Preparation of Zn^{2+}-Chelated Poly(HEMA-MAH) Cryogel for Affinity Purification of Chicken Egg Lysozyme

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ABSTRACT: Supermacroporous poly(2-hydroxyethyl methacrylate) [poly(HEMA)]-based monolithic cryogel column was prepared by radical cryocopolymerization of HEMA with *N*-methacryloyl-L-histidine methyl ester (MAH) as functional comonomer and *N*,*N*′-methylenebisacrylamide (MBAAm) as crosslinker directly in a plastic syringe for affinity purification of lysozyme from chicken egg white. The monolithic cryogel containing a continuous polymeric matrix having interconnected pores of 10–50 μm size was loaded with Zn^{2+} ions to form the metal chelate with poly(HEMA-MAH) cryogel. Poly(HEMA-MAH) cryogel was characterized by swelling studies, FTIR, scanning electron microscopy, and elemental analysis. The equilibrium swelling degree of the poly(HEMA-MAH) monolithic cryogel was 5.62 g H_2O/g cryogel. Poly(HEMA-MAH) cryogel containing 45.8 μmol MAH/g was used in the adsorption/desorption of lysozyme from aqueous solutions. The nonspecific adsorption of lysozyme was very low (7.5 mg/g). The maximum amount of lysozyme adsorption from aqueous solution in phosphate buffer was 209 mg/g at pH 7.0. It was observed that lysozyme could be repeatedly adsorbed and desorbed with the poly (HEMA-MAH) cyogel without significant loss of adsorption capacity. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 2905–2913, 2008

Key words: affinity chromatography; protein purification; metal chelate; histidine; cryogels

INTRODUCTION

Lysozyme is found in a variety of vertebrate cells and secretions, such as spleen, milk, tears, and egg white. Lysozyme lyses certain bacteria by hydrolyzing the linkages between the muramic acid and *N*-acetylglucosamine of the mucopolysaccharides, which are present in the bacterial cell wall. Lysozyme is a commercially valuable enzyme, which has tremendous potential for application in pharmaceutical and food industries. Its common applications are as a cell-disrupting agent for the extraction of bacterial intracellular products, as an antibacterial agent in ophtalmologic preparations, as a food additive in milk products and as a drug for treatment of ulcers and infections.[1] The potential for its use as an anticancer drug has been demonstrated by animal and *in vitro* cell-culture experiments.[2] In a recent article, it has been reported that lysozyme can be used for increasing the production of immunoglobulin by hybridoma technology.[3] The large-scale applications require more efficient and cost-effective techniques for its isolation.[4]

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In respect of ease preparation, economy, adsorption capacity, and stability of immobilized metal affinity chromatography (IMAC) offer several advantages over other conventional adsorbents.[5–9] IMAC is a sensitive technique for protein separation that enables distinguishing between proteins differing by only a single histidine residue on the surface.[10–14] It is assumed that proteins interact mainly through the imidazole group of histidine and, to a lesser extent, the indole-group of tryptophan and the thiol group of cysteine. Aromatic amino acids and the amino terminal of the peptides also contribute.[15] The low cost of metals and the ability to reuse adsorbents hundreds of times without any detectable loss of metal-chelating properties are the attractive features of metal-affinity separation.

Conventional packed-bed columns possess some inherent limitations such as the slow-diffusional mass transfer and the large void volume between the beads.[16] Although some new stationary phases such as the nonporous polymeric beads[17] and perfusion chromatography packings are designed to resolve these problems, these limitations cannot be overcome in essence.[18] Recently, cryogel materials are considered as a novel generation of stationary phases in the separation science.[19–25] Cryogels are a very good alternative to protein purification with many advantages. Several advantages of cryogels are large pores, short diffusion path, low pressure drop,

and very short residence time for both adsorption and elution. Cryogels are also cheap materials, and they can be used as disposable avoiding cross-contamination between batches.

In this study, supermacroporous poly(2-hydroxyethyl methacrylate-*N*-methacryloyl-L-histidine methyl ester) [poly(HEMA-MAH)] monolithic cryogel column was prepared by radical cryocopolymerization of HEMA with *N*-methacryloyl-L-histidine methyl ester (MAH) as functional comonomer and *N*,*N*′-methylene-bisacrylamide (MBAAm) as crosslinker directly in a plastic syringe for affinity purification of lysozyme from chicken egg white. Poly(HEMA-MAH) cryogel was characterized by scanning electron microscopy (SEM), FTIR, elemental analysis, and swelling tests. Then Zn^{2+} ions were chelated through imidazole groups on the MAH reactive functional groups of the polymeric structure. The ability of the monoliths to adsorb lysozyme from aqueous solutions containing different lysozyme concentrations at different pHs was investigated. Desorption of lysozyme and material stability also was tested.

