Poly(2-Hydroxyethylmethacrylate)/Chitosan Dye and Different Metal-Ion-Immobilized Interpenetrating Network Membranes: Preparation and Application in Metal Affinity Chromatography

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Received 24 September 2002; accepted 24 September 2002

ABSTRACT: Composite membranes were synthesized with 2-hydroxyethylmethacrylate and chitosan (pHEMA/chitosan) via an ultraviolet-initiated photopolymerization technique in the presence of an initiator (α,α'-azobisisobutyronitrile). The interpenetrating network (IPN) membranes were improved by the immobilization of dye molecules via hydroxyl and amino groups on the membrane surfaces from the IPNs. A triazidine dye (Procion Green H-4G) was covalently immobilized as a ligand onto the IPN membranes. The protein showed various affinities to different chelated metal ions on the membrane surfaces that best matched its own distribution of functional sites, resulting in a distribution of binding energies. In support of this interpretation, two different metal ions, Zn(II) and Fe(III), were chelated with the immobilized dye molecules. The adsorption and binding characteristics of the different metal-ion-chelated dye-immobilized IPN membranes for the lysozyme were investigated with aqueous solutions in magnetically stirred cells. The experimental data were analyzed with two adsorption kinetic models, pseudo-first-order and pseudo-second-order, to determine the best fit equation for the adsorption of lysozyme onto IPN membranes. The second-order equation for the lysozyme–dye–metal-chelated IPN membrane systems was the most appropriate equation for predicting the adsorption capacity for all the tested adsorbents. The reversible lysozyme adsorption on the dye-immobilized and metal-ion-chelated membranes obeyed the Temkin isotherm. The lysozyme adsorption capacity of the pHEMA/chitosan dye, pHEMA/chitosan dye–Zn(II), and pHEMA/chitosan dye–Fe(III) membranes were 2.54, 2.85, and 3.64 mg cm^{-2}, respectively. The nonspecific adsorption of the lysozyme on the plain pHEMA/chitosan membrane was about 0.18 mg cm^{-2}. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 1843–1853, 2003

Key words: interpenetrating networks (IPN); membranes; adsorption

INTRODUCTION

In comparison with traditional column chromatography, affinity membrane chromatography offers a number of advantages, such as higher flow rates, faster binding kinetics, much lower pressure drops, and higher productivity. Several types of ligands, including biological ligands, pseudospecific ligands, and metal ions, have been immobilized onto membrane supports for the affinity separation and purification of biomolecules.[1–4] The pseudospecific ligands (e.g., triazine dyes) offer advantages over biological ligands in terms of economy, ease of immobilization, stability, and high adsorption capacity. The only drawback of the dyes appears to be their moderate selectivity for target proteins; this problem can be overcome by the introduction of new selectively interacting materials on the basis of their affinities for chelated metal ions.[5–8] The immobilized-metal affinity chromatography (IMAC) of proteins, with the metal chelate linked to Sepharose (Taufkirchen, Germany), was first described by Porath et al.[9] They reported a model system with Zn(II) and Cu(II) columns in tandem for the fractionation of human serum proteins. Such separations are generally based on the selective interactions between proteins containing binding residues (i.e., histidine, cysteine, and trytophan) and chelated metal ions.[10] The selectivity of the separation can be tailored through the choice of the metal ion, the solvent conditions, or the modification of the target protein (e.g., the addition of histidine-rich residues).[11,12] The nature of protein binding has important implications for the design of efficient separations and new materials for IMAC supports. The selection of the support material and its preparation constitute the dominant factors affecting chromatographic performance.[13,14] Accordingly, several studies have been performed to develop novel affinity supports (membranes or beads).[15,16] The materials used for affinity membranes can roughly be divided into two groups,

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Journal of Applied Polymer Science, Vol. 88, 1843–1853 (2003)

that is, natural and synthetic polymers. Natural macromolecular materials such as agarose, dextrin, cellulose, and chitosan are good affinity matrices but are mechanically weak and often difficult to process as membranes. Poly(2-hydroxyethylmethacrylate) pHEMA, is a hydrogel because the matrix retains a large amount of water, and it possesses a high mechanical strength.[17] Much attention has been paid to improving the chemical and physical properties of hydrogels in the swollen state. The main effort has focused on the introduction of a second component, a hydrophobic or hydrophilic polymer, into hydrogels to form so-called interpenetrating networks (IPNs). For the combination of the useful properties of synthetic pHEMA and natural chitosan, a new IPN matrix is prepared from these polymers. In addition, the presence of hydroxyl and amino groups on the prepared IPNs offer easy attachment sites for a variety of ligands.[10–14]

Equilibrium binding studies have shown that a simple Langmuir type may not adequately describe protein binding to IMAC supports: binding heterogeneity, as evidenced by nonlinear Scatchard plots, is often observed for tightly binding proteins. This model, therefore, cannot explain the observed increase in the limiting capacity with different functional heterogeneous surface contents. On the basis of these observations, Hutchens et al. proposed that protein binding might involve simultaneous interactions between multiple sites on the protein and the IMAC support.[18] Protein adsorption is better described by another model also widely used in studies of gas adsorption, the Temkin isotherm.[19]

In this study, pHEMA/chitosan hydrogel membranes were prepared by ultraviolet (UV)-initiated photopolymerization. A ligand of Procion Green H-4G (Reactive Green 5) was immobilized onto the pHEMA/chitosan membranes via nucleophilic substitution reactions under alkaline conditions. The immobilized dye molecule had a phthalocyanine moiety, seven sulfone groups, and three secondary amino groups, and one of the components (i.e., chitosan) of the IPN matrix also had amino groups; those groups showed a high binding affinity to metal ions. Two different metal ions, Zn(II) and Fe(III), were chelated with the immobilized dye molecules. The resulting metal-chelated affinity surfaces of the IPNs were then tested for their propensity to adsorb lysozymes in a batch system. The adsorption conditions (i.e., the initial concentration of the lysozyme, temperature, and medium pH) were varied to evaluate their effects on the performances of the affinity membranes. The adsorption kinetics of the lysozyme on the dye-immobilized and metal-ion-chelated IPN membranes were studied to characterize the surface complexation reaction. The adsorption isotherm was measured to evaluate the discrepancy between the experimental data and the theoretical equilibrium capacity predicted by the kinetic equations.

EXPERIMENTAL

Materials

Lysozyme (chicken egg white, EC 3.2.1.7) was supplied by Sigma Chemical Co. (St Louis, MO) and was used as received. 2-Hydroxyethylmethacrylate (2-HEMA) was obtained from Fluka AG (Taufkirchen, Germany), distilled under reduced pressure in the presence of hydroquinone, and stored at 4°C until use. α,α'-Azobisisobutyronitrile (AIBN), chitosan, and Procion Green H-4G (Reactive Green 5) were obtained from Sigma Chemical. All other chemicals were analytical-grade and were purchased from Merck AG (Darmstadt, Germany).

