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Preparation of surface molecularly imprinted polymeric microspheres and their recognition property for basic protein lysozyme

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ABSTRACT

The surface imprinting of basic protein lysozyme (Lys) was carried out by designing a new route. The copolymerization of N-vinylpyrrolidone (NVP) and 2-hydroxyethyl methacrylate (HEMA) was first conducted in an inverse suspension polymerization system, and the crosslinked copolymeric microspheres HEMA/NVP were prepared. Subsequently, the esterification reaction of methacryloyl (MAO) chloride with the hydroxyl groups on the surfaces of HEMA/NVP microspheres was performed, and the modified microspheres MAO–HEMA/NVP, on which a mass of polymerisable double bonds were introduced, were obtained. In the presence of lysozyme, by initiating of $K_2S_2O_8$–$NaHSO_3$, the monomer methacrylic acid (MAA) in the solution was crosslink-polymerized on the surfaces of MAO–HEMA/NVP microspheres, resulting in the surface imprinting of lysozyme. After removing the template molecules, the lysozyme molecule-surface-imprinted material MIP-HEMA/NVP was obtained. Because there were strong interactions between lysozyme and monomer MAA, electrostatic interaction and hydrogen bonding, the lysozyme molecule-surface imprinting was successfully realized. The MIP-HEMA/NVP microspheres have very high binding affinity for lysozyme, and the binding capacity gets up to 216 mg/g. It is more important that MIP-HEMA/NVP microspheres have specific recognition selectivity for lysozyme, and the selectivity coefficient for lysozyme with respect to bovine hemoglobin (BHb), which was used as a contrast protein in the experiments, actually reaches 31.07. In the respect of protein imprinting, the imprinting material with such high performance is unwonted.

1. Introduction

Since low-density lipoproteins (LDL) is the main carrier of cholesterol in the circulation and cholesterol deposition is one of the factors equated with the onset of coronary disease [1,2]. LDL is recognized as a major risk factor for the development of atherosclerosis and coronary heart diseases. At present, hemoperfusion treatment for familial hyperlipidemia (FH) and serious cardiovascular diseases has been developed as an effective means to reduce LDL in patient's plasma concentration which is difficult to achieve by diet and drugs [3,4]. The key problem of developing the hemoperfusion technique to eliminate LDL from blood is to prepare adsorbents with high performance including high adsorption capacity for LDL and biocompatibility. There are positively charged amino acid residues of lysine and arginine on the surface of LDL, and LDL is a basic protein carrying positive charge. Therefore, the polymeric materials bearing carboxyl groups or sulfonic acid groups have strong adsorption properties towards LDL [5–10]. Thus, it is a promising route for preparing high effective adsorbents for LDL apheresis to try to make carboxyl groups or sulfonic acid groups to be chemically attached to the surfaces of the biocompatible polymeric matrix materials.

Polyvinylpyrrolidone (PVP) and poly(hydroxyethyl methacrylate) (PHEMA) are two kinds of polymeric materials with fine biocompatibility or rather with blood compatibility [11,12], and they have important applications in various biotechnology areas such as tissue engineering, controlled drug release, separation of biomacromolecules as well as biosensor [13–16]. In this work, we first prepared the crosslinked copolymer microspheres (HEMA/NVP) of 2-hydroxyethyl methacrylate (HEMA) and N-vinylpyrrolidone (NVP) via inverse suspension polymerization. The microspheres HEMA/NVP have biocompatibility probably [17,18]. The main aim of this work is to prepare the adsorption material with high performance towards basic proteins with HEMA/NVP as matrix microspheres and by introducing carboxyl groups onto the surface of HEMA/NVP microspheres. As a matter of convenience, we used lysozyme as a basic protein model, or as an analogous of LDL, investigated the adsorption property of the prepared adsorption material towards basic proteins.

For common adsorption materials, the specific adsorption selectivity for the target substance is always absent. There are two kinds of solid adsorbents with high adsorption selectivity for the

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target substance, and they are molecularly imprinted materials and immunoadsorbents. Molecularly imprinted polymers (MIPs) are an artificially synthesized macromolecular material, in which a great deal of specific cavities designed for a target molecule (namely, the template molecule) is distributed and these cavities are complementary in shape, size and functional groups to the target molecule. Therefore, MIPs have specific molecular recognition ability and high binding affinity [19–22] for the target molecule, and are described as artificial antibodies or receptors. In recent years, MIPs as highly selective solid adsorbents have been widely used in various fields, especially in the separation and purification area. We conceive that molecularly imprinted materials can be introduced into blood purification treatment, and they will be able to effectively remove endogenous toxic molecules including baneful proteins from blood. Based on such consideration, we investigated and prepared lysozyme-imprinted material (lysozyme was used as analogous of LDL as described above) with HEMA/NVP as matrix microspheres and with mathacrylic acid as functional monomer by using a new surface imprinting method.

The conventional method to prepare MIPs, entrapment way, has some disadvantages, such as time-consuming and complicated preparation process, less recognition sites inside matrix particles obtained via crushing and grinding the imprinted polymeric monolith, and greater diffusion barrier for the template molecules coming from thick matrices, and these shortcomings lead to the poor binding capacity and lower binding kinetic rate of MIPs towards the template molecules. In order to overcome these drawbacks, many researchers are developing surface-molecular imprinting techniques [19,23–25], and try to place the imprinted caves on the surfaces of solid particles. The surface-imprinted materials are more effective for recognizing the template molecules than the imprinted materials prepared with the conventional imprinting method.