EXPERIMENTAL

Materials

Lysozyme (chicken egg white,EC3.2.1.7), L-histidine methylester, methacryloyl chloride, and ammonium persulfate (APS) were supplied by Sigma (St. Louis, MO). Hydroxyethyl methacrylate (HEMA) was obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) was obtained from Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731) and then a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

Synthesis of MAH

The synthesis and characterization of MAH were performed as described previously.[26] In the experimental procedure for the synthesis of MAH, 5.0 g of L-histidine hydrochloride and 0.2 g of hydroquinone were dissolved in 100 mL of a dichloromethane solution, which was cooled to 0°C. Then, 12.7 g of triethylamine was added to the solution, followed by the addition of 5.0 mL of methacryloyl chloride. Then, this solution was stirred magnetically at room temperature for 2 h, after which hydroquinone and unreacted methacryloyl chloride were extracted with a 10% NaOH solution. The aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was crystallized in an ether-cyclohexane mixture and then dissolved in ethyl alcohol. ^{1}H NMR, performed in $CDCl_3$ on a JEOL GX-400 300 MHz instrument, was used to determine if the MAH structure was synthesized. The residual nondeuterated solvent ($CHCl_3$) was served as an internal reference. Chemical shifts are reported in parts per million downfield relative to $CHCl_3$. The ^{1}H NMR spectrum shows the characteristic peaks of the groups in the MAH monomer as follows: ^{1}H NMR ($CDCl_3$): δ = 1.99 (t; 3H, J = 7.08 Hz, CH_3), 1.42 (m; 2H, CH_2), 3.56 (t; 3H, O—CH_3) 4.82–4.87 (m; 1H, methin), 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl); 6.86 (δ; 1H, J = 7.4 Hz, NH), 7.82 (δ; 1H, J = 8.4 Hz, NH), 6.86–7.52 (m; 5H, aromatic).

Preparation of poly(HEMA-MAH) cryogel

Monomers (50 mg of MBAAm and 0.040 mg MAH) were dissolved in deionized water, and 300 μL HEMA was added. The cryogel was produced by free radical polymerization initiated by TEMED (20 μL) and APS (100 μL) (10% (w/v). After adding APS, the solution was cooled in an ice bath for 2–3 min. TEMED was added, and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into a plastic syringe (2 mL, id. 0.8 cm). The polymerization solution in the syringe was frozen at –12°C for 24 h and then thawed at room temperature. For the removal of unconverted monomers and initiator, washing solutions (i.e., a dilute HCl solution and a water–ethanol mixture) were recirculated through the monolithic cryogel column, until cryogel column is clean. Purity of the monolithic cryogel was followed by observing the change of optical densities of the samples taken from the liquid phase in the recirculation system and also from the DSC thermograms of the cryogel obtained by using a differential scanning microcalorimeter (Mettler, Switzerland). Optical density of the original monolithic cryogel was 1.67. But after the applying of cleaning procedure, this value was reduced to 0.02. In addition, when the thermogram of the uncleaned monolithic cryogel was recorded, it has a peak around 50°C. This peak might be originated from TEMED. But after applying this cleaning procedure, between 30 and 100°C, any peak was not observed on this thermogram. After washing, the cryogel was stored in buffer-containing 0.02% sodium azide at 4°C until use.

Incorporation of Zn^{2+} ions

The investigation of Zn^{2+}-chelation was carried out in a recirculating system equipped with a water

jacket for temperature control. The monolith was washed in 30 mL of water. Then 40 mL of a Zn^{2+} solution [50 mg/L (pH 6.0), adjusted with HCl and NaOH] was pumped through the column under recirculation at room temperature for 2 h. A 1000-ppm atomic absorption standard solution (containing 10% HNO_3) was the source of the Zn^{2+} ions. The concentration of the Zn^{2+} ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (GFAAS, Analyst 800/PerkinElmer, USA). The instrument response was periodically checked with known metal solution standards. The experiments were performed in triplicate, as were analyses of the samples. The Zn^{2+} concentrations in the initial and final solutions were used to calculate the amount of Zn^{2+} ions adsorbed.

Zn^{2+} leakage from the poly(HEMA-MAH) cryogel was investigated in media whose pH varied between 5.0 and 8.0 and also in a medium-containing 1.0*M* NaCl. The monolith was stirred for 24 h at room temperature. Then the concentration of Zn^{2+} ions in the supernatants was determined using an atomic absorption spectrophotometer.

