chitosan-*g*-poly(MAA) membrane were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry sample (about 0.01 g) was mixed with KBr (0.1 g) and pressed into a tablet form. The FTIR spectrum was then recorded. The water content of the chitosan-*g*-poly(MAA) membrane was determined at room temperature in phosphate buffer (50 m*M*, pH

7.0) with a gravimetric method. They were weighed on a sensitive balance ($\pm 1.0 \times 10^{-4}$ g; Shimadzu, Model AX 120).

Adsorption experiments

Different quantities of chitosan-*g*-poly(MAA) membranes, varying from 0.06 to 0.30 mL membrane disk in each 5.0 mL of trypsin solution (0.5 mg mL^{-1}), were stirred at 100 rpm for 3 h at pH 7.0 and at 25°C. Adsorption of trypsin from aqueous solution on the chitosan-*g*-poly(MAA) membrane disks was studied at various pHs, in either acetate (5.0 mL, 50 m*M*, pH 4.0–5.0) or phosphate (5.0 mL, 50 m*M*, pH 6.0–8.0). Adsorption experiments were conducted in stirrer cells at 25°C for 3.0 h with continuous stirring. The effect of temperature and ionic strength on adsorption system was studied in phosphate buffer (50 m*M*, pH 7.0) for chitosan-*g*-poly(MAA) membrane at different temperatures (between 4 and 37°C) and NaCl concentrations (between 0.05 and 1.0*M*), respectively. All experiments were conducted in triplicates with 0.18 mL membrane disks, and initial concentration of protein was 0.5 mg mL^{-1} in each set of experiments. To determine the adsorption capacity of the chitosan-*g*-poly(MAA) membrane for trypsin, the initial and final concentrations of trypsin in the medium were measured at 280 nm by using a double-beam UV/vis spectrophotometer (Tokyo, Japan, Model 1601). Calibration curves were prepared using trypsin as standard (0.1–2.0 mg mL^{-1}). The amount of adsorbed protein was calculated using the following expression:[13]

$$c = [(C_0 - C) \cdot V]/M \quad (2)$$

where c is the amount of protein adsorbed onto the membrane (mg mL^{-1}); C_0 and C are the concentrations of the protein in the initial solution (mg mL^{-1}) before and after adsorption, respectively; V is the volume of the aqueous phase (L) and M is the volume of the membrane (mL).

The stability of chitosan-*g*-poly(MAA) membrane in repeated use

To investigate the stability and reproducibility of chitosan-*g*-poly(MAA) membranes, the adsorption–desorption cycle was repeated six times with the same ion-exchange membranes. The desorption experiments were carried out in a buffer solution at pH 4.0. The trypsin-adsorbed (at pH 7.0) chitosan-*g*-poly (MAA) membrane was placed in the desorption medium for 2 h with stirring at 150 rpm and at 25°C. The trypsin concentration within the desorption medium was determined as described earlier. The elution ratio was calculated from the amount of trypsin adsorbed on the chitosan-*g*-poly(MAA) membranes and the amount of trypsin desorbed.

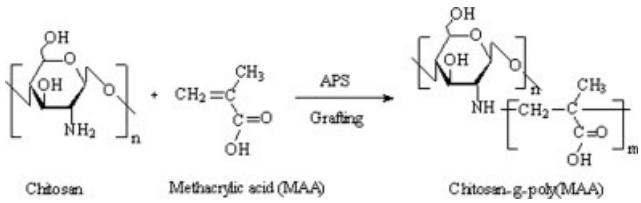

Figure 1 The schematic representation of the grafting reaction of the crosslinked chitosan membrane.

RESULTS AND DISCUSSION

Properties of chitosan-*g*-poly(MAA) ion-exchange membranes

Preparation of chitosan-*g*-poly(MAA) membrane was carried out in two sequential steps: in the first step, chitosan membrane was prepared by phase-inversion method and crosslinked with epichlorohydrin; in the second step, graft copolymerization of methacrylic acid onto crosslinked chitosan membrane was achieved by free radical polymerization in the presence of APS. The complex formed by the reaction between —NH_2 and —OH groups of chitosan decomposed to generate the free radical sites at about 60°C, facilitating the reaction site for the MAA monomer.[28] The schematic representation of grafting reaction is presented in Figure 1.