Synthesis of the pHEMA/chitosan IPNs

The IPN synthesis was achieved by the mixing of a chitosan solution (1.0% chitosan in 1.0% acetic acid, 4.0 mL) with 2-HEMA monomer (2.0 mL) containing 20 mg of AIBN. After the nitrogen flash, the solution was poured into a round, glass mold (diameter = 9.0 cm), sealed, and exposed to UV for 1.0 h at the ambient temperature. The nitrogen atmosphere was maintained during the UV irradiation. After the polymerization period, the resultant product was washed first with a 1.0% NaOH solution and then with distilled water. The IPN membrane was cut into circular pieces (diameter = 0.75 cm) with a perforator and was left in the wet state at 4°C.

Procion green H-4G immobilization onto the IPN membranes

Procion Green H-4G was covalently immobilized onto the IPNs via the nucleophilic reaction between the chloride of its triazine ring and the amide and hydroxyl groups of the IPNs under alkaline conditions. Procion Green H-4G (300 mg) was dissolved in distilled water (10 mL) and transferred into the same medium (80 mL) in which IPN membrane disks (15 g) were equilibrated. Sodium hydroxide (1.0*M*, 10 mL) was then added to the medium (ca. pH 13) and heated at 80°C for 4 h in a sealed reactor. After the reaction period, the solution cooled to room temperature, and IPN membrane disks were washed several times with distilled water, 2.0*M* NaCl, and 10% methanol. During the last stage, IPN membrane disks were washed again with distilled water. The Procion Green H-4G-immobilized IPN membrane disks were stored at 4°C until use.

Incorporation of the metal ions onto the immobilized procion green H-4G

To screen the lysozyme binding characteristic of the adsorbents, we chelated Procion Green H-4G-immo-

bilized IPN membranes in combination with the soft Lewis metal ion Zn(II) or the hard Lewis metal ion Fe(III) with the functional groups of IPN membranes (i.e., the phytalocyanine moiety and sulfone groups of the immobilized dye molecules and the amino groups of the chitosan molecules). A 100 ppm solution of each metal ion was prepared from nitrate salts in distilled water at pH 4.1.The medium pH was adjusted with 0.1*M* HCl at the beginning of the experiment, and it was not checked afterward. The dye-immobilized IPN membranes were placed in the metal-ion solution with stirring at 100 rpm at 25°C for 1 h. After this period, the dye–metal-ion-chelated membrane disks were washed several times with a phosphate buffer (50 m*M*, pH 7.0) and then stored in the same fresh buffer at 4°C until use.

The concentration of each metal ion in the resulting solution was determined with an AA 6800 flame atomic absorption spectrophotometry (AAS; Shimadzu, Tokyo, Japan). The metal-ion desorption experiments were performed in a buffer solution containing 25 m*M* ethylenediaminetetraacetic acid at pH 4.9. The metal-ion-chelated IPN membranes were placed in a medium with stirring at 100 rpm at 25°C for 60 min. The final metal-ion concentration in the medium was determined by AAS.

Effect of the pH and temperature on the lysozyme adsorption

The adsorption of lysozyme from an aqueous medium onto the dye-immobilized and metal-ion-chelated [i.e., Zn(II) and Fe(III)] IPN membranes was studied at various pHs, in either acetate (7.5 mL, 50 m*M*, pH 4.0–5.5) or a phosphate buffer (7.5 mL, 50 m*M*, pH 6.0–8.0). The initial concentration of the lysozyme was 1.0 mg mL^{-1} in each corresponding buffer solution. The adsorption experiments were conducted for 2 h at 25°C with continuous stirring. At the end of this period, the IPN membrane disks were removed from the lysozyme solution. The amount of adsorbed lysozyme on the dye-immobilized and metal-ion-chelated IPN membranes was determined from the initial and final concentrations of the lysozyme within the adsorption medium. A calibration curve was prepared with the lysozyme as a standard (0.05–3 mg mL^{-1}). The concentration of the lysozyme was measured at 280 nm with a Shimadzu 1601 double-beam ultraviolet–visible spectrophotometer.

To evaluate the effect of the temperature on the adsorption rate and capacity, we performed the adsorption experiments at four different temperatures (5, 15, 25, and 35°C)

Adsorption kinetics

To determine the adsorption capacities of the dye-immobilized and metal-ion-chelated IPN membranes, we varied the initial concentration of the lysozyme between 0.05 and 4.0 mg mL^{-1}. In a typical adsorption experiment, the lysozyme was dissolved in a phosphate buffer (7.5 mL, 50 m*M*, pH 7.0), and the dye-immobilized or metal-ion-chelated membranes were added. The adsorption experiments were carried out for 2 h at 25°C at a stirring rate of 100 rpm. The time to reach equilibrium adsorption with continuous stirring was found to be 90 min, and in the rest of the study, a 120-min adsorption duration was, therefore, employed. The amount of the adsorbed lysozyme was obtained with the following equation:

$$q = [(C_o - C)V_s]/S \quad (1)$$

where q is the amount of the lysozyme adsorbed onto IPN membranes (mg cm^{-2}); C_0 and C are the concentrations of the lysozyme in the initial solution and in the aqueous phase after adsorption (mg mL^{-1}), respectively; V_s is the volume of the aqueous solution (mL); and S is the plain surface area of the IPN membranes in the adsorption medium (cm^2).

Pseudo-first-order and pseudo-second-order equations

To examine the controlling mechanism of adsorption processes such as mass transfer and chemical reactions, we used kinetic models to test the experimental data. A large number of different chemical groups on the adsorbent surfaces were created during the preparation of the dye–metal-chelate IPN membranes [i.e., —NH_2, ═NH, —SO_3H, —OH, hydrophobic groups, Zn(II), and Fe(III)], and this implied that there were many types of lysozyme–dye–metal-ion–matrix interactions. The kinetic models (the pseudo-first-order and pseudo-second-order equations) could be used in this case under the assumption that the measured concentrations were equal to the adsorbent surface concentrations. The first-order-rate equation of Lagergren is one of the most widely used for the adsorption of a solute from a liquid solution.[20] It may be represented as follows:

$$dq_t/dt = k_1(q_{eq} - q_t) \quad (2)$$

where k_1 is the rate constant of pseudo-first-order biosorption (min^{-1}) and q_{eq} and q_t denote the amounts of adsorbed lysozyme at equilibrium and at time t (mg cm^{-2}), respectively. After integration by the application of boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$ give

$$\log(q_{eq}/q_{eq} - q_t) = (k_1 \cdot t)/2.303 \quad (3)$$

Equation (3) can be rearranged to obtain a linear form:

$$\log(q_{eq} - q_t) = \log q_{eq} - (k_1 \cdot t)/2.303 \quad (4)$$

A plot of $\log(q_{eq} = q_t)$ against t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process, $\log q_{eq}$ should be equal to the intercept of a plot of $\log(q_{eq} - q_t)$ against t.