Characterization of cryogel

Swelling test

The swelling degree of the cryogel (S) was determined as follows: cryogel sample was washed on porous filter until washing was clear. Then it was sucked dry and then transferred to preweighed vial and weighed (m_{wetgel}). After drying to constant mass in the oven at 60°C, the mass of dried sample was determined (m_{drygel}). The swelling degree was calculated as

$$S = (m_{\text{wet gel}} - m_{\text{dry gel}})/m_{\text{dry gel}} \quad (1)$$

Surface morphology

The morphology of a cross section of the dried cryogel was investigated by SEM. The sample was fixed in 2.5% glutaraldehyde in 0.15*M* sodium cacodylate buffer overnight, postfixed in 1% osmium tetroxide for 1 h. Then the sample was dehydrated stepwise in ethanol and transferred to a critical point drier temperated to +10°C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to + 40°C and the pressure to ~ 100 bar. Liquid CO_2 was transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage. Release of the pressure at a constant temperature of +40°C resulted in dried cryogel sample. Finally, it was coated with gold-palladium (40 : 60) and examined using a JEOL JSM 5600 SEM (Tokyo, Japan).

FTIR

FTIR spectrum of poly(HEMA-MAH) cryogel was obtained using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogel (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany) and pressed into a tablet, and the spectrum was then recorded.

Elemental analysis

Elemental analysis (Leco elemental analyzer, Model CHNS-932, USA) was performed to evaluate how much MAH comonomer was incorporated into the poly(HEMA-MAH) cryogel.

Chromatographic procedures

Lysozyme adsorption from aqueous solutions

Investigation of lysozyme adsorption was carried out in a recirculating system equipped with a water jacket for temperature control. The cryogel was washed with 30 mL of water and then equilibrated with 0.02*M* phosphate buffer (pH 7.0). Then the prepared lysozyme solution (i.e., 50 mL) was pumped through the column under recirculation for 2 h. The adsorption was followed by monitoring the decrease in UV absorbance at 280 nm. The effects of flow rate, ionic strength, lysozyme concentration, and pH of the medium on adsorption capacity were studied.

Desorption and repeated use

In all cases, adsorbed lysozyme molecules were desorbed using 0.1*M* phosphate buffer containing 1*M* NaCl (pH 8.0). In a typical desorption experiment, 50 mL of desorption agent was pumped through the cryogel at a flow rate of 1.0 mL/min for 30 min. The final lysozyme concentration in the desorption medium was spectroscopically determined. When desorption was achieved, the cryogel was cleaned with 1*M* NaOH and then re-equilibrated with 0.02*M* phosphate buffer (pH 7.0). The desorption ratio was calculated from the amount of lysozyme adsorbed on the cryogel and the final lysozyme concentration in the desorption medium. To test the repeated use of poly(HEMA-MAH) cryogel, lysozyme adsorption–desorption cycle was repeated for 20 times using the same cryogel column. To regenerate and sterilize, after desorption, the cryogel was washed with 1*M* NaOH solution.

Purification of lysozyme from chicken egg white

One chicken egg white was filtered through a double layer of cheesecloth into a beaker. Five milliliters of filtrate was transfered into another beaker and diluted with 35 mL of 0.02*M* phosphate buffer (pH

7.0) containing 0.05*M* NaCl. Five milliliters of the prepared egg-white solution was passed through Zn^{2+}-chelated poly(HEMA-MAH) cryogel monolitic column for two cycle at 25°C, at a flow rate of 1 mL/min. Then, lysozyme adsorbed column was eluted with 20 mL of 0.1*M* phosphate buffer (pH 8.0) containing 1*M* NaCl. In the purification experiments, the activity of lysozyme was determined spectrophotometrically at 620 nm, the decrease in the turbidity of culture of *M. lysodeikticus* cells suspended in phosphate buffer (0.1*M*, pH 7.0) was followed for 5 min after the addition of lysozyme. One unit lysozyme activity was defined as the amount of enzyme causing a decrease of 0.001 optical density value per minute at 25°C and pH 7.0. The purity of lysozyme in the purified samples was assayed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separating gel (9 cm × 7.5 cm), and 5% stacking gels were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid-methanol–water (1 : 5 : 5, v/v/v) and destained in ethanol-acetic acid-water (1 : 4 : 6, v/v/v). Electrophoresis was run for 2 h with a voltage of 110 V.

