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Chemically modified chitosan beads as matrices for adsorptive separation of proteins by molecularly imprinted polymer

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Abstract

A simple molecularly imprinted polymer (MIP) was prepared using hemoglobin as the imprinted molecule and acrylamide as the functional monomer. The MIP was achieved by grafting of the selective soft polyacrylamide gel to the maleic anhydride modified chitosan beads by letting the monomers and the protein diffuse into the pores of the chemically modified chitosan matrix before starting the polymerization. The chitosan beads were freed from the surrounding polyacrylamide gel by washing. Langmuir analysis showed that an equal class of adsorption was formed in the MIP, the adsorption equilibrium constant and the maximum adsorption capacity were evaluated to be 1.47 and 35.7 mg/g wet MIP beads, respectively. The MIP has much higher adsorption capacity for hemoglobin than the non-imprinted polymer with the same chemical composition, and also has a higher selectivity for the imprinted molecule. The MIP can be reused and the reproducibility was approximately 100% at low concentration.

Keywords: Chemical modified chitosan beads; Polyacrylamide; Grafting; Molecular imprinting

1. Introduction

Molecularly imprinted polymer (MIP) represents a new class of materials that have artificially created receptor structures (Piletsky, Panasyuk, Piletskaya, Nicholls, & Ulbricht, 1999; Wulff & Sarhan, 1972). Since its discovery in 1972, MIP has attracted considerable interest from scientists and engineers. Usually, only relatively low molecular weight compounds such as amino acid derivatives, certain drugs and pesticides (Andersson, Sellergren, & Mosbach, 1984; Fisher, Muller, & Ekberg, 1991; Vlatakis, Andersson, Müeller, & Mosbach, 1993; Yoshida, Hatate, Uezu, Goto, & Furusaki, 2000) are used as imprinted molecules; the use of biomacromolecules such as protein is seldom (Kempe & Mosbach, 1995; Rachkov & Minoura, 2001; Shi, Tsai, Garrison, Ferrari, & Ratner, 1999). Hjertén (Liao, Wang, & Hjertén 1996) in 1996 introduced an alternative method based on polymerization of the non-charged monomer (acrylamide and *N*,*N*'-methylenebisacrylamide) in the presence of the protein of interest. The selectivity of the polyacrylamide gels for proteins was high, for instance, myoglobin from horse could be separated from myoglobin from whale, even though their 3-D structure is similar (Hjertén et al., 1997). But the polyacrylamide gel was soft and its mechanical strength was not well, so Tong (Tong, Hetényi, Bikádi, Gao, & Hjertén, 2001) improved the properties of the gels. This was achieved by entrapment of the selective soft polyacrylamide gel in the pores of a rigid inert gel by letting the monomers and the protein diffuse into the pores of agarose beads before starting the polymerization. The agarose beads were freed from the surrounding polyacrylamide gel by stirring. But there are no chemical bonds between the agarose matrix and the polyacrylamide gel, the polyacrylamide gel could be

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washed out of the pores of the agarose beads after multiple times.

Chitosan is an N-deacetylated derivative of chitin, a cationic polysaccharide composed of β-D-glucosamine and *N*-acetyl-β-D-glucosamine residues with a 1,4 linkage. Due to its excellent biocompatibility and bioadsorbility, chitosan has been widely used in biomedical applications (Dinesh & Alok, 1998; Zileinski & Aebischer, 1994). Chitosan is readily processible into films, beads and sponges from aqueous acid solution. A wet phase-inversion (immersion precipitation induced phase-inversion) method is a suitable technique to prepare macroporous gels with the desired morphology and pore size (Chandy & Sharma, 1993; Mi, Shyu, Chen, & Lai, 2002; Ngah, Endud, & Mayanar, 2002). Several methods have been used to modify chitosan either physically (Maraca, Suder, & Wightman, 1982; Shi et al., 1999; Shu & Zhu, 2001) or chemically (Amiji, 1997; Koyama & Taniguchi, 1986; Zeng & Ruckenstein, 1998); these modifications were proposed in order to improve pore size, mechanical strength, chemical stability, hydrophilicity and also biocompatibility.