In addition, a pseudo-second-order equation based on the adsorption equilibrium capacity may be expressed as follows:

$$dq_t/dt = k_2(q_{eq} - q_t)^2 \quad (5)$$

where k_2 (cm^{-2} mg^{-1} min^{-1}) is the rate constant of pseudo-second-order adsorption. Integrating eq. (5) and applying the boundary conditions lead to

$$(1/(q_{eq} - q_t)) = (1/q_{eq}) + k_2 t \quad (6)$$

or, equivalently for a linear form, lead to

$$(t/q_t) = (1/k_2 q^2_{eq}) + (1/q_{eq})t \quad (7)$$

A plot of t/q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant (k_2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively.

Lysozyme desorption and stability of the IPN membranes after repeated use

The lysozyme elution experiments were performed in a buffer solution containing 1.0*M* KSCN at pH 8.0. The lysozyme adsorbed onto dye-immobilized and metal-chelated IPN membranes was placed in the desorption medium with stirring at 100 rpm at 25°C for 2 h. The final lysozyme concentration within the desorption medium was determined by spectrophotometry. The elution ratio was calculated from the amount of the lysozyme adsorbed onto the dye-immobilized IPN membranes and the amount of the lysozyme desorbed.

To determine the reusability of the dye-immobilized and metal-ion-chelated IPN membranes, we repeated the adsorption and desorption cycle five times with the same IPN membrane. The desorption ratios of the lysozyme were calculated with the following expression:

$$\text{Desorption ratio} = \frac{[\text{amount of lysozyme released} \times 100]}{[\text{amount of lysozyme adsorbed on the IPNs membranes}]} \quad (8)$$

Characterization studies

Elemental analysis

The amount of covalently bound Procion Green H-4G on the membrane was evaluated with an elemental analysis instrument (Leco, CHNS-932, USA) by a consideration of the nitrogen and sulfur stoichiometry.

Fourier transform infrared (FTIR) spectra

FTIR spectra of the pHEMA/chitosan and Procion Green H-4G-immobilized IPN membranes were obtained with a model 1000 FTIR spectrophotometer (Mattson, Paisley, England). The dry membrane (ca. 0.1 g) was mixed with KBr (0.1 g) and pressed into a tablet form. The FTIR spectrum was then recorded.

Scanning electron microscopy

The dried IPN membranes were coated with gold under reduced pressure, and their scanning electron micrographs were obtained with a JEOL JSM 5600 scanning electron microscope (Tokyo, Japan).

Water content of the pHEMA membranes

The water content of the IPN membranes was determined at room temperature in a saline solution (0.85%) with a gravimetric method. The water content was defined as the weight ratio of water contained within a swollen-to-dry membrane. The water content of the membranes was calculated with the following expression:

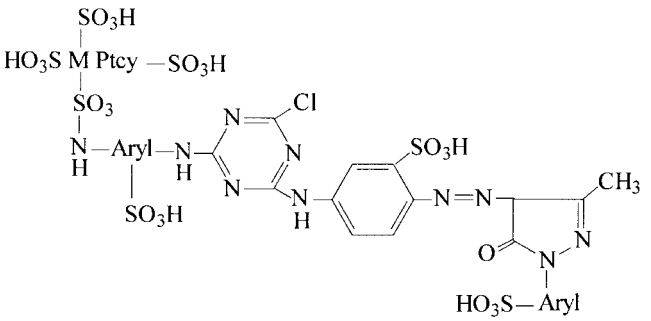
$$\text{Swelling ratio } \% = \{(W_s - W_d)/W_d\} \cdot 100 \quad (9)$$

where W_d and W_s are the dry and swollen weights of the membranes, respectively.

RESULTS AND DISCUSSION

Properties of the dye-immobilized pHEMA/chitosan IPN membranes

Dye immobilization requires a hydrophilic support that possesses chemically modifiable groups. The most frequently used matrices are naturally occurring polysaccharide polymers: agarose, dextran, cellulose derivative, and chitosan. Only the last is resistant to microbial degradation, and the others can undergo microbial degradation. However, for some applications, they show insufficient mechanical strength and porosity. In comparison with other supports, composite pHEMA/chitosan polymeric networks, because of their nature, are very inert toward microbial degradation. The porosity can be modified by variations in the



Ptcy = Phthalocyanine moiety

M = Metal ion complex with Ptcy moiety

Reactive Green 5

Figure 1 Chemical structure of Procion Green H-4G.

concentration of the pore forming agent in the polymerization mixture.

Procion Green H-4G is an aromatic polysulfonated dye that contains seven acidic sulfonate groups and three basic secondary amino groups (7:3). It also has a phthalocyanine moiety (Fig. 1). The binding of Zn(II) and Fe(III) ions to immobilized dye molecules occurs especially through oxygen and nitrogen atoms. The strong binding of Procion Green H-4G to proteins occurs largely at binding sites for the substrate, coenzymes, and other prosthetic groups.

The hydroxyl and amino groups of the IPN matrix can react with the chloride of the triazine ring of Procion Green H-4G under alkaline conditions, giving rise to covalent bounds. Elemental analyses of the plain and Procion Green H-4G-immobilized pHEMA/chitosan membranes were performed, and the immobilized dye on the membrane was found to be 0.018 μmol cm^{-2} from nitrogen and sulfur stoichiometry.

Studies aimed at detecting the leakage of Procion Green H-4G, Zn(II), and Fe(III) ions from dye-immobilized and metal-ion-chelated IPN membranes revealed no leakage in any of the adsorption media and implied that the washing procedure was satisfactory for the removal of the physically adsorbed dye molecules and metal ions from the IPN membranes. Dye leakage also was not observed from the dye-immobilized membranes during a long period of storage time (>4 months).

The main physical and morphological properties of the pHEMA/chitosan IPN membranes were as follows: the water content of the dye-immobilized IPN membrane was 50 $\pm$ 2%, the thickness in the wet state of the IPN membrane was 0.06 cm, the density of the IPNs in the dry state was 1.26 g cm^{-3}, and the plain surface area of the 1.0-mL wet IPN membrane was 38.5 cm^2.

The microstructures of the IPN surfaces and cross sections were investigated with scanning electron microscopy and are presented in Figure 2(A,B), respectively. The IPN membrane displayed a smooth, channel-like, and porous bulk and surface structure. The microporous surface structure of the IPNs could lead to high internal surface areas (i.e., high ligand immobilization and protein adsorption capacity) with low diffusional resistance in the matrix (implying a high adsorption rate). Achieving both a high adsorption capacity and a high adsorption rate was the main concern in the preparation of the affinity membranes for protein separation in this work.