The effect of methacrylic acid concentration on the grafting efficiency of the chitosan membrane is presented in Figure 2. As seen in this figure, the grafting efficiency of the crosslinked chitosan membranes was increased with increasing methacrylic acid concentration (from 0.2 to 0.5 mol L^{-1}). On the other hand, further increase above 0.5 mol L^{-1} causes a significant

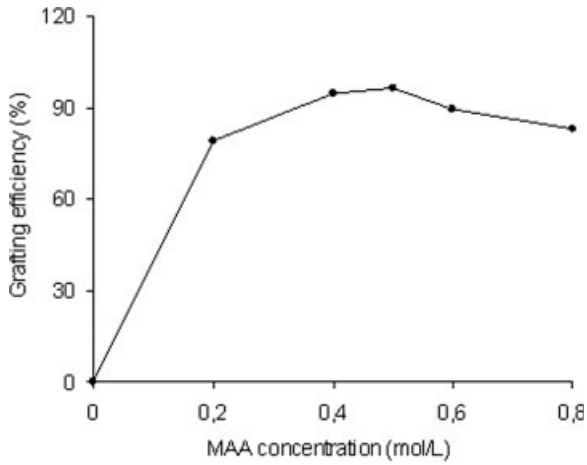

Figure 2 Effect of initial methacrylic acid concentration on the grafting efficiency of the membrane.

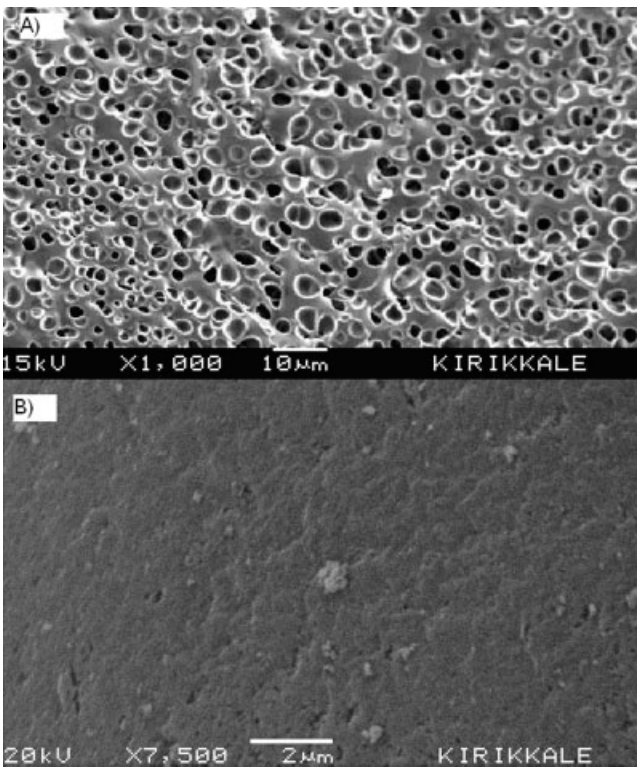

Figure 3 The SEM micrograph of (A) crosslinked chitosan membrane; (B) chitosan-*g*-poly(MAA) membrane.

decrease in the grafting efficiency of the chitosan membrane. After certain limit, the increase in MAA concentration accelerates the homopolymerization reaction rather than graft polymerization. Similarly, Wang et al.[29] studied the grafting of *N*-isopropylacrylamide into tubular-type porous polyethylene membranes. The grafting amounts are found to be proportional to the monomer concentration in the range from 0 to 15%. They reported that the grafting efficiency will depend on the monomer concentration. The grafting efficiency increases with monomer concentration up to a certain limit and then decreases with further increase in the monomer concentration. Therefore, in the remaining study, the initial concentration of methacrylic acid in the polymerization reaction was 0.5 mol L^{-1}. The free amino group content of the crosslinked chitosan membrane was determined by potentiometric titration before grafting, and the amount of free amino group on the crosslinked chitosan membrane was found to be as 2.86×10^{-3} mmol g^{-1} membrane. After grafting with methacrylic acid, there was not a detectable free amino group on the membrane surface. On the other hand, the amount of available carboxyl groups on the chitosan-*g*-poly(MAA) membrane surface was determined by potentiometric method and was found to be 9.72 mmol g^{-1}. From these data, the number of repeating units in poly(MAA) can be calculated at around 3400 units. The equilibrium water content of the chitosan-*g*-poly(MAA) membrane was found to be about 98% and it is moderate water content for a chromatographic support. The surface morphology of the crosslinked chitosan and grafted membranes is exemplified by SEM micrographs in Figure 3(A,B). The crosslinked chitosan membrane had a porous surface structure [Fig. 3(A)]. After grafting with poly(MAA) the pores of the crosslinked chitosan membrane were filled with grafted polymer, and the membrane surface was smoother when compared with bare counterpart membrane. As seen in Figure 3(B), poly(MAA)-grafted chitosan membranes did not show any porous surface structure.