RESULTS AND DISCUSSION

The imidazole nitrogen donor atom incorporated into the MAH group was the most common-binding site for metal ions. The amount of chelated Zn^{2+} on poly (HEMA-MAH) cryogel was measured as 51.3 μmol/g polymer. Mass stoichiometric analysis showed that one incorporated MAH molecule interacted around one Zn^{2+} ion (45.8 μmol MAH/g: 51.3 μmol Zn^{2+}/g). Because MAH has two coordinating sites of nitrogen atoms, it could form a ternary complex that was coordinated water molecules at vacant coordination sites of the Zn^{2+}-MAH complexes. Investigation of leakage of Zn^{2+} from the poly(HEMA-MAH) cryogel detected no leakage in any of the adsorption and desorption media, suggesting that the washing procedure was satisfactory for the removal of the nonspecific adsorbed Zn^{2+} ions from the cryogel.

Surface morphology

A supermacroporous cryogel was produced by polymerization in the frozen state of monomers, 2-hydroxyethyl methacrylate (HEMA) and *N*-methacryloyl-L-histidine methyl ester (MAH) in the presence of APS/*N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) as initiator–activator pair. The SEM micrographs of the internal structure of the cryogel are shown in Figure 1. Poly(HEMA-MAH) cryogel has large continuous interconnected pores (10–50 μm in diameter) that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the protein molecules, allowing them to pass easily. Lysozyme has a molecular size of 3.0 × 3.0 × 4.6 nm and a molecular diameter of 27.3 A°. As a result of the convective flow of the solution through the pores, the mass-transfer resistance is practically negligible.

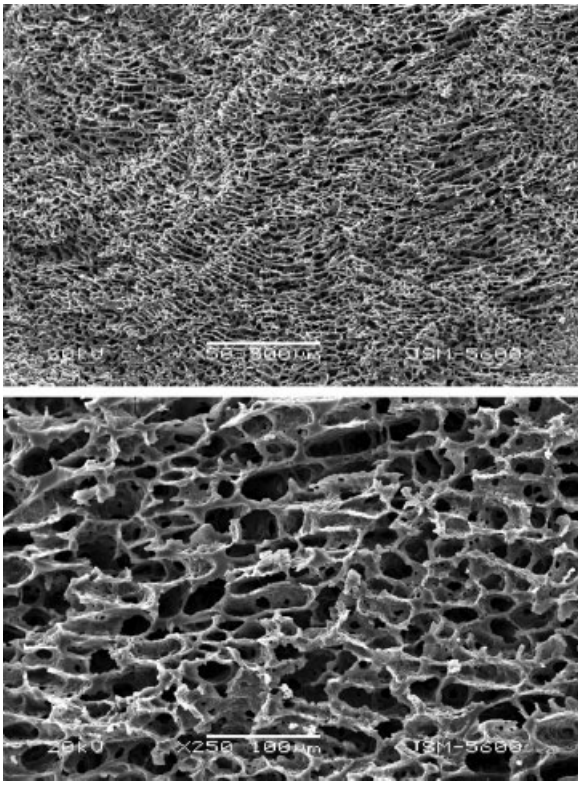

Figure 1 SEM micrographs of poly(HEMA-MAH) cryogel.

Swelling test

The equilibrium swelling degree of the poly(HEMA-MAH) cryogel was 5.62 g H_2O/g dry cryogel. Poly (HEMA-MAH) cryogel is opaque, spongelike, and elastic. This cryogel can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogel was submerged in water, it soaked in water and within 1–2 s restored its original size and shape.

FTIR

The FTIR spectrum of the poly(HEMA-MAH) cryogel had the characteristic stretching vibration bands of hydrogen-bonded alcohol, O—H, around 3481 cm^{-1} and carbonyl, at 1727 cm^{-1}, and the absorption bands of amide II, at 1516 cm^{-1} (Fig. 2).

Lysozyme adsorption from aqueous solutions

Effects of pH

Figure 3 shows the effect of pH on the adsorption of lysozyme on Zn^{2+}-chelated poly(HEMA-MAH) cryo-