To examine the nature of the interactions between the dye (Procion Green H-4G) and the pHEMA/chitosan IPNs, we obtained FTIR spectra of the plain IPNs and dye-immobilized IPNs. As shown in Figure 3, the FTIR spectra of dye-immobilized pHEMA/chitosan IPNs showed an absorption band different than that of pHEMA/chitosan at 1575 cm^{-1}. It was characteristic of an aromatic ring stretching vibration band (C$\cdots$C) arising from immobilized dye molecules on the IPNs. The bands at 3300, 1580, 1090, 1160, and 1260 cm^{-1}, representing N—H stretching, N—H bending (scissoring), symmetric stretching of S═O, asymmetric stretching of S═O, and aromatic C—N vibration, respectively, were due to the Procion Green H-4G dye bonded to pHEMA/chitosan. These bands, however,

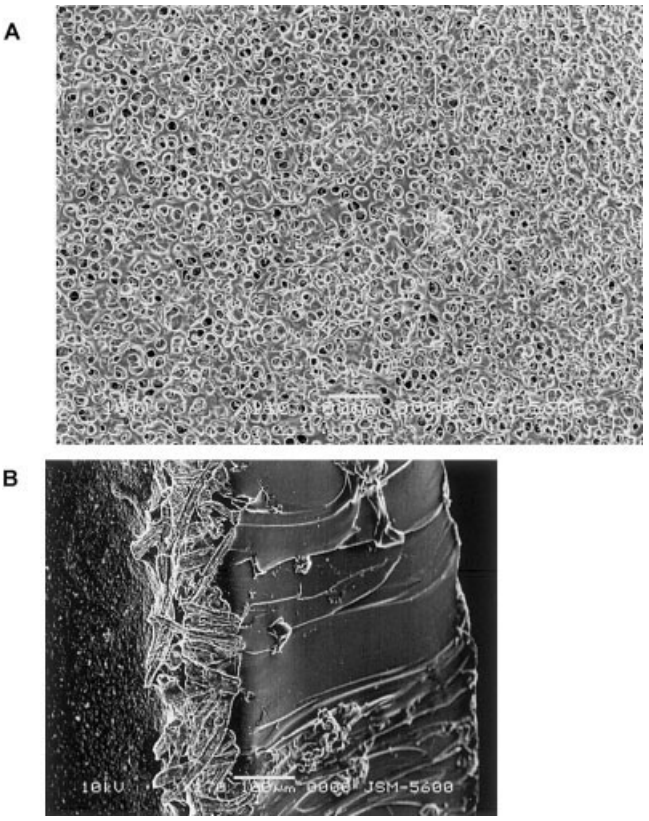


Figure 2 SEM micrographs of a Procion Green H-4G-immobilized pHEMA/chitosan IPN membrane: (A) surface and (B) cross section.

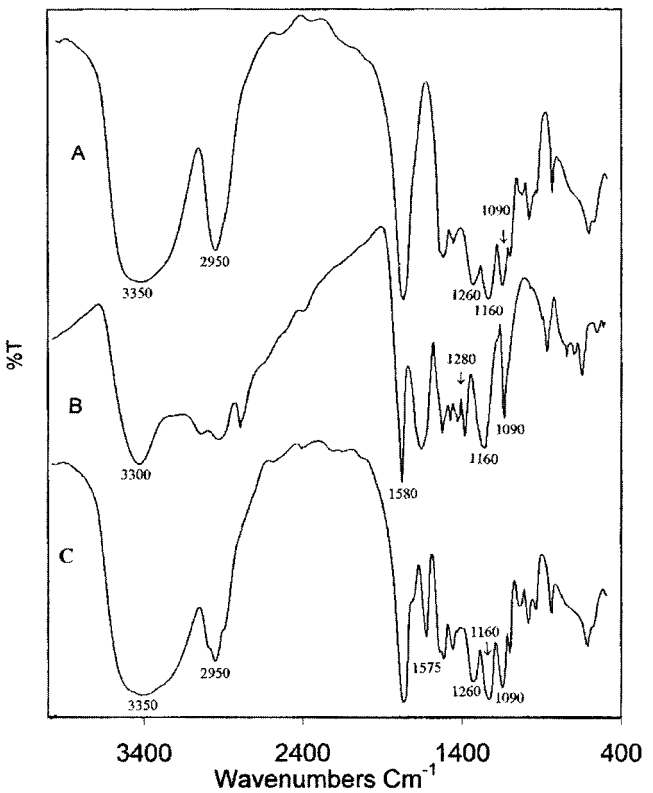


Figure 3 FTIR spectra of (A) a pHEMA/chitosan membrane, (B) Procion Green H-4G, and (C) a Procion Green H-4G-immobilized pHEMA/chitosan IPN membrane.

did not appear because plain pHEMA/chitosan also had some absorption bands in the same region. Therefore, the absorption bands of pHEMA/chitosan overlapped with those of Procion Green H-4G-immobilized IPNs around these wave numbers.

Effect of the pH and temperature on the lysozyme adsorption

The optimal pH values for the adsorption of the lysozyme onto the dye-immobilized and metal-ion-chelated membranes were investigated for the pH range 4.0–8.0. Electrostatic interactions are important for protein retention in dye and metal-chelate chromatography. As observed in Figure 4, at pH 6.0, with dye-immobilized/metal-chelated [i.e., Zn(II) and Fe(III)] IPN membranes, 1.33 and 1.37 mg cm^{-2} lysozyme adsorption was obtained, respectively, whereas with a dye-immobilized membrane, it was 1.24 mg cm^{-2}. Significantly lower lysozyme adsorption was obtained for all the adsorbents below pH 6.0. These results indicated that the pH of the medium had an important effect on the adsorption equilibrium of the lysozyme, and there was a preferential interaction between the lysozyme and dye or different dye–metal-chelated IPN membranes at pH 6.0. The isoelectronic value of the lysozyme was 11.0. The lysozyme molecules would be cationic at pH values below 11.[14] Because proteins are amphoteric, the number of charges on the surface of a protein will vary with the pH of the medium. In principle, it is possible to calculate the number of charges on a protein at a particular pH if the pK_a values of all its ionizable groups are known. For the lysozyme, the number of such groups and their pK_a values were determined by Tanford and Wagner.[21] At an acidic pH, the numbers of positive and negative charges of the lysozyme calculated in this way were 19 and 8, respectively. The ion-exchange properties of a protein cannot be satisfactorily explained by the net charge concept, and the process is primarily governed by the distribution of charges on the proteins.[22] The surface charge distribution of seven closely related lysozyme variants was studied by Fausnaugh-Pollitt et al.[23] The lysozyme variants had amino acid substitutions broadly distributed on their surfaces. Most of the lysozyme variants were resolved by a strong cation exchanger, and this indicated that the cation exchange of the lysozyme involved nearly the whole external surface of the protein. The dye Procion Green H-4G contained on the surface seven acidic sulfonate groups and bore seven negative charges under neutral and basic conditions. Divalent metal ions such as Cu(II), Ni(II), and Zn(II) are considered soft Lewis acids and interact with soft Lewis bases such as nitrogen and sulfur, as in cysteine and histidine. The presence of one histidine residue on the surface of lysozyme molecules provides an affinity for the Zn(II) ion on the dye ligand and through the imidazole group of the histidine residue. However, trivalent metal ions such as Al(III) and Fe(III) are considered hard Lewis acids and interact with hard