FTIR spectra of the chitosan and chitosan-*g*-poly(MAA) membranes are presented in Figure 4. The FTIR spectra of poly(MAA)-grafted chitosan membranes had an absorption band different from that of chitosan at 1765 cm^{-1}. It is the characteristic adsorption of carboxyl groups of the poly(MAA)-grafted chitosan membrane. The appearance of the new peak evidenced the successful grafting of poly(MAA) on the chitosan membrane. The broad bands at $\sim$ 3400 cm^{-1} corresponds to the associated —OH stretching vibration of the hydroxyl groups, and the peak at 1650 cm^{-1} corresponds to the N—H deformation, is due to the amino groups of chitosan in the copolymer structure. A relatively high-intense peak at around 1100 cm^{-1} is because of the characteristic peak of polysaccharides.

Protein adsorption studies

The adsorption capacities and rates for the binding of the trypsin to the chitosan-*g*-poly(MAA) membrane were investigated at different pH values, and the results are presented in Figure 5. The adsorption capacities gradually increased, while the adsorption rates gradually decreased with the adsorption time until adsorption equilibrium was reached. The time at

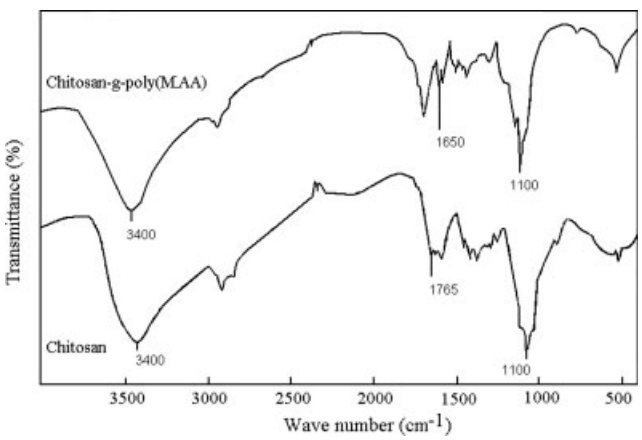

Figure 4 FTIR spectra of the chitosan and poly(methacrylic acid)-grafted chitosan.

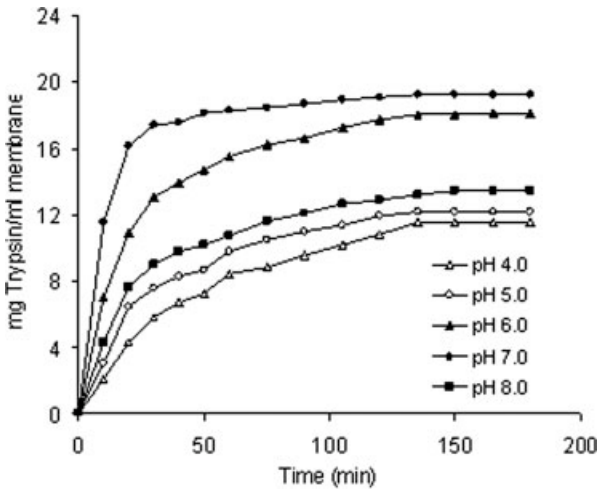

Figure 5 The adsorption rate of trypsin on the chitosan-*g*-poly(MAA) membrane at different pH values.

which trypsin reached adsorption equilibrium was about 150 min. After this equilibrium period, the amount of adsorbed protein on the chitosan-*g*-poly (MAA) membranes did not significantly change with time.

The effect of the solid/liquid ratio on the adsorption capacity of the chitosan-*g*-poly(MAA) membranes was studied for an initial concentration of trypsin (0.5 mg mL^{-1}) and the content of solid 0.06–0.30 mL membrane in adsorption medium (5 mL), and is presented in Figure 6. The resulted increase in the amount of trypsin adsorption from the medium with increase of the solid ratio can be explained by the augment of the number of functional sides in the adsorption medium. An increase in solid ratio from 0.06 to 0.18 mL membrane in 5-mL adsorption medium leads to increase of about 15% in trypsin removal from the medium. In the remaining study, 0.18-mL membrane disks were used in 5.0-mL adsorption medium.

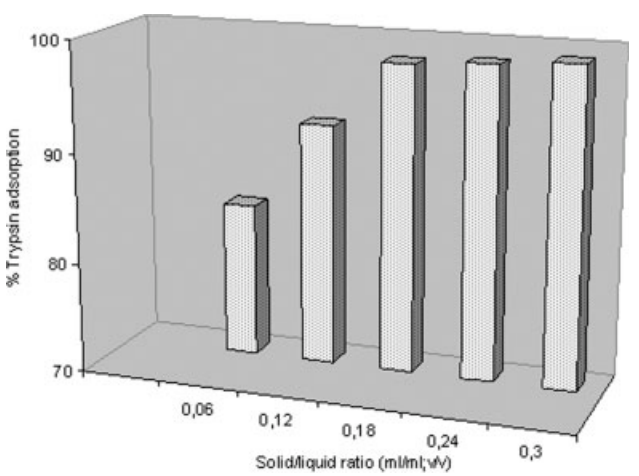

Figure 6 Effect of solid/liquid ratio on the adsorption capacity of the chitosan-*g*-poly(MAA) membrane.