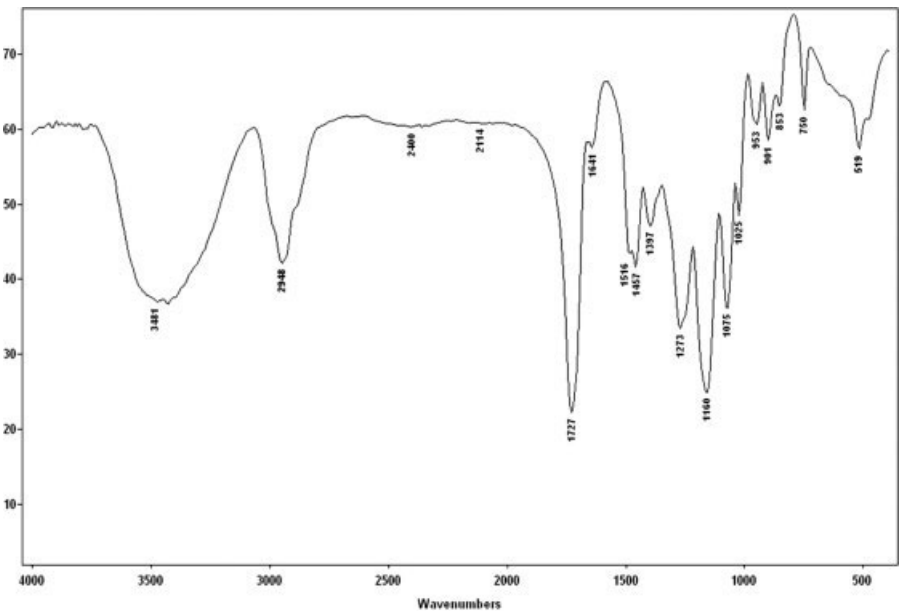

Figure 2 FTIR spectrum of poly(HEMA-MAH) cryogel.

gel. The maximum adsorption of lysozyme was observed at pH 7.0 above and below pH 7.0, the lysozyme adsorption capacity decreased. The protein adsorption on IMAC is mainly based on chelating bonding between metal ions and amino acid residues. The ionization of amino acid residues at a weakly alkaline pH favors the reaction and therefore induces protein adsorption on the metal-immobilized matrices.[27] The lysozyme adsorption in IMAC is mainly through chelation between metal ion and histidine residue of lysozyme, and the pK_a values of histidine residue in most of proteins are in the range of 5.5–8.5.[28–30]

Therefore, the optimal pH for lysozyme adsorption on Zn^{2+}-chelated poly(HEMA-MAH) cryogel is around 7.0. This result has a correlation with lysozyme adsorption on poly(HEMA-MAH) beads.[31] It should also be noted that nonspecific adsorption (i.e., adsorption on Zn^{2+}-chelated poly(HEMA-MAH) cryogel) was independent of pH, and it was observed at the same at all the pH values studied.

Effects of lysozyme concentration

Figure 4 shows the lysozyme adsorption isotherm of the plain and Zn^{2+}-chelated poly(HEMA-MAH) cryogels. Lysozyme adsorption on plain poly(HEMA-MAH) cryogel was low (about 7.5 mg/g), although the adsorption of lysozyme molecules on Zn^{2+}-chelated poly(HEMA-MAH) cryogel through Zn^{2+} ions was significant (up to 209.0 mg/g). As expected, the amount of lysozyme coupled to cryogel almost reached a plateau of around 5.0 mg/mL because of saturation of the active binding sites.

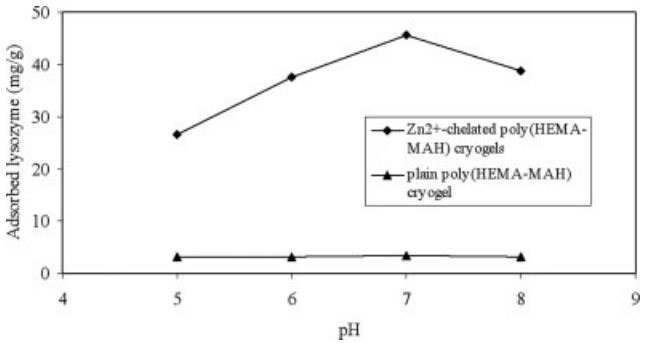

Figure 3 Effect of pH on lysozyme adsorption. MAH content: 45.8 μmol/g; Zn^{2+} loading: 51.3 μmol/g; initial lysozyme concentration: 0.7 mg/mL; flow rate: 1 mL/min; *T*: 25°C.

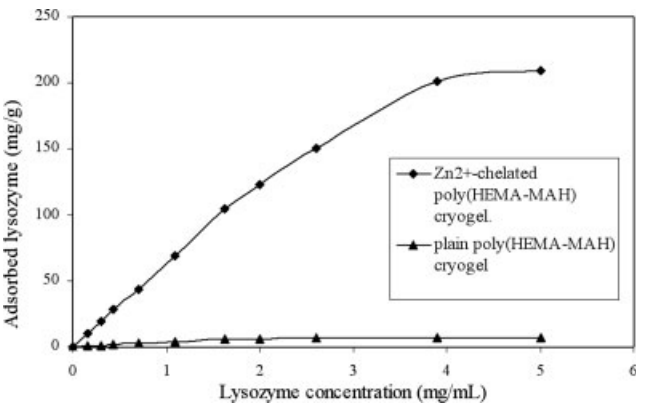

Figure 4 Effect of lysozyme concentration on adsorption. MAH content: 45.8 μmol/g; Zn^{2+} loading: 51.3 μmol/g; pH 7 (phosphate buffer); flow rate: 1 mL/min; *T*: 25°C.