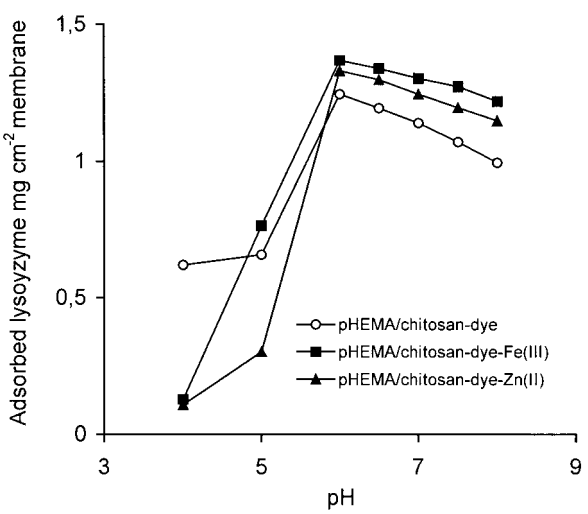


Figure 4 Effects of pH on the lysozyme adsorption for a Procion Green H-4G-immobilized IPN membrane (initial concentration of lysozyme = 1.0 mg mL^{-1}; Procion Green H-4G loading = 0.018 μmol cm^{-2} of IPN membrane; temperature = 25°C).

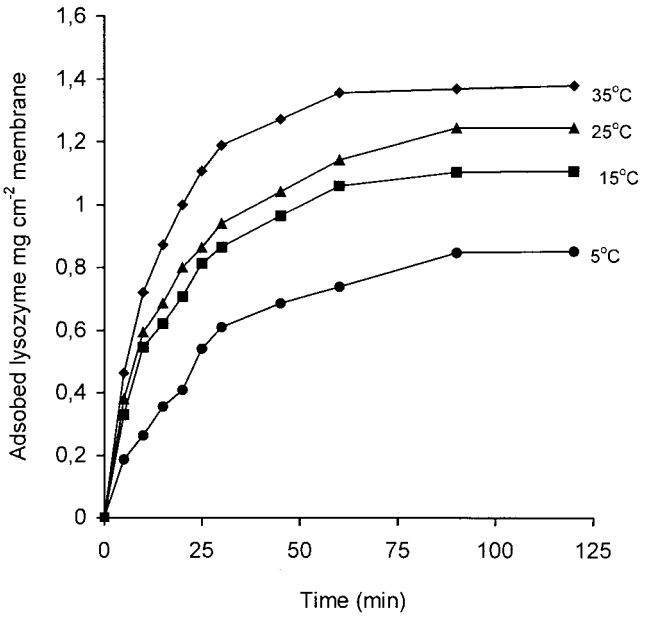


Figure 5 Effect of temperature on the lysozyme adsorption rate and capacity for a dye-immobilized IPN membrane (initial concentration of lysozyme = 1.0 mg mL^{-1}; Procion Green H-4G loading = 0.018 μmol cm^{-2} of IPN membrane; pH = 6.0).

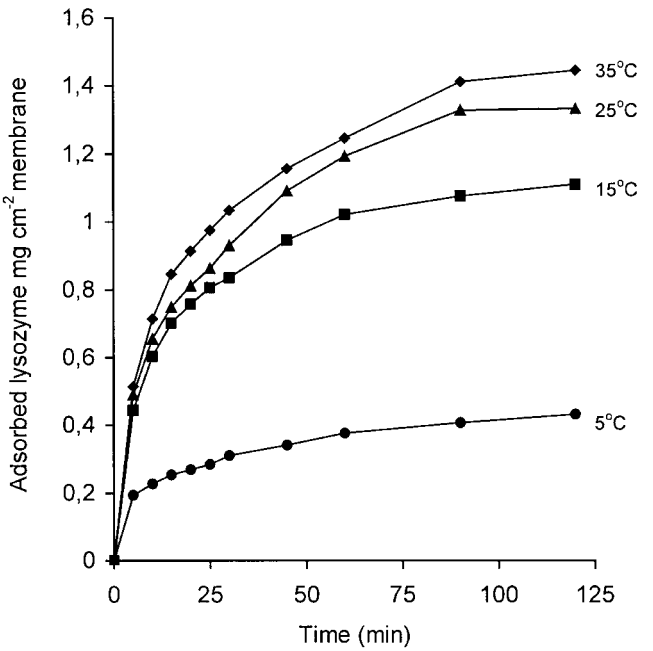


Figure 6 Effect of temperature on the lysozyme adsorption rate and capacity for a dye–Zn(II)-chelated IPN membrane (initial concentration of lysozyme = 1.0 mg mL^{-1}; Procion Green H-4G loading = 0.018 μmol cm^{-2} of IPN membrane; pH = 6.0).

Lewis bases such as oxygen. Molecules that have hard bases, such as phosphates, carboxylates, sulfates, and phenolic groups, have high binding constants. The hard Lewis metal ions [i.e., Fe(III)] were chelated with the immobilized dye molecules. This combination of alternating chelating adsorbent and metal ions could provide a different mode of selectivity for lysozyme adsorption from an aqueous medium. The lysozyme molecule has four aspartic acid, two glutamic acid, and two tyrosine residues (on the basis of its surface-accessible residues). These amino acid side-chain groups of the lysozyme molecules could interact with chelated Fe(III) ions. From this point of view, binding should be a combination of metal coordination and ion-exchange interactions. A secondary interaction between the lysozyme and the ion-exchange membrane may be produced by the cooperative effect of different mechanisms such as hydrophobic interactions, caused by several aromatic structures, on the immobilized dye (Procion Green H-4G) and the amino acid side-chain hydrophobic groups of the lysozyme molecules.[5]

The adsorption isotherms for the binding of lysozyme to Procion Green H-4G-immobilized and Zn(II)- and Fe(III)-chelated IPN membranes at different temperatures are presented in Figures 5–7, respectively. The adsorption isotherms derived from experimental data were steeper at higher temperatures, indicating interactions of greater affinity between the lysozyme and the adsorbents. At higher temperatures, the contact area between the protein and the dye ligand on the matrix should increase, resulting in an increase in the affinity of proteins for the adsorbents. From 5 to 35°C, the adsorption capacity of the dye-immobilized and dye–Zn(II)- and dye–Fe(III)-chelated IPN membranes for the lysozyme increased 1.6-fold,

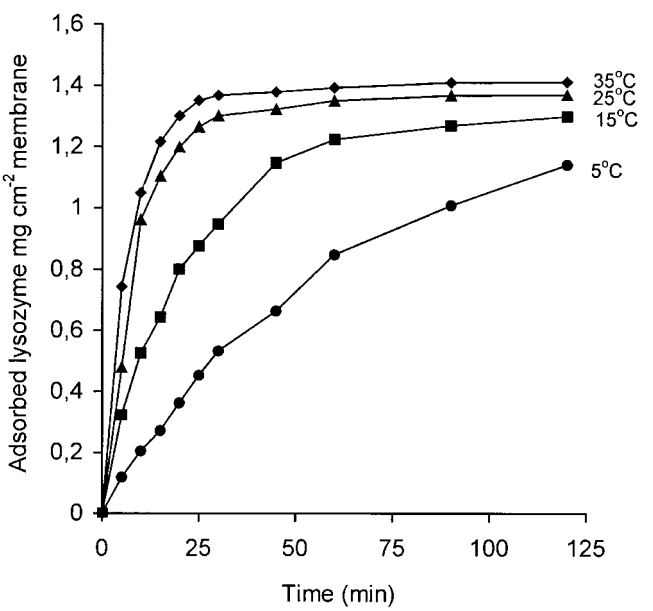


Figure 7 Effect of temperature on the lysozyme adsorption rate and capacity for a dye–Fe(III)-chelated IPN membrane (initial concentration of lysozyme = 1.0 mg mL^{-1}; Procion Green H-4G loading = 0.018 μmol cm^{-2} of IPN membrane; pH = 6.0).

