Preparation and Characterization of Metal-Chelated Poly(HEMA-MAH) Monolithic Cryogels and Their Use for DNA Adsorption

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ABSTRACT: DNA adsorption properties of Zn^{2+}-chelated supermacroporous poly(2-hydroxyethyl methacrylate-*N*-methacryloyl-(L)-histidine methyl ester) [poly(HEMA-MAH)] monolithic cryogel column were investigated for the application of DNA-affinity adsorbents. The monolithic cryogel was loaded with Zn^{2+} ions to form the metal-chelated affinity sorbent. Poly(HEMA-MAH) cryogel was characterized by swelling tests, FTIR, scanning electron microscopy (SEM), and elemental analysis. SEM analysis indicates that the cryogel have a heteroporous structure with interconnected pores of 10–50 μm size, which ascribed to the porogens effect of frozen water crystals. Poly(HEMA-MAH) cryogel containing 45.8 μmol MAH was used in the adsorption/desorption of DNA from aqueous solutions. The maximum amount of DNA adsorption was 32.93 mg/g in Tris buffer at pH 7.0. It was observed that DNA could be repeatedly adsorbed and desorbed with the poly(HEMA-MAH) cryogel without significant loss of adsorption capacity. As a result, these higher amounts of DNA adsorbed poly (HEMA-MAH) cryogels are expected to be good candidate for achieving higher removal of anti-DNA antibodies from systemic lupus erythematosus (SLE) patients plasma. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 116: 1306–1312, 2010

Key words: affinity chromatography; DNA adsorption; metal chelate; histidine; Cryogels

INTRODUCTION

Because immobilized metal affinity chromatography (IMAC) has been introduced by Porath et al.,[1,2] this technique has become a widespread analytical and preparative separation method for the purification of diverse biomolecules, such as enzymes, coenzymes, cofactors, antibodies, antigens, hormones, proteins, and nucleic acids.[3–8] While the interaction of metal ions with nucleic acids is a long-standing and active field of research,[9,10] there are limited studies including the adsorption of nucleic acids to the IMAC columns.[11] 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 immobilized metal affinity separation.

Different polymeric adsorbents have been suggested as supports for immobilization of DNA.[11–18] In order to treat some autoimmune diseases, such as systemic lupus erythematosus (SLE), DNA-affinity adsorbents have been used for removal of anti-double-stranded DNA antibodies from the sera of patients suffering from SLE.[19–22] This adsorbent prepared by us may be considered as a good candidate for achieving higher removal rates for anti-DNA antibodies.

In this study, supermacroporous poly(2-hydroxyethyl methacrylate-*N*-methacryloyl-(L)-histidine methyl ester) [poly(HEMA-MAH)] monolithic cryogels, considered as a novel generation of stationary phases in the separation science,[23–26] were prepared by radical cryo-copolymerization of HEMA with *N*-methacryloyl-(L)-histidine methyl ester (MAH) as functional co-monomer and *N*,*N*′-methylene-bis-acrylamide (MBAAm) as cross-linker directly in a plastic syringe. Then Zn^{2+} ions were chelated through imidazole groups on the MAH reactive functional groups of the polymeric structure.

EXPERIMENTAL

Materials

DNA (from herring testes) was purchased from Sigma (St. Louis, MO), L-histidine methylester, methacryloyl chloride, MBAAm, 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

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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). Deionized water was used in all experiment sets.

Synthesis of MAH

The synthesis and characterization of MAH were performed as described previously.[27] Briefly, 5.0 g of L-histidine methylester 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, which was poured in slowly. After stirring by magnetically at room temperature for 2 h, hydroquinone and unreacted methacryloyl chloride were extracted with 100 mL of 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 ($CHCl_3$) 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 ($CDCl_3$): $\delta = 1.99$ (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 (**m**; 1H, $J = 7.4$ Hz, NH), 7.82 (**d**; 1H, $J = 8.4$ Hz, NH), 6.86–7.52 (m; **2**H, aromatic).

Preparation of poly(HEMA-MAH) cryogel

Supermacroporous monolithic poly(HEMA-MAH) cryogel was prepared by bulk polymerization. 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. After adding TEMED, the reaction mixture was stirred for 1 min and then, this mixture was poured into a plastic syringe (2 mL, id. 0.8 cm) with closed outled at the bottom. The polymerization solution in the syringe was frozen at −12°C for 24 h and then thawed at room temperature. After washing, the cryogel was stored in buffer containing 0.02% sodium azide at 4°C until use.