Adsorption isotherms

The Langmuir adsorption isotherm was used to characterize the interactions of each protein molecule with the adsorbent. This showed a relationship between the concentration of protein in the solution and the amount of protein adsorbed on the solid phase when the two phases were at equilibrium. The Langmuir adsorption model assumes that molecules are adsorbed at a fixed number of well-defined sites, each of which can hold only one molecule. These sites are also assumed to be energetically equivalent and distant from each other so that there is no interaction between molecules adsorbed on adjacent sites.

Langmuir adsorption isotherm is expressed by eq. (2). The corresponding transformations of the equilibrium data for lysozyme gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

$$dq/dt = k_1 C_{eq}(q_m - q_{eq}) - k_2 q_{eq} \quad (2)$$

where q_{eq} is the adsorbed amount of lysozyme (mg/g), C_{eq} is the equilibrium lysozyme concentration (mg/mL), $K_d = k_2/k_1$ is the dissociation constant of the system, q_m is the maximum adsorption capacity (mg/g). At equilibrium, eq. (2) can be reduced to the adsorption isotherm:

$$q_{eq} = q_m C_{eq}/(K_d + C_{eq}) \quad (3)$$

The semireciprocal plot of C_{eq} versus C_{eq}/q_{eq} was used. The semireciprocal transformation of the equilibrium data gave rise to a linear plot with a correlation coefficient (R) of 0.9957 for Zn^{2+}-chelated poly (HEMA-MAH) cryogel, indicating that the Langmuir model could be applied to these affinity adsorbent systems. From the slope and the intercept of the straight line obtained, the values of K_d and q_m are determined. In an ideal adsorbent, the K_d values should be small for the target molecules. The maximum adsorption capacity (q_m) and K_d were found to be 209 mg/g and $4.5 \times 10^{-6} M$ for the lysozyme adsorption on Zn^{2+}-chelated poly(HEMA-MAH) cryogel, respectively.

Adsorption kinetics modeling

To examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction, kinetic models were used to test experimental data.[32] The kinetic models (Pseudo-first- and second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent 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:

$$dq_t/d_t = k_1(q_{eq} - q_t) \quad (4)$$

where k_1 is the rate constant of pseudo-first-order adsorption (1/min) and q_{eq} and q_t denote the amounts of adsorbed protein at equilibrium and at time t (mg/g), respectively. After integration by applying boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, gives

$$\log[q_{eq}/(q_{eq} - q_t)] = (k_1 t)/2.303 \quad (5)$$

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

$$\log(q_{eq} - q_t) = \log(q_{eq}) - (k_1 t)/2.303 \quad (6)$$

a plot of $\log(q_{eq})$ versus 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 interception point of a plot of $\log(q_{eq} - q_t)$ via t.

In addition, a pseudo-second-order equation based on equilibrium adsorption capacity may be expressed in the form

$$dq_t/d_t = k_2(q_{eq} - q_t)^2 \quad (7)$$

where k_2 (g/mg · min) is the rate constant of pseudo-first-order adsorption process. Integrating eq. (7) and applying the boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, leads to

$$[1/(q_{eq} - q_t)] = (1/q_{eq}) + k_2 t \quad (8)$$

or equivalently for linear form

$$(t/q_t) = (1/k_2 q_{eq}^2) + (1/q_{eq})\, t \quad (9)$$

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.

According to the values in Table I, the optimum results are for both the second- and first-order models, with the second-order mechanism R^2 values being the highest. These results suggest that the pseudo-second-order mechanisms are predominant and that chemisorption might be the rate-limiting step that controls the adsorption process. The rate-controlling mechanism may vary during the course of the adsorption process three possible mechanisms may be occurring.[33] There is an external surface mass transfer or film-diffusion process that controls

TABLE I
The First- and Second-Order Kinetic Constants for Zn^{2+}-Chelated Poly(HEMA-MAH) Cryogel

		First-order kinetic			Second-order kinetic		
Co (mg/mL)	Exp q_{eq} (mg/g)	k_1 (1/min)	q_{eq} (mg/g)	R^2	k_2 (g/mg · min)	q_{eq} (mg/g)	R^2
0.16	10.4	0.0866	7.8	0.9303	0.017984	10.9	0.9990
0.3	19.6	0.1099	22.0	0.9971	0.009188	20.7	0.9986
0.4	28.2	0.1085	56.8	0.9222	0.002086	32.6	0.9933
0.7	43.9	0.0489	36.5	0.9821	0.001605	49.3	0.9957
1.1	69.4	0.1340	126.7	0.9558	0.001931	74.6	0.9965
1.6	105.0	0.1575	175.6	0.9420	0.001412	112.4	0.9965
2.0	123.5	0.0456	184.1	0.9410	0.000891	133.3	0.9974
2.6	149.9	0.0366	118.6	0.9907	0.000501	163.9	0.9988
3.9	200.9	0.0419	159.8	0.9810	0.000375	222.2	0.9990
5.0	209.1	0.0507	193.4	0.9951	0.000343	232.6	0.9986

the early stages of the adsorption process. This may be followed by a reaction or constant rate stage and finally by a diffusion stage where the adsorption process slows down considerably.[34]