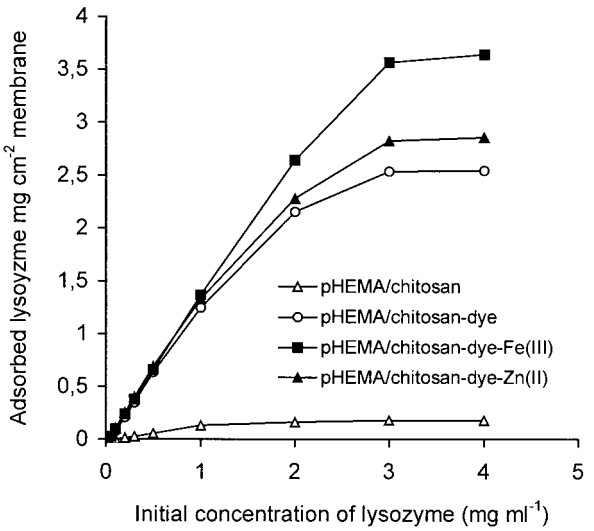


Figure 8 Effect of the lysozyme initial concentration on the lysozyme adsorption for Procion Green H-4G-immobilized and metal-ion-chelated IPN membranes (Procion Green H-4G loading = 0.018 μmol cm^{-2} of IPN membrane; pH = 6.0; temperature = 25°C).

3.3-fold, and 1.2-fold. The increase in the adsorption capacity of the adsorbents for the lysozyme at higher temperatures indicated that to some extent a hydrophobic interaction was present between the lysozyme and hydrophobic groups of the dye molecules.

Adsorption isotherms

The lysozyme adsorption isotherms of the Procion Green H-4G-immobilized and Zn(II)-ion- and Fe(III)-ion-chelated IPN membranes are presented in Figure 8. An increase in the lysozyme concentration in the adsorption medium led to an increase in the amount of the adsorbed lysozyme on all the tested adsorbents, but this relation leveled off around 3.0 mg of lysozyme per milliliter of the adsorption medium. This could be explained by the saturation of interacting groups of the immobilized Procion Green H-4G and/or chelated metal ions with the adsorbed lysozyme molecules; as a result, the maximum adsorption capacity was achieved. A negligible amount of lysozyme adsorbed nonspecifically onto the plain IPN membrane (0.17 mg cm^{-2}). Procion Green H-4G immobilization significantly increased the lysozyme adsorption capacity (ca. 14-fold) of the dye IPN membrane up to 2.54 mg cm^{-2}. Note the dye-immobilized membrane containing 0.018 μmol of Procion Green H-4G per cm^2 of the IPN membrane. The loadings of Zn(II) and Fe(III) ions onto dye-immobilized membranes were 0.059 and 0.043 μmol cm^2 of membrane, respectively. The binding ratio of each metal ion to the immobilized dye molecule was approximately 3 for divalent metal ions and 2 for trivalent metal ions [i.e. Zn(II) and Fe(III)]. As seen in Figure 8, the chelation of metal ions onto dye-immobilized membranes led to a further increases in the adsorption capacity of the IPN membranes. These were 1.12 and 1.43 times higher for Zn(II) and Fe(III) metal–dye-chelated membranes, respectively, than for the dye-immobilized IPN membrane. It was clear that this increase was due to ternary complex formation between the Procion Green H-4G, metal ions, and lysozyme molecules (i.e., the metal ions promoted the adsorption of the lysozyme).[5]

The concept of the hardness and softness of metal ions may serve as a guide for understanding the principles and mechanisms of metal affinity chromatography. According to these principles, the binding of proteins to soft metals [e.g., Zn(II)] occurs via the electron-donating side chains of residues such as histidine and cysteine. The lysozyme has only one histidine residue and does not have a cysteine residue on the surface. This exposed histidine residue should be the dominant binding site in lysozyme adsorption with chelated Zn(II) ions. However, Fe(III) ions have a strong affinity for oxygen-containing functional groups such as carboxylic and phenolic oxygen.[10] The presence of aspartic acid, glutamic acid, and tyrosine residues on the surface of the lysozyme molecules provides an affinity for Fe(III) ions through carboxylate and phenolic functional groups. In this study, the dye–Fe(III)-chelated IPN membrane yielded a higher adsorption capacity than the dye-immobilized and dye–Zn(II)-chelated membranes. Therefore, the number of binding sites on the protein, the types of metal ions, and the ligand–polymer structure influenced the metal affinity protein precipitation.

The Langmuir model is based on assumptions of adsorption homogeneity such as equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed species. Reversible protein adsorption is better described by another model also widely used in studies of gas adsorption, the Temkin isotherm. This model assumes that adsorption is characterized by a uniform distribution of binding energies, up to some maximum binding energy (ΔG_{max}), which results in the following isotherm equation:[19]

$$q = q_T \ln(1 + K_T c) \tag{10}$$

where K_T (mL mg^{-1}) is the equilibrium binding constant corresponding to ΔG_{max} [$K_T = \exp(-\Delta G_{max}/RT$]; ($R$ is the universal constant known as the gas constant (8, 314) Joule mol^{-1} K^{-1} and T is temperature), c (mg mL^{-1}) is the concentration of the protein in the solution at equilibrium, q (mg protein cm^{-2} membrane) is the amount of the protein adsorbed onto the surface, and q_T (mg of protein cm^{-2} of membrane) is the differential surface capacity for protein adsorption per unit of binding energy.

The binding of the lysozyme onto dye-immobilized and Zn(II)- and Fe(III)-chelated IPN membranes was

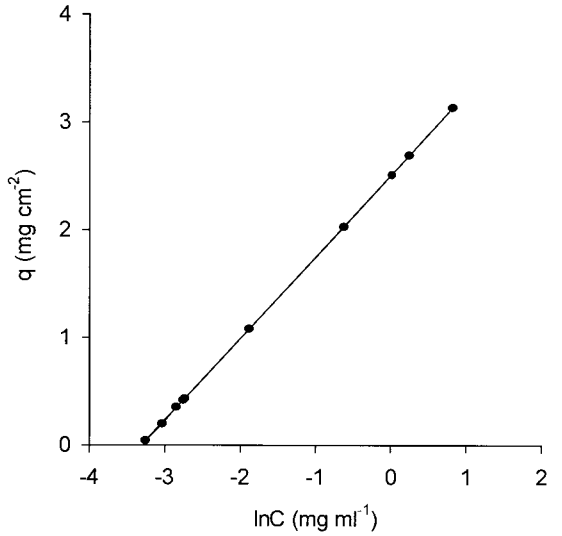


Figure 9 Semilogarithmic plot of the Temkin isotherm for a dye-immobilized IPN membrane.