In this paper, vinyl groups are introduced to chitosan beads, so that polyacrylamide gel could be grafted to the modified chitosan beads. Thereby the interaction forces of the selective polyacrylamide gel and chitosan matrix could be strengthened by chemical bonds. The MIP beads thus obtained were characterized by IR, ESEM, and UV spectrophotometer measurement, and the adsorption capacity and selectivity of the MIP were also discussed.

2. Experimental

2.1. Materials

Chitosan was purchased from Boao Bio-Technology Company, Shanghai (China). Deacetylation degree is 90%. Epichlorhydrin (ECH) and maleic anhydride (MAH) were purchased from Tianjin No. 1 Chemical Reagent Factory. Acrylamide (Am) was purchased from Miou Chemical Factory Tianjin (China) and purified by recrystallization. *N*,*N*'-methylenebisacrylamide (MBA) was purchased from Tianjin Special Reagent Factory. Potassium persulfate (KPS) was obtained from Tianjin No. 3 Chemical Reagent Factory and purified by recrystallization. Hemoglobin (Hb) and bovine serum albumin (BSA) were purchased from Sino-America Biotechnology Company and the protein solutions were prepared using 0.01 M sodium phosphate buffer (pH 6.8). Other chemicals were analytical grade and used as received.

2.2. Chitosan characterization

2.2.1. Molecular weight determination

Molecular weight was determined by a viscometric method (Chen & Hwa, 1996). Chitosan was dissolved in 0.2 M acetic acid in 0.1 M sodium acetate aqueous solution. The relative viscosity, η, of the chitosan solution was measured using an Ubbelohde capillary viscometer at 30 ± 0.1 °C. Specific viscosity was calculated using the equation:

$$\eta_{sp} = (\eta_{solution} - \eta_{solvent})/\eta_{solution}$$

where intrinsic viscosity, $[\eta]$, is defined as the reduced viscosity, η_{red}, extrapolated to a chitosan concentration, C, of zero:

$$[\eta] = (\eta_{sp}/C)_{C\to 0} = (\eta_{red})_{C\to 0}$$

where C is in g/ml. Viscosity average molecular weight was calculated according to the Mark–Houwink equation

$$[\eta] = KM_v^{\alpha}$$

$$K = 1.64 \times 10^{-30} \times \mathrm{DD}^{14},$$

$$\alpha = -1.02 \times 10^{-2} \times \mathrm{DD} + 1.82,$$

where DD is the degree of deacetylation of chitosan (Wang, Bo, Li, & Qin, 1991).

2.3. Preparation of macroporous chitosan beads

Chitosan beads were prepared as reported elsewhere (Chandy & Sharma, 1993; Shu & Zhu, 2001). In this work chitosan (3.0 g) was dissolved in 2% (v/v) acetic acid (100 ml). The solution was dropped through a 7-gauge needle into 2 M sodium hydroxide, and the gelled spheres formed instantaneously. This process was accomplished by using a Model 100 (Qingpu Huxi Instrument Plant, Shanghai, China) push–pull syringe pump. The chitosan beads formed were left in the sodium hydroxide solution for 24 h, and then washed with distilled water. The resultant chitosan beads (18 g) were cross-linked with 0.043 M epichlorhydrin aqueous solution (pH 10) (25 ml) at 60 °C for 5–6 h. Thereafter, the beads were washed extensively with distilled water until the washing solution became neutral. The diameter of the wet bead was approximately 0.85 ± 0.05 mm.

2.4. Characterization of cross-linked chitosan beads

The amine content of the cross-linked chitosan beads was determined titrimetrically. Cross-linked chitosan beads (1.0 g) (filter paper used to absorb the surface water) were added to 0.1 M hydrochloric acid (20 ml); the mixture was left to stand for 16 h in a tightly closed bottle. The mixture was filtered, and the filtrate (5 ml) was titrated with a 0.1 M sodium hydroxide.