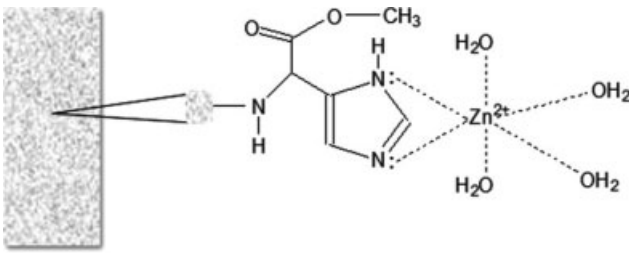

Figure 1 Schematic diagram of Zn^{2+} ions chelated MAH structure.

Incorporation of Zn^{2+} ions

Zn^{2+}-chelation was carried out in a recirculating system. After washing of cryogel with water, 40 mL of a Zn^{2+} solution [50 mg/L (pH 5.0), adjusted with HCl and NaOH] was pumped through the column by recirculating 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/Perkin Elmer, USA). The Zn^{2+} chelation step was depicted in Figure 1. The Zn^{2+} concentrations in the initial and final solutions were used to calculate the amount of Zn^{2+} ions adsorbed.

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 ($m_{\text{wet gel}}$). After drying to constant mass in the oven at 60°C, the mass of dried sample was determined ($m_{\text{dry gel}}$). 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 coated with gold-palladium (40 : 60) and investigated by SEM (JEOL JSM 5600 SEM, Tokyo, Japan).

FTIR

FTIR spectrum of poly(HEMA-MAH) cryogel was obtained by 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, Leco Co., USA) was performed to evaluate how much MAH co-monomer was incorporated into the poly (HEMA-MAH) cryogel.

Chromatographic procedures

DNA adsorption from aqueous solutions

Investigation of DNA adsorption was carried out in a recirculating system equipped with a water jacket for temperature control. After washing of cryogel with water, it was equilibrated with 0.02*M* Tris buffer containing 0.02*M* NaCl (pH 7.0). Then the prepared DNA solution was pumped through the column by recirculating for 2 h. The adsorption was followed by monitoring of the decrease in UV absorbance at 260 nm. The effects of flow rate, DNA concentration, and pH of the medium on adsorption capacity were studied. The flow rate of the solution (i.e., 50 mL of the aqueous DNA solution) was varied in the range of 1.0–2.0 mL/min. To observe the effects of the initial DNA concentration on adsorption, it was varied between 0.5 and 5.0 mg/mL. To determine the effects of pH on adsorption, pH of the medium was varied between 5.0 and 8.0.

All experiments and the samples were performed in replicates of three. For each set of data present, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples in order to determine the margin of error.

Desorption and repeated use

In all cases, adsorbed DNA 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 DNA 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* Tris buffer containing 0.02*M* NaCl (pH 7.0). The desorption ratio was calculated from the amount of DNA adsorbed on the cryogel and the final DNA concentration in the desorption medium. To test the repeated use of poly(HEMA-MAH) cryogel, DNA adsorption-desorption cycle was repeated for 10 times using the same cryogel column. To regenerate and sterilize, after desorption, the cryogel was washed with 1*M* NaOH solution.

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 49.2 μmol/g polymer. Mass stoichiometric analysis showed that one incorporated MAH molecule interacted around one Zn^{2+} ion (45.8 μmol MAH/g: 49.2 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 nonspecifically adsorbed Zn^{2+} ions from the cryogel.

SEM observation

A supermacroporous cryogel was produced by polymerization in the frozen state of monomers, 2-HEMA and MAH, MBAAm as cross linker in the presence of APS/*N*,*N*,*N*′,*N*′- TEMED as initiator/activator pair. The SEM micrographs of the internal structure of the cryogel are shown in Figure 2. 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 DNA molecules, allowing them to pass easily. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible.