In the present investigation, a new surface imprinting method was designed, and surface imprinting of lysozyme was conducted with HEMA/NVP as matrix microspheres and with methacrylic acid as functional monomer, and lysozyme molecule-surface-imprinted material MIP-HEMA/NVP was prepared. The binding characteristic of MIP-HEMA/NVP for lysozyme was investigated in depth. The lysozyme-surface-imprinted material MIP-HEMA/NVP has biocompatibility probably because of the biocompatibility of the matrix microspheres, and furthermore, it is more important that the experimental result indicates that the surface-imprinted material possesses peculiar binding affinity and specific recognition selectivity for lysozyme. Based on the above result, it can be expected that the LDL-surface-imprinted material with high performance can also be obtained with HEMA/NVP as matrix microspheres and with mathacrylic acid as functional monomer via surface-molecular imprinting. Such materials are promising and potential in blood purification treatment. So this investigation result is beneficial for the designing and preparing of new and more efficient LDL adsorbents for hemoperfusion treatment.

2. Experimental

2.1. Materials and instruments

N-vinylpyrrolidone (NPV, Dexiang Medicine Inc., Shanghai, China) was of analytical grade, and was purified by distillation under vacuum prior to use. 2-Hydroxyethyl methacrylate (HEMA, The Tianjin Chemical Reagent Institute, Tianjin City, China) was of analytical grade, and was purified by distillation under vacuum prior to use. *N,N′*-Methylenebisacrylamide (MBA, Xiangzhong Fine Chemical Plant, Province Hunan, China) was of chemical grade. Liquid paraffin (Tianjing BASF Chemical Company, Tianjing City, China) was of chemical grade. Ammonium persulphate (APS, Fushu Chemical Engineering Inc., Shanghai, China) was of analytical grade. Sorbitan monostearate (Span-60, Tianda Chemical Reagent Plant, Tianjin City, China) was of chemical grade. Methacrylic acid (MAA, Ruijinte Chemical Company, Tianjin City, China) was of analytical grade and was purified by distillation under vacuum prior to use. Ammonium persulphate (APS, Fushu Chemical Engineering Inc., Shanghai, China) was of analytical grade. Sodium bisulfite (Fushu Chemical Engineering Inc., Shanghai) was of analytical grade. Lysozyme (Lyz, Hangzhou Chunlei Feed Science and Technology Inc., Hangzhou City, China) was of chemical grade. Bovine hemoglobin (BHb, Xueman Biotechnology Inc., Shanghai, China) was of chemical grade. Other reagents were all commercial chemicals with analytical grade and purchased from Chinese companies.

The instruments used in this study were as follows: PerkinElmer 1700 infrared spectrometer (FTIR, PerkinElmer Company, USA), Unic-2602 UV/Vis spectrophotometer (Unic Company, Shanghai, China), LEO-438VP scanning electronic microscope (SEM, LEO Company, UK), and THZ-92C constant temperature shaker equipped with gas bath (Boxun Medical Treatment Equipment Factory, Shanghai, China).

2.2. Preparation of crosslinked microspheres HEMA/NVP

According to the procedure described in Ref. [26], inverse suspension polymerization method was used for the preparation of crosslinked microspheres HEMA/NVP, and the typical process was as follows. The continuous phase (oil phase) was consisted of 60 mL of liquid paraffin containing 1.30 g of oil-soluble surfactant Span-60 that was used as suspension stabilizer. The oil phase was poured into a four-necked flask equipped with a mechanical stirrer, a reflux condenser and a N_2 inlet. HEMA (2 mL), NVP (2 mL) and redistilled water (2.3 mL) were mixed and dissolved each other, 0.27 g of MBA was dissolved in this solution, and the dispersed phase (water phase) was constituted. After the oil phase was heated to 35 °C, the water phase was added, and the mixture was stirred and sufficiently dispersed for 30 min, while N_2 was bubbled to exclude air. Then the mixture was heated to 65 °C with stirring, and 0.0213 g of APS was added. Under nitrogen atmosphere and with stirring at 450 rpm of rate, the crosslinking copolymerization was carried out at a constant temperature of 65 °C for 8 h. Transparent microspheres with a mean diameter of 90 μm were obtained. The resultant microspheres were washed repeatedly with petroleum ether and acetone, dried under vacuum at 50 °C for 24 h, obtaining the crosslinked microsphers HEAM/NVP. The chemical structure of the microspheres was characterized by FTIR, and the morphology and size were examined by SEM.