The pH value of the solution affected the external charge distribution of trypsin and the chitosan-*g*-poly-(MAA) membrane.[30,31] To investigate the effects of pH on the trypsin adsorption efficiency and capacity of the chitosan-*g*-poly(MAA) membrane, the medium pH was changed between pH 4.0–8.0. Figure 7 shows the effect of pH on the amount of trypsin adsorbed on ion-exchange membrane. As seen here, the electrostatic interaction between trypsin and the chitosan-*g*-poly(MAA) membrane was the strongest at around pH 7.0 and gave the highest adsorption capacity. The isoelectronic (pI) value of trypsin is 10.5. The trypsin molecules would be cationic at pH values below 10.5. The trypsin molecules have net positive charges when medium pH is less than 10.5. On the other hand, the carboxylic groups of the grafted poly(metacrylic acid) brushes have net negative charge at above pH 4.0. At around pH 7.0, the electrostatic interaction between the protein ''trypsin'' and ion-exchange adsorbent should be predominant. Increasing the pH thereafter caused a decrease in adsorption. Specific interactions (electrostatic and hydrogen bonding) between trypsin and ion-exchange membrane at pH 7.0 may result from both the ionization states of several groups on both ion-exchange membrane (i.e., carboxyl, amino, carbonyl, and hydroxyl groups) and amino acid side chains in trypsin. Proteins that change conformation as a function of their environment (pH, salt, temperature, etc.), such as trypsin, which has a molecular mass of 24 kDa, could change conformation upon binding functional surface. Thus, trypsin molecules would expand and contact according to the variation of the ionizable groups on the surfaces. At pH 7.0, the resulting trypsin adsorption may also be due to suitable conformation of trypsin molecules on the chitosan-*g*-poly(MAA) membrane surface.

The functional groups of amino acid side chains of protein surface from which different amino acid

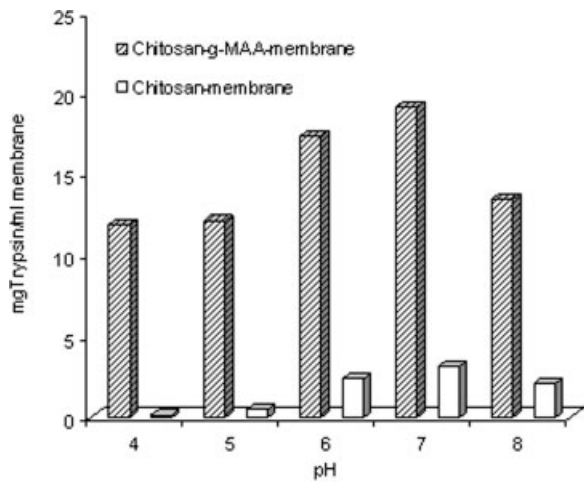

Figure 7 The adsorption of trypsin on the chitosan-*g*-poly (MAA) membrane as a function of pH.

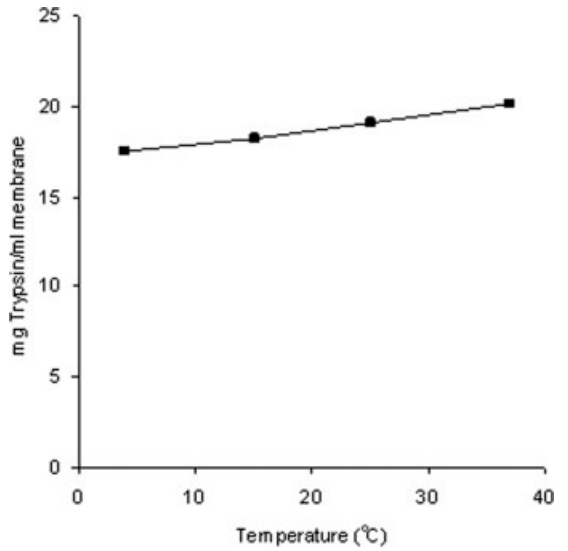

Figure 8 Effect of temperature on the adsorption capacity of the chitosan-*g*-poly(MAA) membrane.