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, sponge like, 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 3421 cm^{-1} and carbonyl stretching (amide I) at 1722 cm^{-1}, and C—N stretching band at 1538 cm^{-1} (Fig. 3). These data confirmed that the poly(HEMA-

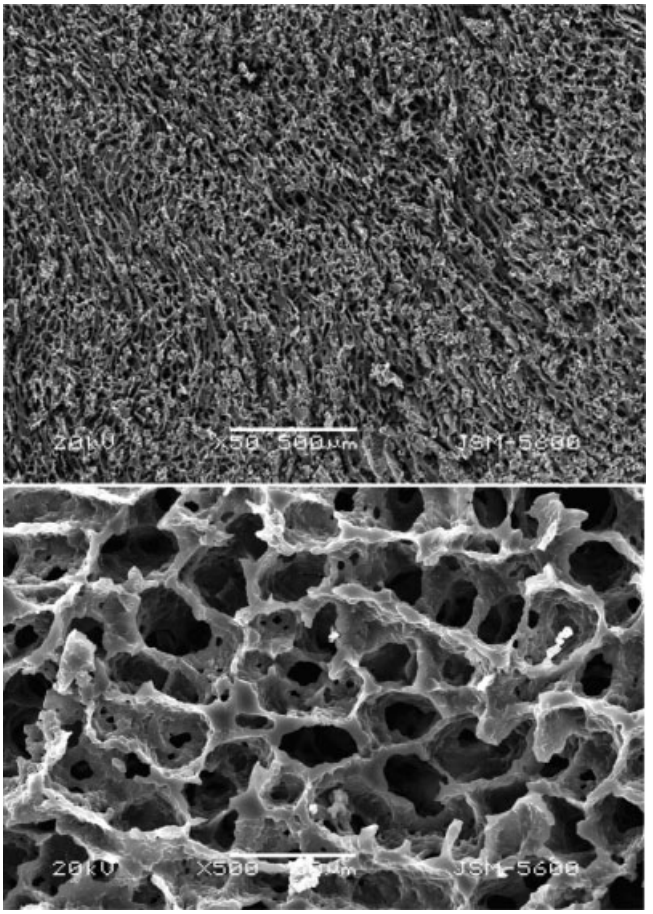

Figure 2 SEM micrographs of poly(HEMA-MAH) cryogel.

MAH) cryogel was formed with functional groups MAH.

DNA adsorption from aqueous solutions

Effects of pH

Figure 4 shows the effect of pH on the adsorption of DNA on Zn^{2+}-chelated poly(HEMA-MAH) cryogel. The maximum adsorption of DNA was observed at pH 7.0 (0.02*M* Tris buffer containing 0.02 M NaCl) above and below the pH 7.0, the DNA adsorption capacity decreased. The DNA adsorption on IMAC

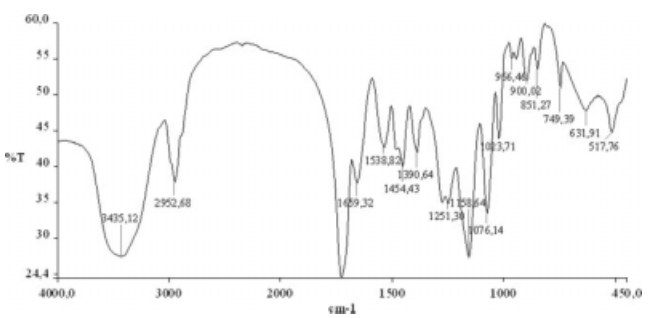

Figure 3 FTIR spectrum of poly(HEMA-MAH) cryogel.

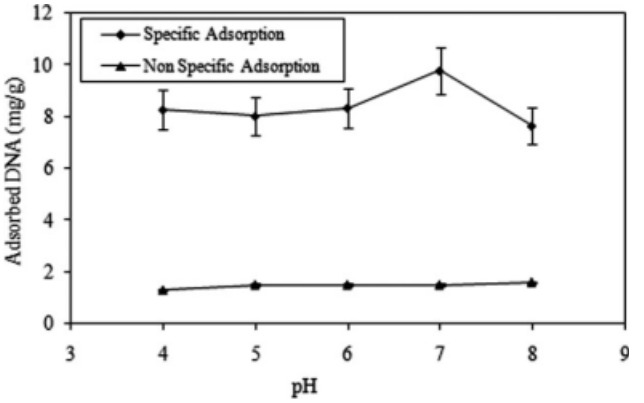

Figure 4 Effect of pH on DNA adsorption. MAH content: 45.8 μmol.g^{-1}; Zn^{2+} loading: 49.2 μmol/g; initial DNA concentration: 1 mg/mL; flow rate: 1 mL/min; *T*: 25ºC.

is mainly through chelation between metal ions and the phosphate backbone or the DNA bases.[28] DNA adsorption in IMAC is mainly at a weakly alkaline pH favors the reaction and therefore induces DNA adsorption on the metal-immobilized matrixes.

The optimal pH for DNA adsorption on Zn^{2+}-chelated poly(HEMA-MAH) cryogel is around 7.0. This result has a correlation with nucleic acid separation by IMAC.[11] It should be also noted that nonspecific adsorption (i.e., adsorption on poly(HEMA-MAH) cryogel) was independent of pH and it was observed at the same at all the pH values studied.