The surface morphology of the wet beads was studied using an environmental scanning electron microscope (ESEM Philips×130). Samples containing water without drying were mounted on metal stubs and at low vacuum

degree ($\sim 10^{-3}$ atm) and relatively low temperature (near 0 °C) observed.

2.5. Modification of the porous chitosan beads with maleic anhydride (MAH-Cs)

The cross-linked chitosan beads were modified chemically with maleic anhydride to introduce vinyl groups. Chitosan beads (21.0 g), maleic anhydride (9.8 g) and 95% ethanol (50 ml) were put into a 100 ml, four-necked flask equipped with a mechanical stirrer and heated to at 70 °C. Then triethylamine was dropped into the mixture and heated to 80 °C for 8 h. The resultant MAH-chitosan beads were washed extensively with distilled water to remove reagents.

2.5.1. Determination of the carboxyl content of the MAH-Cs beads

The carboxyl content of the MAH-Cs beads was estimated as follows: the polymer sample (1.0 g) was left in contact with 0.1 M sodium hydroxide (10 ml) for 4 h, and the unreacted sodium hydroxide content was determined by the titration of a portion of the filtrate (5 ml) with 0.1 M hydrochloric acid.

2.6. Graft copolymerization of acrylamide

To prove that acrylamide could be grafted to the MAH modified chitosan beads, only acrylamide, no cross-linker was added to react with the MAH modified chitosan, for the non-grafted polyacrylamide could be dissolved in water and washed out of the beads by 0.01 M sodium phosphate buffer (pH 6.8) elution. MAH-Cs beads (16.0 g), acrylamide (2.0 g), potassium persulfate (20.1 mg) and 0.01 M sodium phosphate buffer (pH 6.8, 28 ml) were put into a 100 ml four-necked flask which was equipped with a mechanical stirrer and a nitrogen inlet. The mixture was stirred continuously under a nitrogen stream for 45 min, then 0.01 M sodium phosphate buffer (pH 6.8) containing 0.16% w/v sodium bisulphate (5 ml) was added. The mixture was stirred under a nitrogen atmosphere for 2 h at 4 °C. Removal of the unreacted reagents and the ungrafted homopolyacrylamide from the beads was accomplished through a soxhlet extraction with boiling distilled water for 24 h.

2.6.1. IR spectra of chitosan derivatives

The IR spectra of cross-linked chitosan beads and its derivatives were recorded with a Bio-Rad FTS 135 Fourier-transform infrared (FTIR) spectrometer in the range 3500–500 cm^{-1} using KBr pellets.

2.7. Preparation of Hb-imprinted polymer (MIP)

Hb-imprinted polymer (MIP) was prepared in the same way and same amounts as for grafted chitosan beads. Wet MAH-Cs beads (16 g) (filter paper used to absorb the surface water), acrylamide (1.9 g), *N*,*N*'-methylenebisacrylamide (0.1 g), potassium persulfate (20.4 mg), hemoglobin (600 mg), 0.01 M sodium phosphate buffer (pH 6.8, 28 ml) were stirred under nitrogen for 45 min, then 0.01 M phosphate buffer (pH 6.8) containing 0.16% w/v sodium bisulphite (5 ml) was added. The mixture was stirred under the nitrogen atmosphere for 2 h and the gel formed was put into a nylon stocking to press out the surrounding polyacrylamide gel, then the freed chitosan beads were washed with 10% v/v acetic acid containing 10% w/v sodium dodecyl sulfate (SDS) solution to desorb the hemoglobin till the color was pale (but was still somewhat colored). The MIP beads obtained were equilibrated with 0.01 M sodium phosphate buffer (pH 6.8) for 24 h.

The non-imprinted polymer (NIP) was prepared using the this procedure without addition of the hemoglobin and was worked up by the same procedure.

2.8. Characterization of the MIP beads

2.8.1. The morphology of the MIP beads

The surface morphology of the wet MIP beads was also studied using environmental scanning electron microscope (ESEM Philips×130) procedure as the cross-linked chitosan beads.