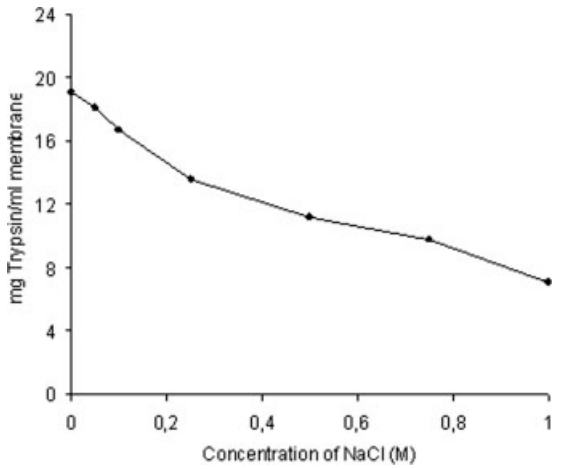

Figure 9 The trypsin adsorption at different NaCl concentrations onto the chitosan-*g*-poly(MAA) membrane. Conditions: Initial trypsin concentration 0.5 mg mL^{-1}; pH: 7.0; Temperature: 25°C.

charge states originate are mainly because of carboxyl and amino groups. At pH below the pK_a of poly (MAA) (4.75), the poly(MAA) chains are closely packed, limiting the interaction of trypsin with carboxylic groups of poly(methacrylic acid) brushes. At pHs above the pK_a value of poly(MAA), the carboxylic groups are ionized and interact with basic protein trypsin. On the other hand, in the basic pH region, the amino groups of the trypsin were deprotonated and the amount of adsorbed trypsin was consequently decreased.

As temperature increases, the contact area between the protein and the ligand on the matrix should increase, resulting in an increase in the affinity of proteins for the adsorbent at higher temperature. Trypsin showed an increased adsorption capacity with the chitosan-*g*-poly(MAA) membranes at higher temperature, and the results are presented in Figure 8. From 4 to 37°C, the absorption capacity of the chitosan-*g*-poly-(MAA) membranes for trypsin increased about 16%.

As seen in Figure 9, the adsorption capacity of the chitosan-*g*-poly(MAA) membrane was decreased for trypsin about 2.69 folds with increasing NaCl concentration from zero to 1.0*M* as the ionic strength increases from 0.0 to 1.0*M*. The decrease in trypsin adsorption capacity of ion-exchange membrane with increasing ionic strength should be resulted from decrease in the electrostatic interactions between trypsin and ion-exchange membrane. This behavior may be explained by the formation of more compact structures of the trypsin molecules at high-ionic strengths because of the conformational changes.[32–36] This result is also consistent with the prediction of the Debye-Hückel theory for the interaction of opposite charged interacting with protein/adsorbent interface.[28] From these observations, the hydrophobic moiety of the ion-exchange membranes did not play an important role for the interaction of the protein ''trypsin'' during the adsorption process.

Equilibrium adsorption isotherms

Figure 10 shows the equilibrium experimental data of trypsin adsorption on the chitosan-*g*-poly(MAA) membrane at four different temperatures. From the equilibrium adsorption isotherm observations, it can be concluded that the amount of trypsin adsorbed by the ion-exchange membranes increased with increasing trypsin concentration in the medium. It should be noted that, with increasing temperature, the amount of trypsin adsorbed on chitosan-*g*-poly(MAA) membrane increased. The maximum trypsin adsorption onto chitosan-*g*-poly(MAA) ion-exchange membranes

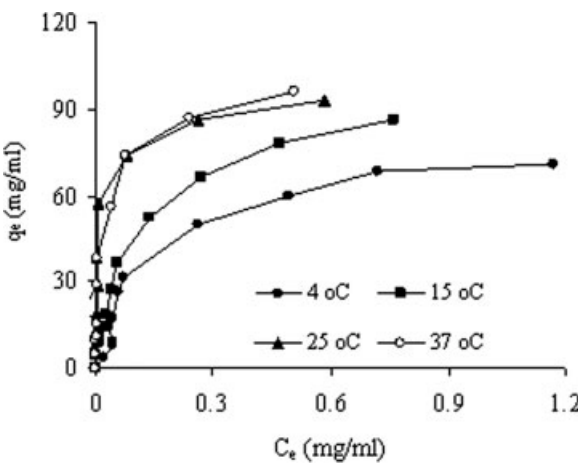

Figure 10 The equilibrium experimental data of trypsin adsorption on the chitosan-*g*-poly(MAA) membrane for different temperature, pH: 7.0; temperature: 25°C.