Effects of DNA concentration

The important role of some divalent cations play in bridging of DNA to the mica has been studied.[29] Affinity of DNA bases to some divalent cations increases throughout this series: Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Cu^{2+}.[30,31] In this study, Zn^{2+} ion was chosen as DNA binding agent to the adsorbent contains MAH molecule as co-monomer. Figure 5 shows the DNA adsorption isotherm of the plain (as

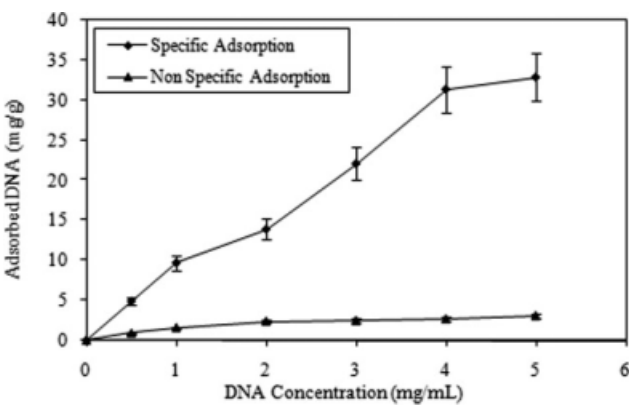

Figure 5 Effect of DNA concentration on adsorption. MAH content: 45.8 μmol/g; Zn^{2+} loading: 49.2 μmol/g; pH 7 (Tris buffer); flow rate: 1 mL/min; *T*: 25°C.

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

Co	Exp. q_{eq}	First-order kinetic			Second-order kinetic		
(mg/mL)	(mg/g)	k_1 (1/min)	q_{eq} (mg/g)	R^2	k_2 ($\times 10^{-3}$ g/mg.min)	q_{eq} (mg/g)	R^2
0.5	04.85	0.0309	3.59	0.980	10.87	5.51	0.999
1.0	09.70	0.0375	8.22	0.997	5.14	11.19	0.999
2.0	16.41	0.0325	11.24	0.973	7.07	18.12	0.998
3.0	22.13	0.0421	18.82	0.996	2.75	25.06	0.999
4.0	31.43	0.0293	25.44	0.995	1.46	36.23	0.999
5.0	32.93	0.0341	26.19	0.993	1.03	37.17	0.999

a reference surface) and Zn^{2+}-chelated poly(HEMA-MAH) cryogels. DNA adsorption on plain poly (HEMA-MAH) cryogel was low (about 3.1 mg/g), although adsorption of DNA molecules on Zn^{2+}-chelated poly(HEMA-MAH) cryogel through Zn^{2+} ions was significant (up to 32.93 mg/g). As expected, the amount of DNA coupled to cryogel almost reached a plateau of around 5.0 mg/mL because of saturation of the active binding sites.

Adsorption Isotherms

During the experiments, adsorption isotherms were used to evaluate adsorption properties. The Langmuir adsorption isotherm used to characterize the binding process of DNA to Zn^{2+}-chelated poly (HEMA-MAH) cryogels is generally formulated by eq. (2), which is utilized in a linear form as in eq. (3).

$$Q = Q_{max} \cdot b \cdot C_e/(1 + b \cdot C_e) \quad (2)$$

$$1/Q = (1/Q_{max} \cdot b)(1/C_e) + 1/Q_{max} \quad (3)$$

where Q is the concentration of DNA bound to the adsorbent (mg/g), C_e the equilibrium DNA concentration in solution (mg/L), b the Langmuir constant (L/mg), and Q_{max} is the maximum adsorption capacity (mg/g). A linear plot of $1/Q$, the reciprocal of the bound DNA concentration versus $1/C_e$, the reciprocal of the unbound DNA concentration gave rise to an intercept of $1/Q_{max}$ and a slope expressed by $1/(Q_{max} \cdot b)$. The maximum adsorption capacity (Q_{max}) for the Zn^{2+}-chelated poly(HEMA-MAH) cryogels was determined from the intercept ($1/Q_{max}$) to be 88.49 mg/g, and the respective Langmuir constant was extracted from the slope ($1/(Q_{max} \cdot b)$) to be 1.0 mg^{-1}. The high correlation coefficient (R^2) was 0.993 for Zn^{2+}-chelated poly(HEMA-MAH) cryogels, indicating that the Langmuir adsorption model can be applied in this affinity adsorbent system.