2.3. Introducing polymerisable double bonds onto HEMA/NVP microspheres

Methacryloyl chloride was synthesized via the reaction of methacrylic acid with thionyl chloride. The crosslinked microspheres HEAM/NVP (2 g) were added into a reactor, followed by adding 40 mL of acetone. The microspheres were allowed to soaked and swelled fully for 12 h. After adding 2 mL of methacryloyl chloride, the temperature of the content in the reactor was slowly heated to 40 °C, a little of Na_2CO_3 as capturing acid reagent was added, and the surface modification reaction of HEAM/NVP microspheres was allowed to be carried out at the constant temperature of 40 °C for 12 h. After finishing the reaction, the resultant microspheres were washed with distilled water repeatedly, dried under vacuum, and the modified microspheres MAO–HEMA/NVP, on which methacryloyl (MAO) groups were bound, were obtained. The content of the polymerisable double bonds on the surfaces of MAO–HEMA/NVP microspheres were determined with KBr–$KBrO_3$

method. The MAO–HEMA/NVP microspheres prepared and used in this study had a double bond content of 1.12 mmol/g. The infrared spectrum of MAO–HEMA/NVP microspheres was determined with KBr pellet method to confirm the bonding of methacryloyl (MAO) groups.

2.4. Preparing lysozyme molecule-surface-imprinted material MIP-HEMA/NVP

Lysozyme (0.14 g), MAA (3.5 g) and MBA (0.3 g) were together dissolved in 70 mL of a buffer solution with pH 9, and these components in the mixed solution were allowed to interact for 4 h. The mixed solution was transferred into a four-necked flask equipped with a reflux condenser, a mechanical agitator and a N_2 inlet, followed by adding 1 g of the modified microspheres MAO–HEMA/NVP. After soaking and swelling the microspheres MAO–HEMA/NVP for 12 h, 20 mg of APS and 10 mg of $NaHSO_3$ were added into the flask, and the crosslinking polymerization of MMA was conducted at a constant temperature of 30 °C under N_2 atmosphere with stirring for 12 h. After finishing the polymerization, the resultant microspheres were filtered out, and soaked and washed with $NaHCO_3$ solution to elute the template molecules, lysozyme, until without lysozyme in the eluent (detected by UV spectrophotometry). The product microspheres were again washed with distilled water repeatedly, and dried under vacuum, resulting in the lysozyme molecule-surface-imprinted microspheres MIP-HEMA/NVP. The infrared spectrum of MIP-HEMA/NVP microspheres was determined to confirm their structure.

In the absence of lysozyme, the crosslink-polymerization of MMA was also conducted on the surface of the modified microspheres MAO–HEMA/NVP, and the blank microspheres CPMAA-HEMA/NVP, on which crosslinked polymethacrylic acid (CPMAA) was only chemically attached, but there were no imprinted caves of lysozyme, were obtained. Apparently, the microspheres CPMAA-HEMA/NVP are a non-imprinted material in comparison with the microspheres MIP-HEMA/NVP.

2.5. Investigating binding characteristic of MIP-HEMA/NVP for lysozyme

2.5.1. Evaluating binding property of MIP-HEMA/NVP

The binding behavior of MIP-HEMA/NVP for lysozyme was examined with batch method (static method) and column method (dynamic method), respectively.

2.5.1.1. Batch method. Based on the determination of the binding dynamics behavior of MIP-HEMA/NVP for lysozyme (the equilibrium binding time was 5 h), the combining isothermal experiments were carried out. Numbers of 50 mL of lysozyme solutions with different concentrations in a range of 0.4–3 mg/mL were transferred into a number of conical flasks, respectively. The MIP-HEMA/NVP microspheres weighted accurately (about 0.2 g) were added into these solutions, respectively. These mixtures were shaken for 5 h on a constant temperature shaker at 30 °C, centrifugalized after reaching binding equilibrium, and the equilibrium concentrations of lysozyme in the supernatants were determined with UV spectrophotometry at 280 nm. The equilibrium binding amounts of MIP-HEMA/NVP towards lysozyme was calculated according to Eq. (1), and the binding isotherm was plotted.

$$Q_e = \frac{V(C_0 - C_e)}{m} \quad (1)$$

where Q_e (mg g^{-1}) was the equilibrium binding amount of lysozyme, C_0 and C_e (mg/mL) were the initial and final concentrations of lysozyme, respectively, V (mL) was the volume of the lysozyme solution, and m (g) was the mass of MIP-HEMA/NVP microspheres.

2.5.1.2. Column method. For the column method, the experimental procedures are explained as follows. MIP-HEMA/NVP microspheres (0.984 g) were packed into a piece of glass pipe with an internal diameter of 1.0 cm, and the bed volume (BV) of the packed column was 2 mL. The lysozyme solution with a concentration of 1.0 mg/mL was allowed to gradually flow through the packed column at a rate of three bed volumes per hour (3 BV/h) in a countercurrent manner. The effluents with two bed volume (2 BV) interval were collected, respectively, and the lysozyme concentrations in these effluents were determined with spectrophotometry. The dynamic binding curve was plotted, and the leaking adsorption amount and saturated adsorption amount of lysozyme were calculated with the data of the lysozyme concentrations and bed number of those effluents, respectively.

2.5.2. Selectivity experiments

In this work, bovine hemoglobin (BHb) was selected as a contrast protein to examine the recognition selectivity of MIP-HEMA/NVP microspheres towards lysozyme. For the sake of comparing the binding selectivity of MIP-HEMA/NVP for lysozyme, the static binding isotherm and dynamic binding curve of MIP-HEMA/NVP for BHb as well as that of CPMAA-HEMA/NVP for BHb were also determined with spectrophotometry at 405 nm, separately.