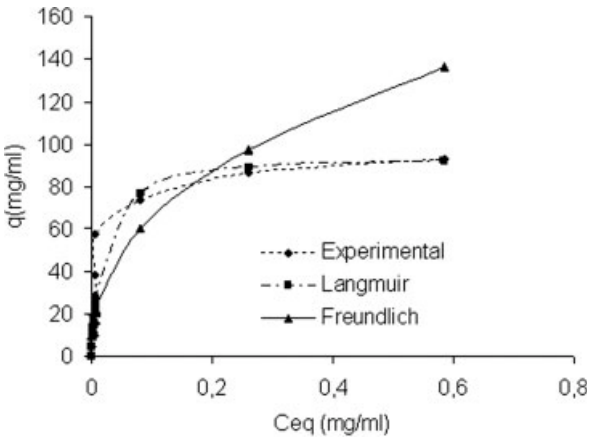

Figure 11 Comparison of the equilibrium experimental and the adsorption isotherms obtained from the Langmuir and the Freundlich models for trypsin adsorption on the membrane. Conditions: pH: 7.0; temperature: 25°C.

was observed as 92.8 mg mL^{-1} at 25°C. The maximum adsorption capacity of the crosslinked chitosan membranes for trypsin was found to be 6.9 mg mL^{-1}. The grafting of the chitosan membranes with poly(MAA) brush increased the absorption capacity about 13.4 folds.

Two theoretical isotherm models (Langmuir and Freundlich) were used to analyze the experimental data. The Langmuir model is described by the following equation:

$$dq/dt = k_1\, C(q_m - q) - k_2\, q \tag{3}$$

where C is the concentration of protein in solution, q is the amount of protein adsorbed on the membranes, and q_m is the maximum adsorption capacity of the membranes. At equilibrium, eq. (3) leads to

$$q_{eq} = q_m\, C_{eq}/(K_d + C_{eq}) \tag{4}$$

where K_d is the dissociation constant.

The Freundlich isotherm is frequently used to describe the adsorption. It relates the adsorbed concentration as the power function of solute concentration. This empirical equation takes the form:

$$q_{eq} = K_F(C_{eq})^{1/n} \tag{5}$$

where K_F and n are the Freundlich constants characteristic of the system. K_F and n are indicators of the adsorption capacity and adsorption intensity, respectively.

The corresponding semireciprocal plots and Scatchard plots gave rise to linear plots for the chitosan-*g*-poly(MAA) ion-exchange membrane at different temperature, and the correlation coefficients of semireciprocal plots (R^2) was greater than 0.988 for all the temperatures, indicating that the Langmuir model could be applied in this system (Fig. 11). From the slopes, the maximum capacities (q_m) were found to be between 87.63 and 98.05 mg mL^{-1} for trypsin on the chitosan-*g*-poly(MAA) membranes at different temperatures. The fitted curves and the fitted parameter values for the Langmuir model are presented in Figure 11 and Table I, respectively. Since the adsorption of trypsin onto the chitosan-*g*-poly(MAA) membrane can be described in terms of the Langmuir model. The apparent dissociation constant (K_d) estimated from the intercept is a measure of the stability of the complex formed between a protein and an adsorbent under specified experimental conditions. For example, a large K_d value indicates that the protein has a low-binding affinity for the adsorbent. At different temperatures, the K_d values were found to be between 11.48 and $0.73 \times 10^{-6} M$ for the chitosan-*g*-poly(MAA) membranes and, a high-binding affinity was obtained for the ion-exchange membranes.

The Freundlich plots for trypsin adsorption on the chitosan-*g*-poly(MAA) membranes at different temperatures are presented in Table I. The Freudlich constants, K_F and n were found to be between 88.2 and 159.6 and, 1.63 and 2.61, respectively. Values of $n > 1$ for the ion-exchange membranes indicate positive cooperativity in binding and a heterogeneous nature of adsorption. Thus, the adsorption of trypsin onto the chitosan-*g*-poly(MAA) membrane cannot be described in terms of the Freudlich model due to the homogenous adsorption as described by the Langmuir model.

Kinetic modeling

The kinetic models (the first-order and second-order equations) can be used in this case assuming that

TABLE I
Langmuir and Freundlich Constants and Correlation Coefficients for Adsorption of Trypsin on the Chitosan-*g*-poly(MAA) Membranes at Different Temperatures

Temperature (K)	Experimental c_{eq} (mg mL^{-1})	Langmuir constants			Freundlich constants		
		q_m (mg mL^{-1})	$K_d \times 10^6$ (M)	R^2	n	K_F	R^2
277	70.65	87.63	11.48	0.988	1.63	88.2	0.948
288	86.11	90.92	3.68	0.994	2.07	112.8	0.982
298	92.86	95.24	0.82	0.998	2.40	170.7	0.943
310	95.84	98.05	0.73	0.997	2.61	159.6	0.983