2.8.2. Mechanical properties determination

Mechanical strength studies of the MIP beads were completed using a mechanical stirrer. 100 MIP beads and water (40 ml) were put in a 50 ml flask, and stirred at different speeds. After 30 min, the fragments were separated and the MIP beads were renumbered. The mechanical strength was determined by noting the percent loss in number of the MIP beads. The samples of reference were cross-linked chitosan beads and polyacrylamide beads. The polyacrylamide beads were prepared using inverse suspension polymerization, the total monomer concentration was 6% (w/v) and cross-linking concentration was 5% (w/w), in the same way as the MIP beads. The range of 0.8–1.0 mm diameter polyacrylamide beads was selected to study mechanical strength.

2.8.3. Adsorption dynamics of Hb on MIP

To investigate the adsorption dynamics of the MIP beads, the wet beads (0.5 g) (filter paper used to absorb the surface water) was placed in a 50 ml conical flask and mixed with 0.4 mg/ml Hb solution (25 ml). The conical flask was oscillated in a constant temperature bath at 25 °C for different times. The concentration of Hb in the solution was determined using a spectrophotometer at 280 nm. The adsorbance Q, was calculated based on the difference of Hb concentration before and after adsorption, the volume of aqueous solution, and the weight of the MIP beads,

according to:

Adsorbance $Q = (C_0 - C_t)\, V/W$

where C_0 is the initial Hb concentration (mg/ml), C_t is the Hb concentration (mg/ml) at different times, V is the volume of Hb solution (ml), and W is the weight of the MIP beads (g).

2.8.4. Adsorption experiments

The wet MIP beads (0.5 g) (filter paper used to absorb the surface water) were placed in a 50 ml conical flask and mixed with (10 ml) Hb solution of a known concentration. The conical flask was oscillated at 25 °C for 16–17 h, and the concentration of Hb in the solution was determined using a spectrophotometer at 280 nm and a calibration curve constructed from use of standards.

3. Results and discussion

3.1. The characterization of the chitosan beads and MIP beads

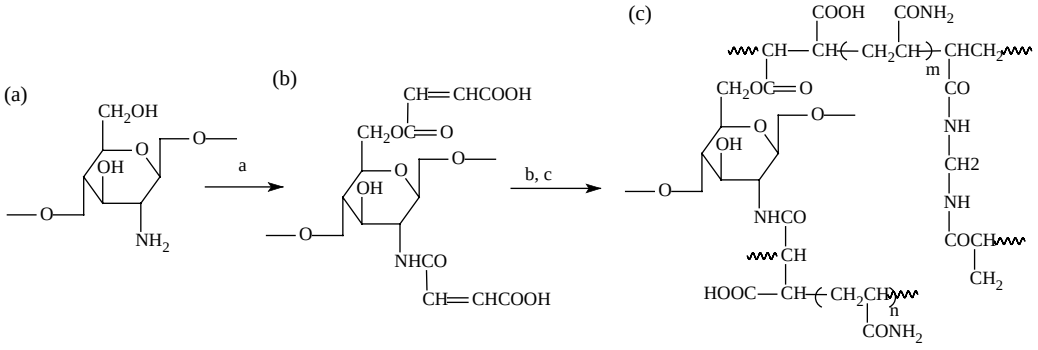
Although the average molecular weight can be determined by several methods, viscometry is claimed to be the simplest, most rapid, and probably the most precise determination method (Rinaudo & Domard, 1988). Thus, the viscosity average molecular weight of the chitosan used in our lab was determined to be 503,495 by this method.

In the first step, chitosan beads were prepared by phase inversion. A uniform particle size (0.85 ± 0.05 mm) of the macroporous chitosan gel was obtained and the average pore size of the beads was valued to be 4.9 μm. An analysis of the cross-linked chitosan beads gave 0.21 mmol of amine content per gram wet beads (the water content was 95.4%); that means about 23% amine groups were cross-linked with epichlorhydrin. Obviously, this analytical result indicates that there are enough amine groups in the accessible positions of the cross-linked matrix. The analysis of MAH-Cs gave 0.28 mmol of carboxyl content per gram wet beads (water content was 87.7%). That means that the vinyl group contents were above 0.28 mmol/g and that most amine had reacted with maleic anhydride.