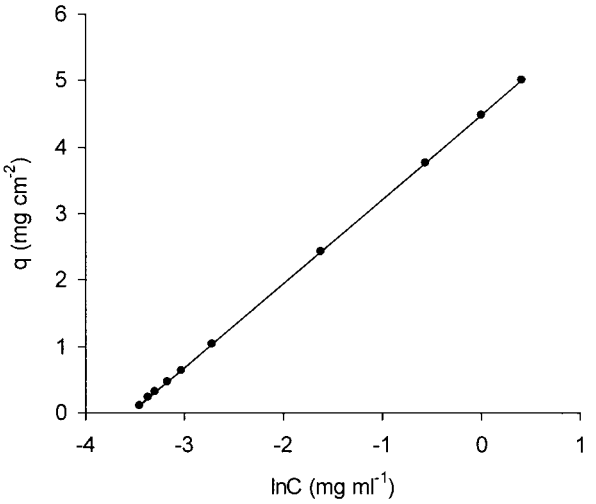


Figure 11 Semilogarithmic plot of the Temkin isotherm for a dye–Fe(III)-chelated IPN membrane.

measured, and semilogarithmic plots of the experimental data demonstrated that the Temkin model fit the individual isotherms (Figs. 9–11). The Temkin model indicated that the binding energy decreased with an increasing amount of the adsorbed protein on the adsorbent surface. In this case, ΔG_{max} decreased from -8.18 to -8.73 kJ mol^{-1} as the adsorbed lysozyme increased on the adsorbent surface (Table I). These results indicated that there was a relationship between the surface interactions of lysozyme groups [i.e., Zn(II) ions with histidine and Fe(III) with aspartic acid, glutamic acid, and tyrosine residues] and the types of metal ions on the dye-immobilized IPN membrane surfaces. The molecular basis of these results was very apparent when Fe(III) ions chelated with a dye-immobilized IPN membrane. In this case, the q_T value was about 1.67 times higher for the Fe(III)-chelated IPN membrane than for the dye-immobilized membrane. The q_T values should depend on the types of metal ions and the number of interacting functional groups on the IPN membrane surface.

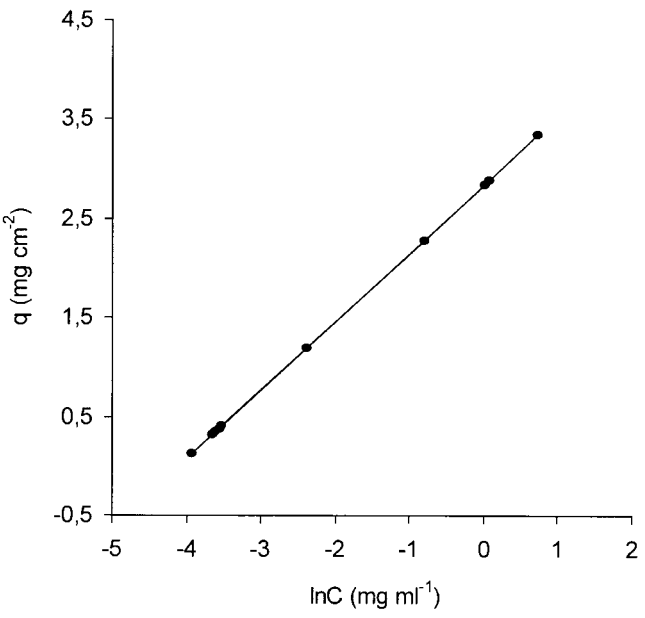


Figure 10 Semilogarithmic plot of the Temkin isotherm for a dye–Zn(II)-chelated IPN membrane.

A wide variety of adsorbents with a wide range of adsorption capacities have been reported in the literature for lysozyme adsorption. Denizli et al.[15] reported that 135 μg cm^{-2} lysozyme adsorbed onto a Cibacron Blue F3GA-immobilized pHEMA membrane. Ratnayake and Regnier[24] reported that 12.2–21.6 μg cm^{-2} lysozyme adsorbed onto adsorbents based on acrylate-grafted silica membranes. Finette et al.[11] reported that 30 μg cm^{-2} lysozyme adsorbed onto Cibacron Blue F3GA-immobilized porous silica. Buijs and Hlady[25] achieved a 4–25 μg cm^{-2} adsorption capacity with quaternary aminopropyl dimethlsilyl methylated silica. Champluvier and Kula[26] used microfiltration membranes as pseudoaffinity adsorbents and achieved 78–122 μg cm^{-2} adsorption capacities with the Ultipor membrane containing different dyes such as Cibacron Blue F3GA, Procion Blue MX 5B, and Procion Red HE3B. Tennikova et al.[27] reported that 260 μg cm^{-2} lysozyme adsorbed with sulfone-modified poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) macroporous polymeric membranes. Ruckenstein and

TABLE I
Temkin Parameters for Adsorption Isotherms

Type of membranes	ΔG_{max} (kJ/mol)	q_t (mg/cm^2)
pHEMA/chitosan dye	−8.18	0.761
pHEMA/chitosan dye–Zn(II)	−8.36	1.034
pHEMA/chitosan dye–Fe(III)	−8.73	1.268

TABLE II
First-Order and Second-Order Kinetic Constants for pHEMA/Chitosan Dye and Different Metals-Ion-Immobilized IPN Membranes

	Experimental	First-order kinetics			Second-order kinetics		
IPN membrane	q_{eq} (mg/cm^2)	$k_1 \times 10^2$ (min^{-1})	q_{eq} (mg/cm^2)	R^2	$k_2 \times 10^2$ (g mg^{-1} min^{-1})	q_{eq} (mg/cm^2)	R^2
pHEMA/chitosan dye	2.54	3.72	1.97	0.978	2.41	2.85	0.999
pHEMA/chitosan dye–Zn(II)	3.64	6.60	1.99	0.967	3.50	4.1	0.996
pHEMA/chitosan dye–Fe(III)	2.85	3.20	2.06	0.957	2.72	2.99	0.998

Zeng[16] used macroporous chitin affinity membranes for the separation of lysozyme from a mixture of lysozyme and ovalbumin, and they achieved a 50 mg mL^{-1} adsorption capacity. Nash and Chase[28] reported that the lysozyme adsorption capacities of modified poly(styrene divinylbenzene) and Procion Yellow HE-3G attached to poly(vinyl alcohol) were in the range of 0.01–0.02 μg cm^{-2}. The maximum lysozyme adsorption achieved with the adsorbents developed in this study was 3.64 mg cm^{-2} (or 3640 μg cm^{-2}, based on the plain surface area) with the dye–Fe(III)-chelated pHEMA/chitosan membrane, and this was quite comparable with values found in the related literature.