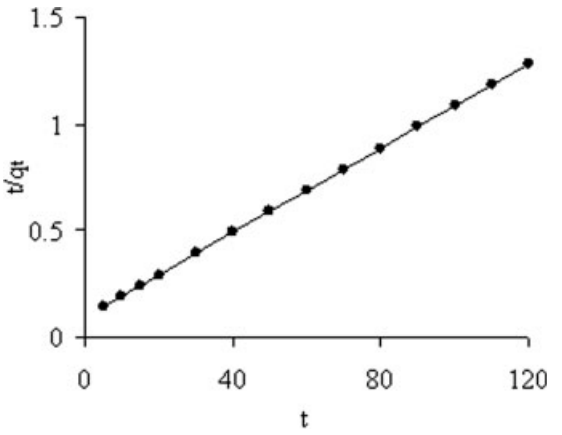

Figure 12 Second-order kinetic plots of the experimental data for the chitosan-*g*-poly(MAA) membrane.

measured concentrations are equal to adsorbents surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$dq_t/dt = k_1(q - q_t) \tag{6}$$

where k_1 is the rate constant of the first-order adsorption (min^{-1}), and q and q_t denotes the amount of adsorbed trypsin at equilibrium and at time t (mg mL^{-1}), respectively.

Equation (5) can be rearranged to obtain a linear form

$$\log(q - q_t) = \log q - (k_1 \cdot t)/2.303 \tag{7}$$

A plot of log ($q - q_t$) against t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process log q should be equal to the intercept of a plot of log ($q - q_t$) against t.

The second-order equation was applied for adsorption of solutes on the adsorbents.[37] The second-order equation based on adsorption equilibrium capacity may be expressed in the form as follows:

$$q_{eq}/(q - q_t) = k_2 t + 1 \tag{8}$$

The linear form of equation is as follows:

$$1/q_t = 1/k_2 q t + 1/q \tag{9}$$

From the eq. (9), a plot $1/q_t$ versus $1/t$ should give a straight line, and the sorption capacity q and the rate constant k_2 can be calculated from the intercept and the slope of the linear second-order equation, respectively.

To analyze the adsorption kinetics of trypsin, the first-order and the second-order kinetics models were applied to the experimental data. The second-order equation fitted well with the experimental data (Fig. 12). The comparison of experimental adsorption capacities and the theoretical values estimated from the first-order equation are presented in Table II. The theoretical q values for the chitosan-*g*-poly(MAA) membranes were very close to the experimental q values in the case of the second-order kinetics. The second-order kinetics best described the data.

In addition, Arrhenius plots in the temperature range from 4 to 37°C obtained from $1/T$ versus ln k_2 (k_2; second-order rate constant) appear linear; activation energy (E_a) was found to be −6.58 for the chitosan-*g*-poly(MAA) membranes. The lower activation energy calculated for the ion-exchange membranes indicate that the adsorption of trypsin on the chitosan-*g*-poly (MAA) membranes is favorable. The binding of trypsin on the chitosan-*g*-poly(MAA) membranes may not require a large conformational deformation, thereby resulting in lower activation energy for the molecule to reorganize and attain the proper conformation for binding to the ion-exchange membranes.

Thermodynamic parameters

The thermodynamic parameters such as free energy changes (ΔG°), enthalpy change (ΔH°), and entropy change (ΔS°) for the adsorption process can be estimated using the following equations:

$$(\Delta G^\circ) = -RT \ln K_a \tag{10}$$

$$\ln K_a = (\Delta S^\circ/R) - (\Delta H^\circ/RT) \tag{11}$$

TABLE II
Kinetics Model Parameters for the Adsorption of Trypsin onto Chitosan-*g*-poly(MAA) Membrane

Temperature (K)	Experimental c_{eq} (mg mL^{-1})	First-order q (mg mL^{-1})	First-order $k_1 \times 10^2$ (1 min^{-1})	First-order R^2	Second-order q (mg mL^{-1})	Second-order $k_2 \times 10^2$ (mL/mg^{-1} min^{-1})	Second-order R^2
277	70.65	85.11	6.18	0.923	76.33	1.37	0.997
288	86.11	120.14	6.50	0.965	90.91	1.12	0.999
298	92.86	122.74	5.43	0.916	100.13	1.03	0.998
310	95.84	158.21	2.84	0.989	101.01	1.01	0.995

TABLE III
Thermodynamic Parameters of the Chitosan-*g*-poly(MAA) Membrane for Adsorption of Trypsin

Temperature (K)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J/mol^{-1} K^{-1})
277	−26.19	63.75	325.91
288	−29.96		
298	−34.72		
310	−36.41		