Then, polyacrylamide was added to the MAH-Cs beads for Hb-specific adsorption. The polyacrylamide gel was anchored by grafting of acrylamide to the vinyl groups of the chemically modified chitosan beads. Preparation of the cross-linked chitosan beads, its modification to yield vinyl groups and subsequent grafting by redox initiation from the surface is depicted in Scheme 1.

Because cross-linked polyacrylamide gel is insoluble in water, to prove that acrylamide could be grafted to the MAH-chitosan beads, only acrylamide, and no cross-linker (MBA) was added to the reaction system, for polyacrylamide could be dissolved in water and the cross-linked polyacrylamide could not. Structure changes of the cross-linked chitosan and its derivatives were confirmed by IR spectra (Fig. 1). The IR spectra for MAH-Cs (Fig. 1(2)) showed new peaks at 1704, 1658, and 1569 cm^{-1}, which were attributed to the maleic acid that reacted with the amino groups of the chitosan beads: the peak at 1569 cm^{-1} was indicative of carbon–carbon double bonds; carbonyl groups and carboxylic groups were confirmed by the adsorptions at 1658 and 1704 cm^{-1}, respectively. The strong peak of 1667 cm^{-1} of Fig. 1(3)—polyacrylamide grafted chitosan beads—showed the carbonyl groups of amides. Thus the conclusion can be drawn that acrylamide could be grafted to the MAH modified chitosan beads.

It can be seen from Table 1 that after imprinting, the mechanical strength of the MIP beads were improved. The reason can be seen from Fig. 2, which indicates that the pores on the surfaces of the MIP bead which used a chitosan bead as matrix had become smaller. That means the pores of the chitosan beads were filled up by the polyacrylamide gel and formed a two-phase structure comprising the rigid chitosan and the soft polyacrylamide gel. The existence of the rigid chitosan beads endowed the strength of the MIP beads, and the soft polyacrylamide gel can be the area of the stress concentration. Thus the mechanical property was better than either the cross-linked chitosan beads or the polyacrylamide gel on their own.



Scheme 1. (a) maleic anhydride; (b) acrylamide; (c) *N,N'*-methylenebisacrylamide.

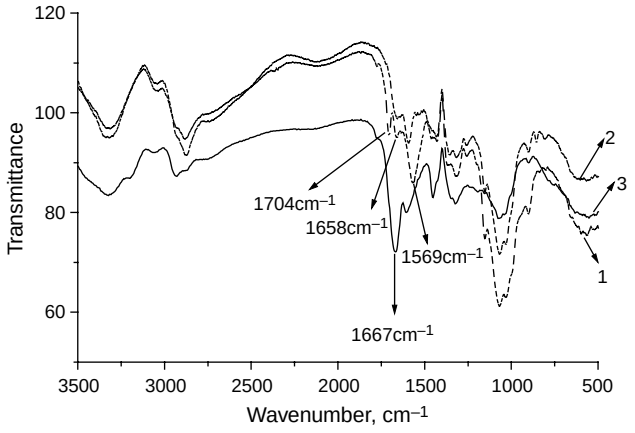


Fig. 1. IR spectra of (1) cross-linked chitosan beads; (2) MAH-Cs beads; (3) polyacrylamide grafted beads.

Table 1
Mechanical strength of the beads

Bead Type	Crumpling ratio % for stirring speed (r min^{-1}) of			
	200	400	600	800
Chitosan beads	4	34	40	52
Polyacryl-amide beads	0	4	28	36
MIP beads	0	0	1	5

3.2. Adsorptive characteristics of MIP

3.2.1. The adsorption dynamic of Hb on MIP

The adsorption dynamic of Hb on MIP was carried out using 0.5 g wet MIP beads. The profile is shown in Fig. 3.