In order to further show the binding specificity of MIP-HEMA/NVP for lysozyme, the competitive adsorption of lysozyme with respect to BHb was studied. A binary mixed solution of BHb/Lys was prepared, and the concentrations of the two proteins in the mixed solution were the same (2 mg/mL). 0.2 g of MIP-HEMA/NVP was added into 50 mL of the mixed solution, and the static adsorption experiments were performed. After binding equilibriums were reached, the concentrations of two proteins in the supernatant were determined with spectrophotometry. In the concentration determination, the two-band method was used, and the two bands were selected as 405 and 280 nm. The distribution coefficient for each substance was calculated according to Eq. (2), and this equation was originated from Ref. [27].

$$K_d = \frac{Q_e}{C_e} \quad (2)$$

where K_d represents the distribution coefficient (ml/g), Q_e (mg/g) is the equilibrium binding amount, and C_e (mg/mL) is the equilibrium concentration.

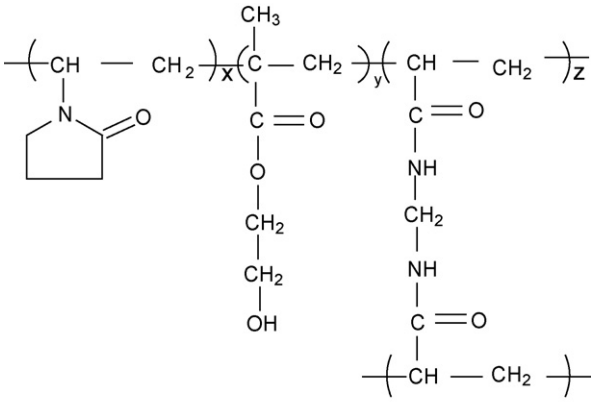
The selectivity coefficient k of MIP-HEMA/NVP for lysozyme with respect to the competition species, BHb, can be obtained from the equilibrium binding data according to Eq. (3), and it can reveal adequately the recognition selectivity of MIP-HEMA/NVP towards lysozyme.

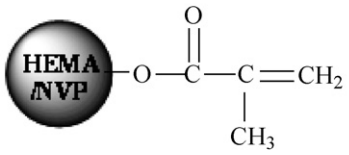
$$k = \frac{K_d(\text{Lys})}{K_d(\text{BHb})} \quad (3)$$

where k is the selectivity coefficient, and the value of k allows an estimation of selectivity of MIP-HEMA/NVP for lysozyme. A relative selectivity coefficient k' is also defined as expressed in Eq. (4) [27], and the value of k' can reveal the enhanced extent of the adsorption affinity and selectivity of the imprinted material MIP-HEMA/NVP towards the template molecule with respect to the non-imprinted material CPMAA-HEMA/NVP.

$$k' = \frac{k_{impr}}{k_{non\text{-}impr}} \quad (4)$$

where k_{impr} is the selectivity coefficient of MIP-HEMA/NVP for lysozyme with respect to the competition species, BHb, and





Scheme 1. Schematic expression of chemical structures of HEMA/NVP and MAO–HEMA/NVP microspheres.

$k_{non\text{-}impr}$ is the selectivity coefficient of CPMAA-HEMA/NVP for lysozyme also with respect to BHb.

2.6. Desorption experiment

A certain amount of MIP-HEMA/NVP microspheres adsorbing lysozyme in a saturation state was packed into a piece of glass pipe with an internal diameter of 1.0 cm, and the bed volume (BV) of the packed column was 2 mL. An aqueous solution of $NaHCO_3$ with a concentration of 0.2 M was used as eluent, and was allowed to gradually flow through the column at a rate of three bed volumes per hour (3 BV/h) in a countercurrent manner. The effluents with two volume (2 BV) interval were collected, respectively, and the lysozyme concentrations of these effluents were determined with spectrophotometry. The dynamic desorption curve was plotted, and the elution property of MIP-HEMA/NVP was evaluated.

3. Results and discussions

3.1. Chemical structure and morphology of HEMA/NVP and MAO–HEMA/NVP microspheres

Inverse suspension polymerization was first carried out with two monomers, HEMA and NVP, and with MBA as crosslinker, resulting in the crosslinked microspheres HEMA/NVP. Subsequently, the esterification reaction of the hydroxyl groups existing on HEMA/NVP microspheres with methyacryloyl chloride was conducted, leading to the introduction of the polymerisable double bonds (methylacryloyl groups) onto the surfaces of the HEMA/NVP microspheres and forming the modified microspheres MAO–HEMA/NVP. The chemical structures of HEMA/NVP and MAO–HEMA/NVP microspheres can be schematically represented in Scheme 1, whereas Fig. 1 gives the infrared spectra of the crosslinked microspheres HEMA/NVP and the modified microspheres MAO–HEMA/NVP. Because the spectrum of the