Biosorption kinetic modeling

To analyze the adsorption kinetics of the lysozyme, we applied the pseudo-first-order and pseudo-second-order kinetic models to the experimental data. The pseudo-second-order equation fit the experimental data well. A comparison of the experimental adsorption capacities and the theoretical values estimated from the previous two equations are presented in Table II. The theoretical q_{eq} values estimated from the first-order kinetic model were significantly different from the experimental values, and the correlation coefficients were also slightly lower (Fig. 12). These results showed that these adsorbent systems were not described by the first-order kinetic model.

The correlation coefficients for the linear plots of t/q_t against t for the second-order equation were greater than 0.995 for all the adsorbents for contact times of 120 min (Fig. 13). The theoretical q_{eq} values for all the tested adsorbent systems were very close to the experimental q_{eq} values for second-order kinetics. The second-order kinetics best described the data.

Desorption and reusability of the adsorbents

The desorption of the adsorbed lysozyme from the Procion Green H-4G-immobilized and Zn(II)- and Fe(III)-chelated IPN membranes was studied in a batch system. The lysozyme-loaded membrane disks were placed within a desorption medium containing 1.0*M* KSCN at pH 8.0, and the amount of the lysozyme released in 2 h was determined (Fig. 14). For all the tested adsorbents, more than 97% of the adsorbed lysozyme was removed when KSCN was used as a

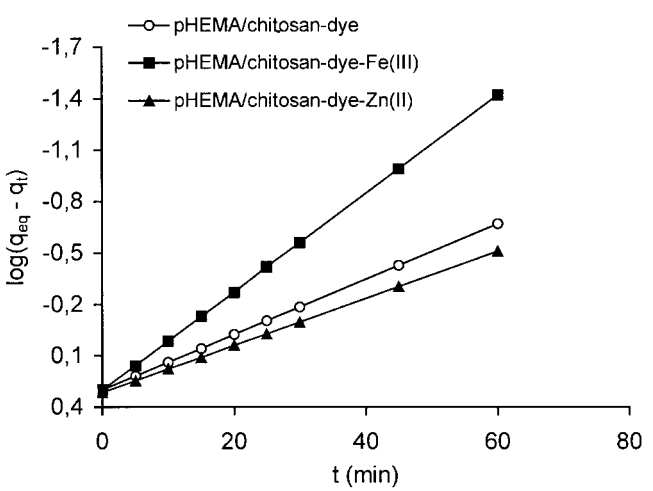


Figure 12 First-order-kinetic plot of the experimental data for the adsorbents.

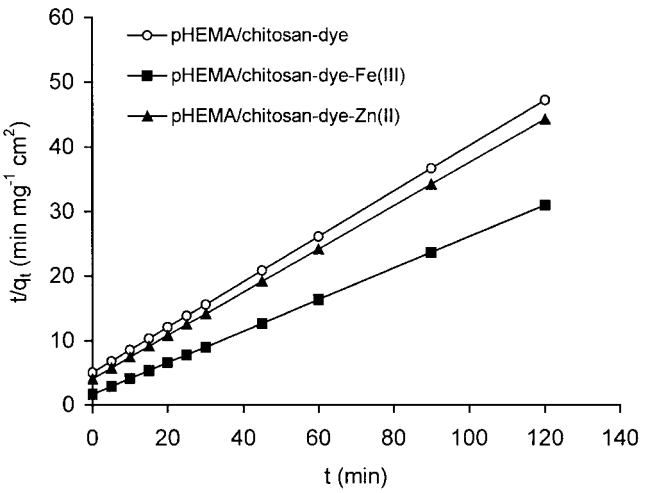


Figure 13 Second-order-kinetic plot of the experimental data for the adsorbents.

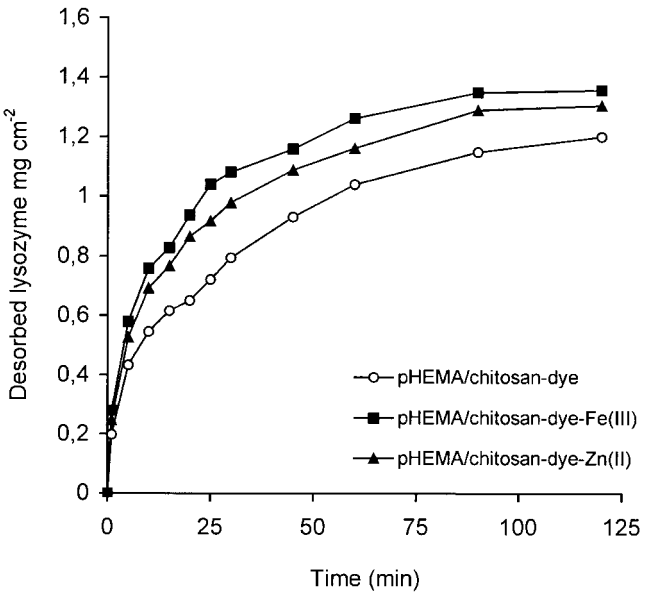


Figure 14 Desorption isotherms for dye-immobilized and metal-chelated IPN membranes.

desorption agent. The desorption results showed that KSCN was a suitable desorption agent for the Procion Green H-4G-immobilized and metal-ion-chelated IPN membranes and that it allowed the repeated use of the modified IPN membranes developed in this study. To demonstrate the reusability of the dye-immobilized and metal-ion-chelated IPN membranes, we repeated the lysozyme adsorption–desorption cycle five times with the same adsorbents, and the adsorption capacity of the dye-immobilized and metal-ion-chelated IPN membranes did not change during the repeated adsorption–desorption operations.

CONCLUSIONS

This study demonstrated the preparation and application of pHEMA/chitosan IPN membranes for the dye-ligand and metal-chelate affinity chromatography of proteins. The pHEMA/chitosan IPN membranes were prepared by UV-initiated photopolymerization. Procion Green H-4G was then covalently immobilized onto the membranes with a membrane phase concentration of 0.018 μmol cm^{-2} as a dye ligand. Zn(II) and Fe(III) ions were chelated with immobilized dye molecules at pH 4.1. Adsorption and desorption studies of the lysozyme on the dye-immobilized and metal-ion-chelated IPN membranes led to the following conclusions. The lysozyme adsorption capacity of the dye-immobilized membranes was 2.54 mg cm^{-2}. The chelation of Zn(II) and Fe(III) ions onto the dye-immobilized membranes led to significant increases in the adsorption capacity, about 12 and 43% of the IPN membranes to the lysozyme, respectively. The adsorbed lysozyme was desorbed up to 99% with 1.0*M* KSCN as the desorption agent. Repeated adsorption and desorption processes showed that these novel IPN membranes had good properties as dye-ligand and metal-chelate affinity adsorbents and would be effective in processing large volumes of biological fluids containing target proteins.

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