It can be seen that sharp increase occurred in the first 6 h, but beyond this point, the adsorbance increased slowly. After about 17 h from the start, the adsorption process reached equilibrium. A relevant graph of ln (C_0/C) vs. t gives two linear plots bisecting around 6 h (Fig. 4). Obviously, the first part of the kinetic plot represents the Hb molecule first adsorbed on the surface of the MIP beads, the second part of the plot with a smaller slope indicates slow adsorption rates. In other words, the Hb adsorption was fast at the beginning, but after the adsorption on to the surface, the penetration of Hb molecule into the MIP beads becomes much more difficult.

3.2.2. Adsorption isotherm

In order to investigate the binding performance of the MIP, its binding isotherm was determined in the 0.2–0.8 mg/ml range of initial concentration of Hb (Fig. 5).

In this range, the binding data obtained were dealt with by linear regression analysis according to the Langmuir adsorption equation:

$$C_e/Q_e = C_e/Q_{max} + 1/bQ_{max}$$

where C_e is the equilibrium or final concentration of Hb (mg/ml), Q_e is the adsorption capacity of Hb adsorbed per unit weight of MIP at equilibrium concentration (mg/g), Q_{max} is the maximum adsorption capacity (mg/g) and b is the Langmuir adsorption equilibrium constant (g/ml). The Langmuir regression equation obtained is $C_e/Q_e = 0.019 + 0.028C_e$, this is the change of the form of Langmuir adsorption equation: from $Q_e = (Q_{max}bC_e)/(1 + bC_e)$ to $C_e/Q_e = C_e/Q_{max} + 1/bQ_{max}$. This is a C_e/Q_e vs. C_e equation and the correlation coefficient $r = 0.9989$ (Fig. 6). This indicates that the Langmuir plot is a line, the slope and intercept of which are equal to Q_{max} and $1/bQ_{max}$, respectively, and that the binding sites in MIP are homogeneous in respect to the affinity for Hb, which were formed due to template effect in the imprinting process, and the non-specific adsorption to MIP could be assumed to be small enough to ignore in this concentration range. The b and Q_{max} values can be calculated to be 1.47 and 35.7 mg/g of wet polymer, from the slope and the intercept of the Langmuir regression equation, respectively.

3.2.3. Freundlich isotherm

The well known Freundlich isotherm used for isothermal adsorption is a special case for heterogeneous surface energy in which the energy term in the Langmuir equation varies as a function of surface coverage strictly due to variation of the sorption. The Freundlich equation is

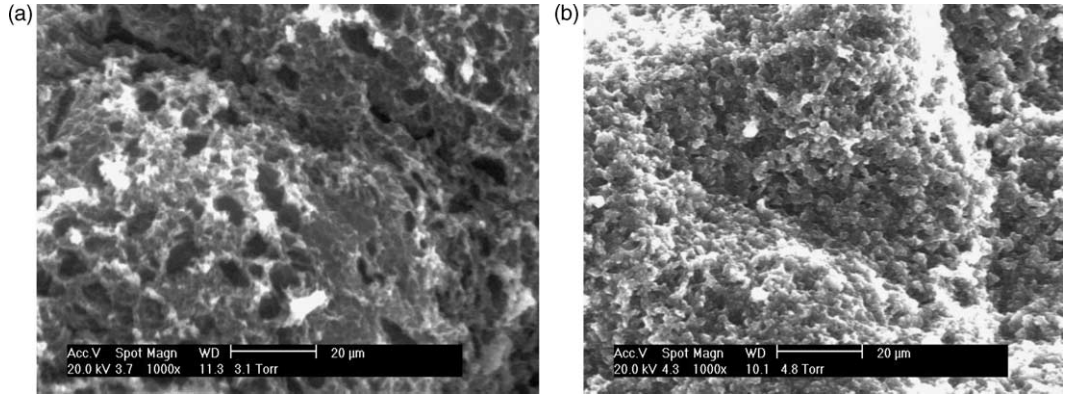


Fig. 2. (a) ESEM microphotograph of cross-linked chitosan bead (×1000 times); (b) ESEM microphotograph of MIP bead (×1000 times).