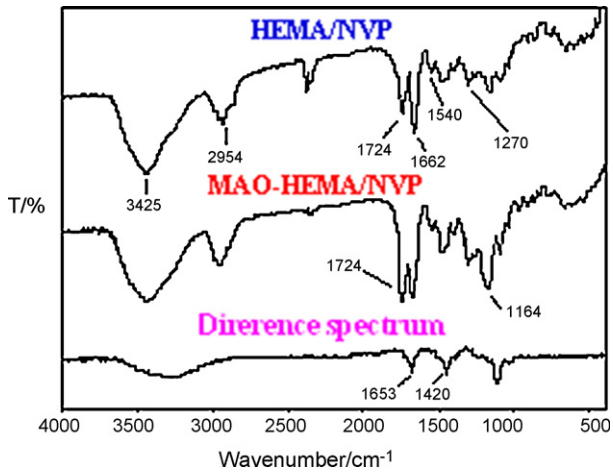


Fig. 1. Infrared spectra of HEMA/NVP and MAO–HEMA/NVP microspheres as well as difference spectrum.

matrix of MAO–HEMA/NVP is identical with that of HEMA/NVP microspheres, the difference spectrum of MAO–HEMA/NVP and HEMA/NVP microspheres was also determined to more clearly confirm the structure of MAO–HEMA/NVP, and it is also given in Fig. 1.

In the spectrum of HEMA/NVP, the characteristic absorption bands of the three kinds of monomer units, HEMA, NVP and MBA, are all displayed. The bands at 1270 and 1662 cm^{-1} are attributed to the characteristic absorptions of C–N bond and C=O bond in NVP unit, respectively; the bands at 1724, 1162 and 3425 cm^{-1} are ascribed to the characteristic absorptions of C=O bond of ester carbonyl group, C–O–C bond of ester group and O–H bond of hydroxyl group of β-hydroxyl groups in HEMA unit, respectively; the band at 1540 cm^{-1} indicates the characteristic absorption of C=O bond of amido carbonyl groups in MBA unit, and the absorption band of $–NH_2$ group of amido groups in MBA unit is also located at about 3425 cm^{-1}, namely it is overlapped by that of O–H bond of HEMA unit to a certain extent; the band at 2954 cm^{-1} represents the characteristic absorption of methyl group $–CH_3$ and methylene group $–CH_2–$ in the main chains of HEMA/NVP.

In the spectrum of the modified microspheres MAO–HEMA/NVP, the band at 1724 cm^{-1} and the band at 1162 cm^{-1}, which represent the characteristic adsorption C=O bond of ester carbonyl group and C–O–C bond of ester group,

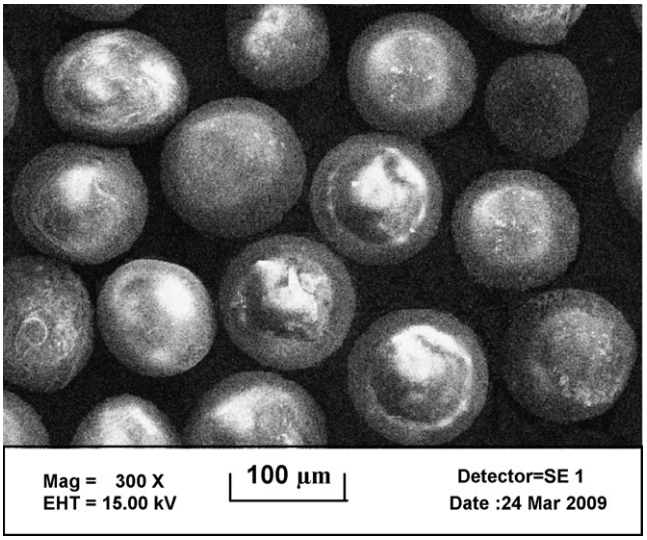


Fig. 2. SEM images of HEMA/NVP microspheres.

respectively, have been strengthened obviously because of the forming of new ester groups.