Effect of flow rate

The adsorption amounts at different flow rates are given in Figure 5. Results show that the lysozyme adsorption capacity on Zn^{2+}-chelated poly(HEMA-MAH) cryogel decreases when the flow rate through the column increases. The adsorption capacity decreased significantly from 48.1 to 31.2 mg/g polymer with the increase of the flow rate from 0.5 to 3.0 mL/min. An increase in the flow rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column. This is due to the decrease in contact time between the lysozyme molecules and Zn^{2+}-chelated poly(HEMA-MAH) cryogel at higher flow rates. These results are also in agreement with those referred to the literature.[35] When the flow rate decreases the contact time in the column is longer. Thus, lysozyme molecules have more time to diffuse to the pore walls of cryogel and to bind to the ligand, hence a better adsorption capacity is obtained. In addition, for column operation the cryogel is continuously in contact with a fresh protein solution. Consequently, the concentration in the solution in contact with a given layer of cryogel in a column is relatively constant.

Effect of temperature

The effect of temperature on lysozyme adsorption was studied in the range of 5–35°C. The equilibrium adsorption of lysozyme on Zn^{2+}-chelated poly (HEMA-MAH) cryogel decreased significantly with increasing temperature and maximum adsorption achieved at 5°C (Fig. 6). From 5–35°C, the adsorption capacity of the cryogel decreased about 20.5% for Zn^{2+}-chelated poly(HEMA-MAH) cryogel. A possible explanation for this behavior is the exothermic nature of the adsorption process.

Desorption and reusability of adsorbents

Desorption of lysozyme from Zn^{2+}-chelated poly (HEMA-MAH) cryogel was also carried out in col-

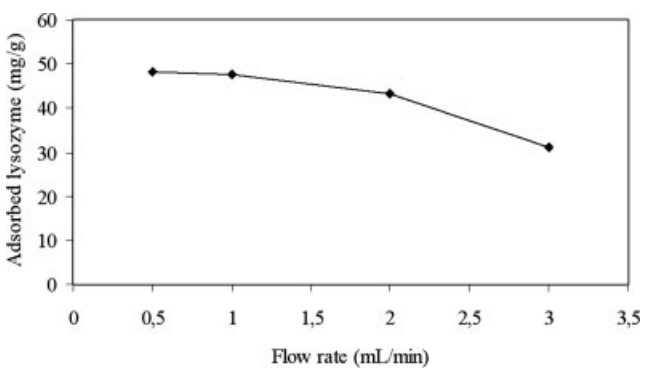

Figure 5 Effect of flow rate on lysozyme adsorption. MAH content: 45.8 μmol/g; Zn^{2+} loading: 51.3 μmol/g; initial lysozyme concentration: 0.7 mg/mL; pH 7 (phosphate buffer); *T*: 25°C.

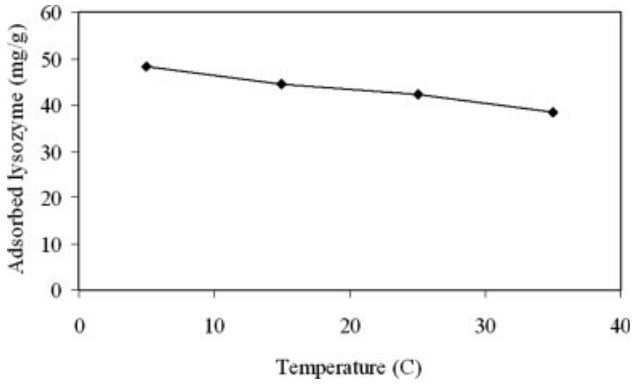

Figure 6 Effect of temperature on lysozyme adsorption. MAH content: 45.8 μmol/g; Zn^{2+} loading: 51.3 μmol/g; initial lysozyme concentration: 0.7 mg/mL; pH 7 (phosphate buffer) flow rate: 1 mL/min.

umn system. The desorption of lysozyme is expressed in percentage of totally adsorbed lysozyme. Because of economic restraints, there is a growing interest in the preparation and use of effective low cost and reusable adsorbents.[36] Up to 97.2% of the adsorbed lysozyme was desorbed by using 0.1*M* phosphate buffer containing 1*M* NaCl (pH 8.0) as elution agent. The addition of elution agent reduced electrostatic interactions, resulting in the release of the lysozyme molecules from the adsorbent. Note that there was no Zn^{2+} release from the cryogel. With the desorption data given earlier, we concluded that 0.1*M* phosphate buffer containing 1*M* NaCl (pH 8.0) is a suitable desorption agent and allows repeated use of the affinity cryogel used in this study.