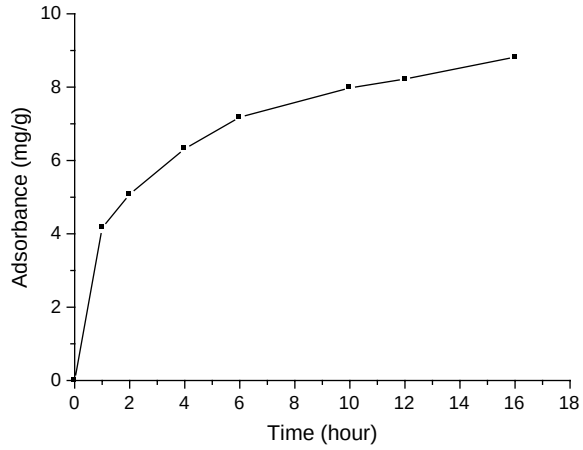


Fig. 3. Influence of adsorbing time to the adsorption capacity $V=25$ ml; $C=0.4$ mg/ml; sample quantity: 0.5 g; $T=25$ °C.

given as:

$$Q_e = Q_f C_e^{1/n}$$

where Q_f is roughly an indicator of the adsorption capacity and $1/n$ is the adsorption intensity, Q_f and $1/n$ can be determined from the linear plot of $\ln Q_e$ vs. $\ln C_e$. The obtained Freundlich regression equation is $\ln Q_e = 3.26 + 0.75 \ln C_e$, correlation coefficient $r=0.9974$. The n and Q_f values can be calculated to be 1.47 and 36.6 mg/g of wet polymer from the slope and the intercept of the Freundlich regression equation, respectively. The magnitude of the exponent $1/n$ gives an indication of the favorability of the adsorption. The value, $n>1$ represents a favorable adsorption condition for Hb adsorption on the MIP beads, and the high correlation coefficients ($r=0.9974$) showed that the Freundlich isotherm agrees well with experimental data also.

3.3. The degree of specificity

The special selectivity test of MIP was carried out using BSA as a reference substrate. Wet MIP and NIP beads (0.5 g each) were put to into 1.0 mg/ml Hb and BSA solutions (10 ml each), respectively, the condition was the same as that of the experiments for adsorption isotherms. The adsorption capacities of Hb and BSA on MIP and NIP, respectively were determined by the equilibrium adsorption method. The distribution coefficient K_D is defined as

$$K_D = C_p/C_s$$

where C_p is the concentration of protein on the MIP (in mg/g) and C_s the concentration of protein in the solution (in mg/ml).

The MIP exhibited high selectivity for Hb compared to BSA, the K_D of which for Hb and BSA were 23.2 and 1.53 ml/g, respectively. However, NIP exhibited too low values of K_D for Hb and BSA to be detected. The evidence indicates that the imprinting method creates a microenvironment based on shape selection and position

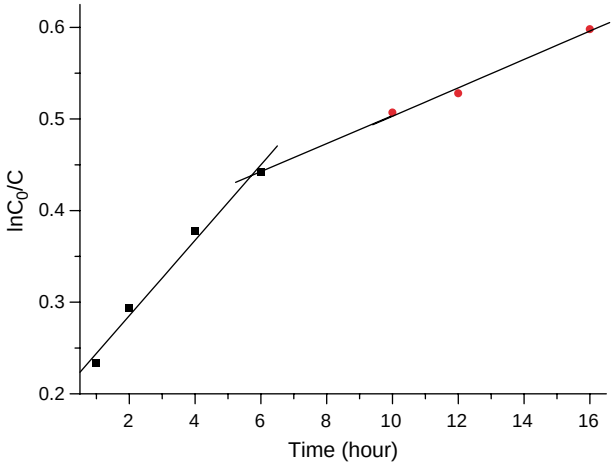


Fig. 4. Adsorption dynamics of the MIP beads.