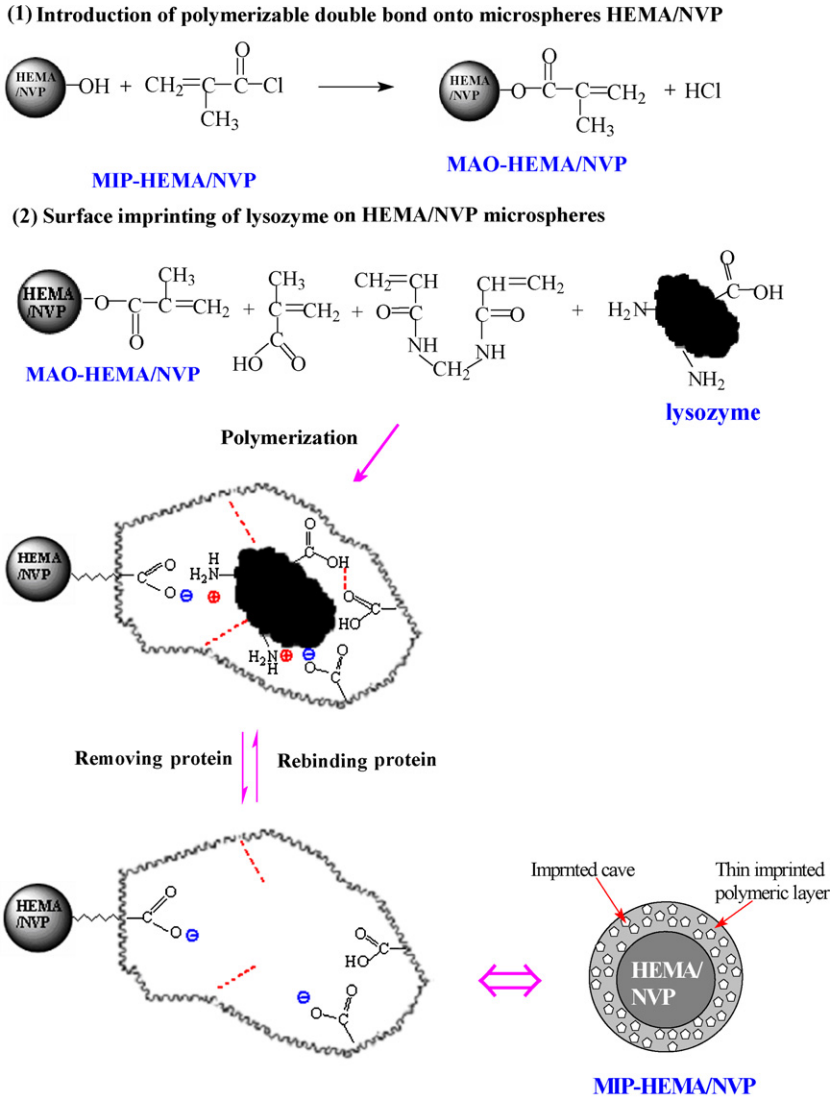
In the spectrum of MAO–HEMA/NVP, the characteristic absorption band of double bond of methacryloyl groups of MAO–HEMA/NVP is covered up and the double bond existence cannot be confirmed. Whereas in the difference spectrum, the characteristic absorption of the double bond of methacryloyl (MAO) groups has been exposed at 1653 cm^{-1}, and the band at 1420 cm^{-1} should be attributed to the bending vibration absorption of =C–H bond of MAO groups.

The above results show that the esterification reaction of the hydroxyl groups on HEMA/NVP microspheres with methacryloyl chloride has occurred, and the modified microspheres MAO–HEMA/NVP have been formed.

Fig. 2 presents the SEM image of the crosslinked microspheres HEMA/NVP. It can be found that the sphericity of the microspheres prepared by inverse suspension polymerization method is excellent, and the grain size is well-proportioned.

3.2. Preparing processes and structure of MIP-HEMA/NVP microspheres

It was confirmed in our previous study that there were strong interactions between polymethacrylic acid (PMAA) and lysozyme molecules, and they were electrostatic interaction and hydrogen bonding as well as hydrophobic interaction [28]. The electrostatic interaction was originated from the attract force of the dissociated carboxyl group of PMAA with the negative charge towards lysozyme molecules, which is a basic protein and in a general pH range is positively charged, whereas the hydrogen bonding was formed by the interaction between those carboxyl groups of PMAA and the amino acid residues of lysozyme such as amino groups. It is certain that the two strong interactions also exist between monomer MAA and lysozyme molecules, and such interactions also were confirmed by other investigations [29,30]. In the solution of MAA and lysozyme, which was used as template, many MAA molecules will be combined around every lysozyme molecule via electrostatic interaction and hydrogen bonding. On the other hand, a great number of polymerisable double bonds exist on the surfaces of the modified microspheres MAO–HEMA/NVP. In this way, by initiating of APS/$NaHSO_3$, the monomers MAA combined with template molecules in the solution are crosslink-polymerized on the surfaces of MAO–HEMA/NVP microspheres at a lower temperature of 30 °C (so that the protein denaturation was avoided during the imprinting process), resulting in the realization of lysozyme imprinting. After washing away the template molecules, lysozyme molecule-surface-imprinted microspheres MIP-HEMA/NVP are obtained. On the surface of MIP-HEMA/NVP



Scheme 2. Schematic expression of preparation process of MIP-HEMA/NVP.

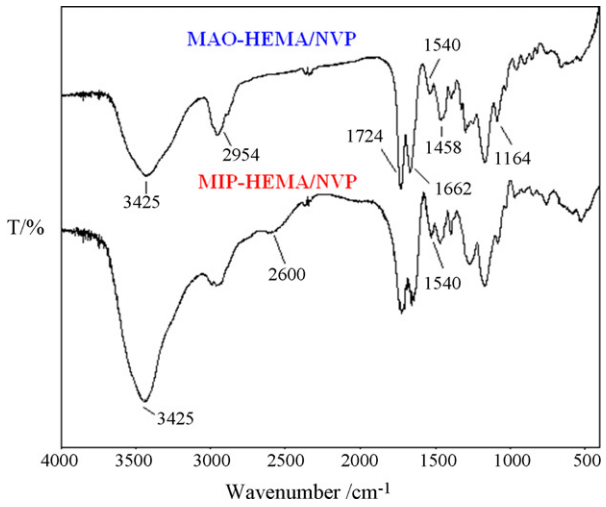


Fig. 3. Infrared spectra of MAO–HEMA/NVP and MIP-HEMA/NVP microspheres.