Adsorption kinetics modeling

In order to examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction, kinetic models were used to test experimental data.[23] 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:

$$dq_t/dt = K_1(q_e - q_t) \quad (4)$$

where K_1 is the rate constant of pseudo-first order adsorption (1/min) and q_e and q_t denote the amounts of adsorbed protein at equilibrium and at time (mg/g), t respectively. After integration by applying boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, gives

$$\mathrm{Log}(q_e - q_t) = \mathrm{Log}(q_e) - (K_1 t)/2.303 \quad (5)$$

a plot of Log(q_e) versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process logq_e should be equal to the interception point of a plot of Log($q_e - q_t$) via t.

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

$$dq_t/dt = K_2(q_e - q_t)^2 \quad (6)$$

Where K_2 (g/mg·min) is the rate constant of pseudo first order adsorption process. Integrating eq. (6) and applying the boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, leads to

$$(t/q_t) = (1/K_2 q_e^2) + (1/q_e)t \quad (7)$$

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_e) 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

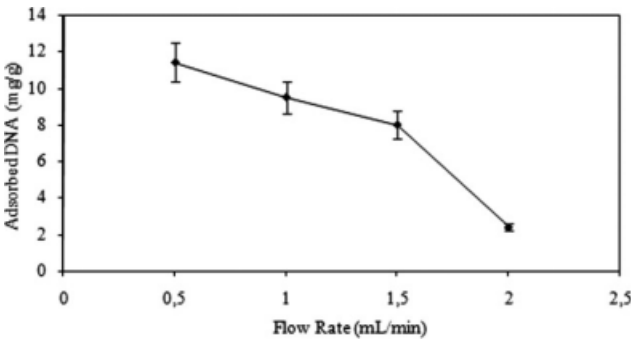

Figure 6 Effect of flow rate on DNA adsorption. MAH content: 45.8 μmol/g; Zn^{2+} loading: 49.2 μmol/g; initial DNA concentration: 1 mg/min; pH 7 (Tris buffer); *T*: 25ºC.

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.[29] There is an external surface mass transfer or film diffusion process that controls 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.[32]

Effect of flow-rate

The adsorption amounts at different flow-rates are given in Figure 6. The results show that the DNA 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 11.4 to 2.4 mg/g polymer with the increase of the flow-rate from 0.5 to 2.0 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 decrease in contact time between the DNA 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.[33] When the flow-rate decreases the contact time in the column is longer. Thus, DNA 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.

Desorption and reusability of adsorbents

Desorption of DNA from Zn^{2+}-chelated poly (HEMA-MAH) cryogel was also carried out in column system. The desorption of DNA is expressed in percentage of totally adsorbed DNA. Due to economic restraints, there is a growing interest in the preparation and use of effective low-cost and reusable adsorbents.[22] Up to 97.9% of the adsorbed DNA 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 DNA 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 10 times using the same cryogel. There was no remarkable decrease in the adsorption capacity of the cryogel. The DNA adsorption capacity decreased only 4.7% after 10-cycle.

CONCLUSION

A wide variety of functional molecules, (i.e. enzymes, antibodies, proteins, and nucleic acids) may be used as ligands in the design of novel adsorbents.[34,35] These ligands are extremely specific in most cases. However, they are expensive, due to high cost of production and/or extensive purification steps. The main advantage of IMAC consists in its simplicity, universality, stability, and cheapness of the chelating supports.[36] Experiment results of these new metal-immobilized affinity adsorbent prepared by chelateing Zn^{2+} on poly(HEMA-MAH) cryogel indicated that this novel adsorbent can effectively adsorb DNA from aqueous solution, and the excellent adsorption-desorption (i.e., up to 32.93 mg/g-97.9%) of the adsorbent promise it to be useful in practical applications. It can be concluded that Zn^{2+} plays a key role for the adsorption of DNA on poly(HEMA-MAH) cryogel. Therefore, the formation of coordinated compound between DNA and Zn^{2+} should be considered to be the major binding mode. DNA immobilized affinity sorbents is commonly used in the therapy of some autoimmune diseases by removal of anti-DNA antibodies from patient sera.[12,20,22] The correlation of amount of DNA immobilized on affinity sorbents with anti-DNA antibody adsorption capacity was exhibited.[16,17] Therefore, our prepared adsorbents carrying higher amounts of DNA may be considered as a good candidate for achieving higher removal rates for anti-DNA antibodies.

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