The dependency of the equilibrium association constant, K_a, ($K_a = 1/K_d$, from Langmuir constant) versus $1/T$ for the binding of trypsin on the chitosan-*g*-poly (MAA) membranes was analyzed in terms of Van't Hoff plots. The ΔG° values for trypsin adsorbed on the chitosan-*g*-poly(MAA) membranes were calculated for each temperature and tabulated in Table III. The negative ΔG° values for each temperature indicated that adsorption of trypsin on the chitosan-*g*-poly(MAA) membranes were a favorable process and those were ranged between −26.19 and −36.41 kJ mol^{-1}. The ΔS° value for the adsorption of trypsin to the chitosan-*g*-poly(MAA) membranes was 325 J/mol^{-1} K^{-1}. At lower temperatures, due to lower kinetic motion of the carboxylic groups, the value of the phase ratio will be different to that at higher temperatures. The positive values of ΔS° show the increased randomness at the solid/solution interface during adsorption. The calculated ΔH° value of the system for the interaction of the trypsin with the pendant carboxylic groups of the poly(MAA) was 63.75 kJ mol^{-1}. The positive value of ΔH° indicates the endothermic nature of adsorption.

Desorption

The desorption of adsorbed trypsin from the chitosan-*g*-poly(MAA) ion-exchange membranes was studied in a batch system. The trypsin-adsorbed chitosan-*g*-poly(MAA) membranes were placed within the desorption medium containing 0.5*M* glutamic acid at pH 4.0, and the amount of trypsin released in 120 min was determined. For all the tested adsorbents more than 97% of the adsorbed trypsin was desorbed. To show the reusability of the chitosan-*g*-poly(MAA) membranes, adsorption–desorption cycle of trypsin was repeated six times by using the same chitosan-*g*-poly(MAA) membranes (Fig. 13). The adsorption capacity of the ion-exchange membrane did not change significantly after six times use in the repeated adsorption–desorption operations.

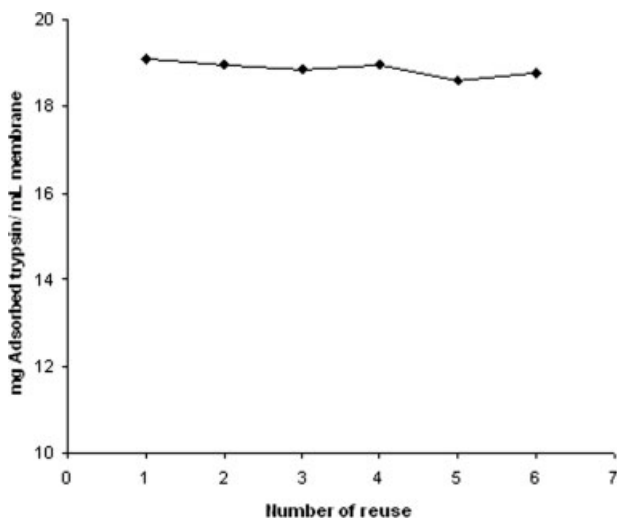

Figure 13 Reusability of the chitosan-*g*-poly(MAA) membrane during adsorption/desorption of trypsin.

CONCLUSION

An ion-exchange membrane matrix was prepared by grafting of poly(MAA) on the epichlorohydrin crosslinked chitosan membranes. The poly(acrylic acid) brushes on the membrane to form films capable of binding monolayer of proteins. In addition, poly(acrylic acid) film provides a hydrophilic microenvironment for the guest protein. The adsorption behaviors of the model protein ''trypsin'' under chromatographic conditions onto chitosan-*g*-poly(MAA) membranes have been investigated for detailed characterization of poly (acrylic acid) brushes layers on the membrane surfaces. It has been shown that the liquid/solid ratio, pH, ionic strength, and temperature can have important effects on the adsorption equilibrium. The pH of the medium has an important effect on the adsorption equilibrium of trypsin, and there is a preferential interaction between trypsin and chitosan-*g*-poly(MAA) membranes at pH 7.0. The trypsin adsorption capacity of the chitosan-*g*-poly(MAA) membranes was decreased with increasing ionic strength. The theoretical q_{eq} value of the ion-exchange membrane system was very close to the experimental q_{eq} values in the case of second-order kinetics. The ion-exchange groups of the chitosan-*g*-poly(MAA) membrane showed excellent performance in the electro-driven adsorption of trypsin, but the protonation of carboxylic groups in the pH range < 4.0 can be a limitation. Finally, the hydrogel hybrid membrane prepared from crosslinked chitosan and poly(methacrylic acid) brushes can be a useful candidate as an ion-exchange adsorbent in the separation of basic protein from biological fluids.

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