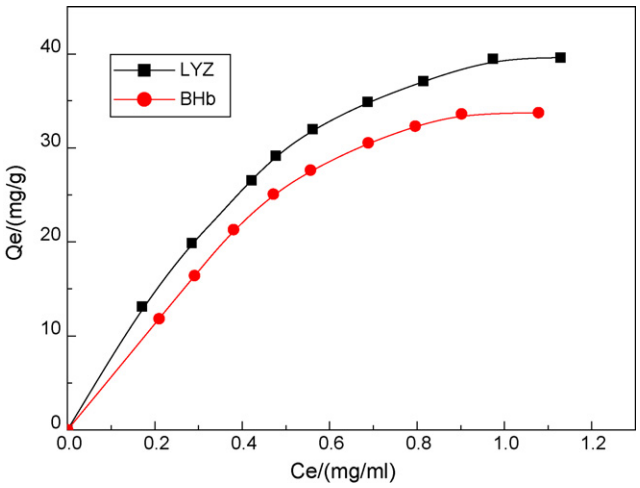


Fig. 4. Adsorption isotherms of CPMAA-HEMA/NVP for lysozyme and BHb. Temperature: 30 °C; pH 9.

microspheres, there is a thin polymeric film, within which large numbers of lysozyme-imprinted caves is distributed, and apparently, the MIP-HEMA/NVP microspheres is a surface imprinting material. The entire chemical process of preparing MIP-HEMA/NVP microspheres can be schematically expressed in Scheme 2.

Fig. 3 gives the infrared spectrum of MIP-HEMA/NVP, and for comparison, the infrared spectra of MAO–HEMA/NVP microspheres is also presented in Fig. 3. In the spectrum of MIP-HEMA/NVP, in comparison with that of MAO–HEMA/NVP, the band at 1540 cm^{-1} that is the characteristic absorption of C=O bond of amido carbonyl groups in MBA unit as shown in Fig. 1 is strengthened obviously due to the further incorporation of the crosslinker MBA units into MIP-HEMA/NVP during the imprinting process. At the same time, the band at about 3425 cm^{-1}, which is ascribed to the characteristic absorption of $-NH_2$ group of amido groups in MBA unit, is also strengthened with the same reason. In the infrared spectrum of MIP-HEMA/NVP, the adsorption bands of the carboxyl groups of the crosslinked polymethacrylic acid are covered up basically, but then, a band at about 2600 cm^{-1} has been appeared. This band should be corresponding to the synergism vibration absorption of C–O bond and O–H bond of the carboxyl groups of the crosslinked polymethacrylic acid.

The above spectrum changes reveal that the crosslink-polymerization of MAA has occurred on the surfaces of MAO–HEMA/NVP microspheres in the presence of lysozyme, and the molecularly imprinted material MIP-HEMA/NVP has been formed.

3.3. Binding characteristic of MIP-HEMA/NVP for lysozyme

3.3.1. Binding isotherms and dynamics binding curves

The isothermal adsorption experiments in the batch method were first performed, and Figs. 4 and 5 give the adsorption isotherms of CPMAA-HEMA/NVP (non-imprinting material) and the binding isotherms of MIP-HEMA/NVP (imprinting material) for lysozyme and BHb, respectively.

It can be seen from Fig. 4 that the adsorption capacities of CPMAA-HEMA/NVP for lysozyme and BHb are similar and the adsorption capacity difference is not notable, namely, CPMAA-HEMA/NVP microspheres have no adsorption selectivity for lysozyme. However, for the imprinted microspheres MIP-HEMA/NVP, a remarkable difference of the binding capacity for the two proteins is fully displayed, as shown in Fig. 5. The maximum binding amount of lysozyme gets up to 216 mg/g, showing the excellent binding affinity of MIP-HEMA/NVP for lysozyme. Such large and wonderful binding capacity is reported for the first time, and it is nearly 10–20 times higher than that of other lysozyme-imprinted materials previously reported [31–33], fully displaying the high efficiency of this novel surface imprinting method. In contrast, the corresponding binding amount of MIP-HEMA/NVP for BHb is only 32 mg/g, indicating that MIP-HEMA/NVP microspheres nearly do not bind and do not recognize BHb basically. The above experimental fact clearly demonstrates that the lysozyme-imprinted material MIP-HEMA/NVP has excellent binding behavior for the template lysozyme, very excellent binding affinity and very high recognition selectivity. This result also shows clearly that this surface-molecular imprinting technique designed in this investigation is very successful. By right of electrostatic interaction and hydrogen bonding between the functional monomer MAA and lysozyme molecule, the effective surface imprinting of lysozyme is realized on the surfaces of the modified microspheres MAO–HEMA/NVP.

In order to further study the binding characteristic of MIP-HEMA/NVP for lysozyme, adsorption experiments in the column method were also performed. Figs. 6 and 7 display the dynamic adsorption curves of CPMAA-HEMA/NVP and the dynamic binding curves of MIP-HEMA/NVP for lysozyme and BHb, respectively.