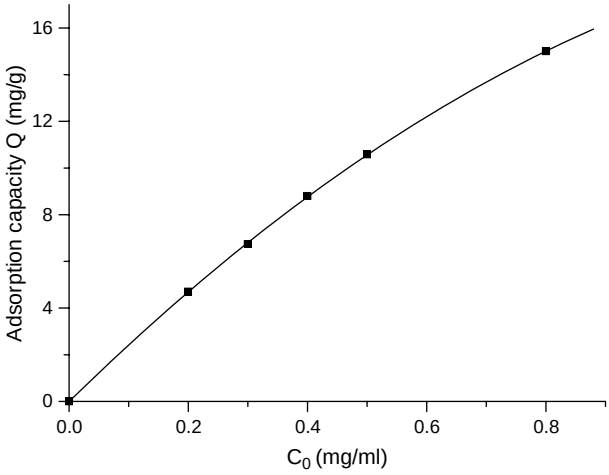


Fig. 5. Adsorption isotherms of Hb on MIP beads $V=25$ ml; $C=0.2$–0.8 mg/ml; sample quantity: 0.5 g; $T=25$ °C; adsorption time = 16–17 h.

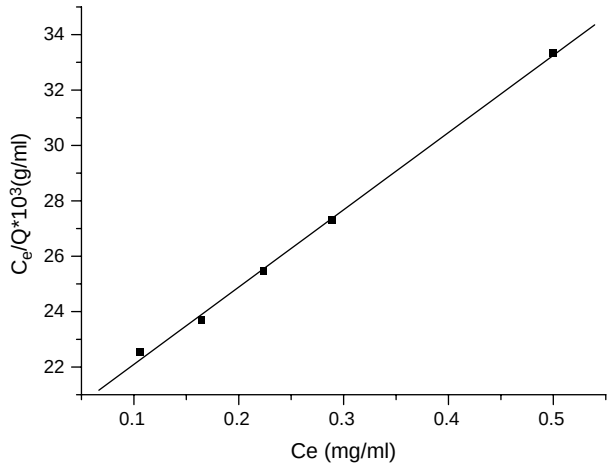


Fig. 6. Adsorption isotherms of Hb on MIP beads, linearized according to the Langmuir equation. $V=25$ ml; $C=0.2$–0.8 mg/ml; the sample quantity: 0.5 g; $T=25$ °C; adsorption time = 16–17 h.

Table 2
Reproducibility of MIP beads

Hb initial concentration	Adsorption capacity (mg/g)		
	The first time	The second time	Reproducibility (%)
0.4 (mg/ml)	8.36	7.44	95.2
1.0 (mg/ml)	20.4	19.34	91.6

of functional groups that recognizes the Hb template molecule.

Though hemoglobin and bovine serum albumin are both global proteins, Hb is a tetrameric protein composed of pairs of two different polypeptides and has a biconcave shape, whereas BSA consists of one polypeptide and has an ellipsoidal shape. Since the cavities formed of MIP are matched to the size of Hb, it is very difficult for the molecules with other dimensions to enter the cavities, therefore the distribution coefficient of MIP to BSA is lower correspondingly.

3.4. Desorption and reproducibility

Desorption studies will help to elucidate the nature of the adsorption process and to regenerate the MIP beads so that they can be used again. 10% v/v Acetic acid containing 10% w/v dodecyl sodium sulfate is used to desorb the Hb, then the beads were washed with 0.01 M sodium phosphate buffer pH 6.8 until the pH of the effluent reached 6.8 and there was no precipitate of dodecyl sulfate detectable upon addition of potassium chloride. In order to study the stability and recovery of the MIP, two cycles of adsorption/elution/regeneration were performed at different concentrations of hemoglobin. The results (Table 2) indicated that the MIP beads are very stable, and maintain their adsorption capacity at almost constant value.

4. Conclusion

In the present study, a novel method of polyacrylamide gel grafting to MAH-modified chitosan beads was developed to prepare Hb-imprinted polymer. The mechanical strength of the selective polyacrylamide gel could be improved as the MAH modified chitosan beads were used as matrix. The Hb-imprinted polymer prepared by molecular imprinting exhibited high selective adsorption for Hb in a molecular recognition process and Hb could be effectively adsorbed. Therefore such a MIP can be used to remove the protein from different solutions, and it is also promising for use in the area of biosensor materials.

Acknowledgements

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