To show the reusability of Zn^{2+}-chelated poly (HEMA-MAH) cryogel, the adsorption–desorption cycle was repeated 20 times using the same cryogel. There was no remarkable reduce in the adsorption capacity of the cryogel. The lysozyme adsorption capacity decreased only 5.2% after 20 cycle.

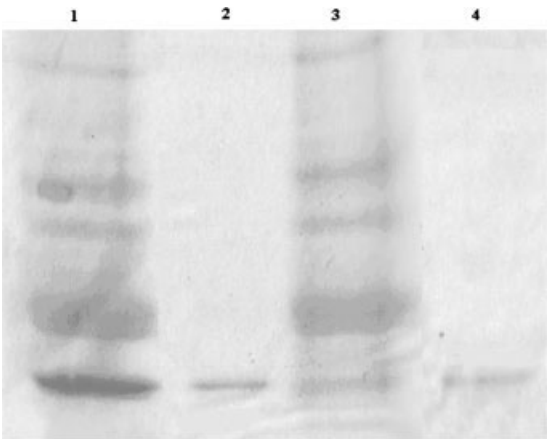

Figure 7 SDS/PAGE of lysozyme. The purity of lysozyme was assayed by SDS/PAGE using 10% separating gel (9 cm × 7.5 cm) and 5% stacking gels were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid-methanol-water (1 : 5 : 5, v/v/v) and destained in ethanol-acetic acid-water (1 : 4 : 6, v/v/v). Lane 1, egg white before adsorption; lane 2, desorbed lysozyme; lane 3, egg white after adsorption; lane 4, commercial lysozyme. Equals amounts of sample were applied to each line.

Adsorption of lysozyme from egg-white

The chicken egg white is an important source for lysozyme purification, because it contains about 3.5 wt % lysozyme. The classical lysozyme purification method required several steps, such as precipitation, centrifugation, and affinity adsorption.[30] In this study, the purification lysozyme from egg white was studied with Zn^{2+}-chelated poly(HEMA-MAH) cryogel in column system. The purity of the lysozyme eluted from polymer was determined by SDS-PAGE (Fig. 7). The purity of the desorbed lysozyme was about 88.6% with recovery about 80.5%. Zn^{2+}-chelated poly(HEMA-MAH) cryogel provided an efficient method to purify lysozyme from diluted egg white, showing high adsorption capacity and high selectivity for lysozyme. The specific activity of the purified lysozyme with Zn^{2+}-chelated poly(HEMA-MAH) cryogel was 42.381 U/mg. It should be noted that the specific activity of native lysozyme was 46.500 U/mg. As seen here there was no drastic decrease in specific activity during the purification studies.

CONCLUSIONS

A new metal-immobilized affinity adsorbent was prepared by chelateing Zn^{2+} on poly(HEMA-MAH) cryogel. Experiment results indicated that this novel adsorbent can effectively adsorb lysozyme from aqueous solution, and the excellent adsorption–desorption behaviors of the adsorbent promise it to be useful in practical applications. The adsorption of lysozyme showed a greater dependence on pH and flow rate and temperature of the adsorption medium. The maximum adsorption of lysozyme was observed at pH 7.0 above and below pH 7.0, the lysozyme adsorption capacity decreased. The lysozyme adsorption capacity on Zn^{2+}-chelated poly (HEMA-MAH) cryogel decreased when the flow-rate through the column increased. The equilibrium adsorption of lysozyme on Zn^{2+}-chelated poly (HEMA-MAH) cryogel decreased significantly with increasing temperature and maximum adsorption achieved at 5°C. The lysozyme adsorption capacity decreased only 5.2% after 20 cycle. Compared with poly(HEMA-MAH) cryogel, the adsorption capacity of Zn^{2+}-chelated poly(HEMA-MAH) cryogel is greatly increased. It can be concluded that Zn^{2+} plays a key role for the adsorption of lysozyme on poly(HEMA-MAH) cryogel. Therefore, the formation of coordinated compound between protein and Zn^{2+} should be considered to be the major binding mode. However, electrostatic and hydrophobic interactions may be also involved in the adsorption of lysozyme on Zn^{2+}-chelated poly(HEMA-MAH) cryogel.

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