The following facts can be observed from Fig. 6. As the solutions of lysozyme and BHb with the same concentration flows

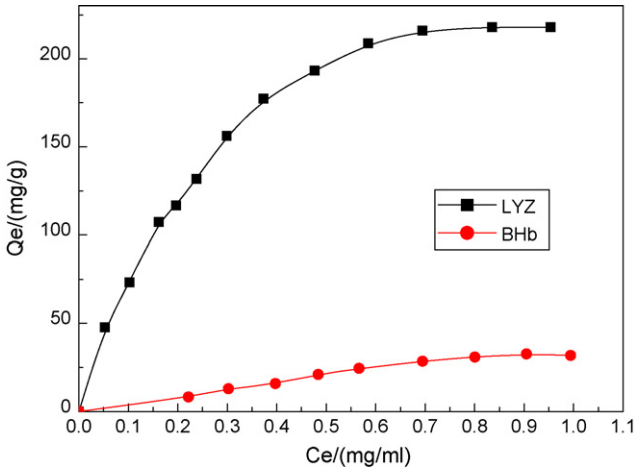


Fig. 5. Binding isotherms of MIP-HEMA/NVP for lysozyme and BHb. Temperature: 30 °C; pH 9.

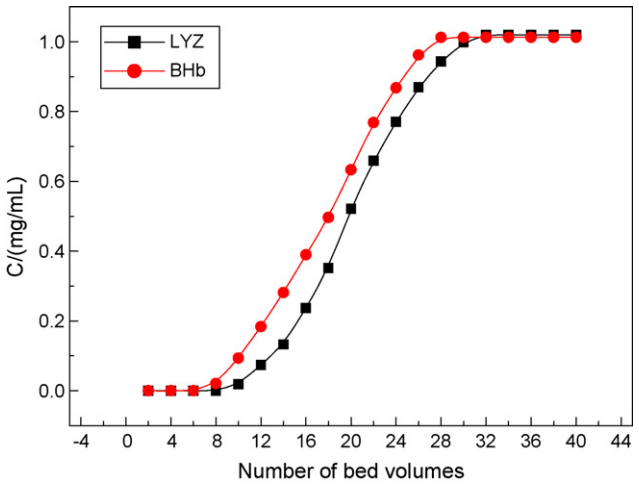


Fig. 6. Dynamic adsorption curves of CPMAA-HEMA/NVP for lysozyme and BHb. BV: 2 mL; temperature: 20 °C; initial concentration: 1 mg/mL; flow rate: 3 BV/h.

upstream through the column packed with CPMAA-HEMA/NVP microspheres, respectively, the leaking volumes are approximately identical and are about 10 BV and 8 BV, respectively. This fact again shows that CPMAA-HEMA/NVP has no adsorption selectivity for lysozyme. However, it is observed in Fig. 7 that the dynamic binding curve of MIP-HEMA/NVP for lysozyme is obviously different from that for BHb. The leaking volume of lysozyme is 84 BV and far greater than that of BHb (only about 6 BV). By calculating, the leaking and saturated adsorption amounts of lysozyme are 170 and 223.9 mg/g, respectively, whereas for BHb, they are only about 8.3 and 44 mg/g, respectively. Obviously, MIP-HEMA/NVP microspheres nearly do not recognize and do not bind BHb molecules. In contrast, they have excellent recognition selectivity for lysozyme, and it will be further discussed below.

3.3.2. *Recognition selectivity of MIP-HEMA/NVP for lysozyme*

Competitive adsorption experiments of MIP-HEMA/NVP microspheres were conduced in a binary solution of lysozyme and BHb. In Table 1, the data of the distribution coefficients K_d, selectivity coefficients k and the relative selectivity coefficient k' are summarized.

From the data in Table 1, the following facts can be found: (1) the selectivity coefficient of the non-imprinting material, CPMAA-HEMA/NVP, for lysozyme in relation to BHb is very low,

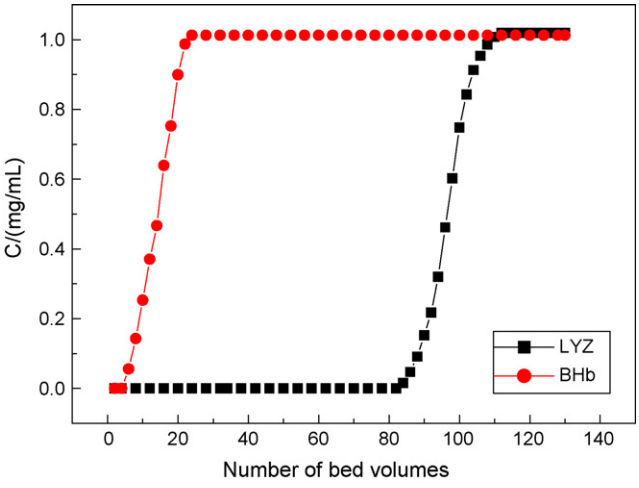


Fig. 7. Dynamic binding curves of MIP-HEMA/NVP for lysozyme and BHb. BV: 2 mL; temperature: 20 °C; initial concentration: 1 mg/mL; flow rate: 3 BV/h.

Table 1
Distribution coefficient and selectivity coefficient data.

	Adsorb material			
	CPMAA-HEMA/NVP		MIP-HEMA/NVP	
	Lys	BHb	Lys	BHb
K_d (mL/g)	42.412	35.27	661.859	21.302
k	1.202		31.07	
k'	25.849			

and is only 1.202. This implies that the adsorption abilities of CPMAA-HEMA/NVP for the two proteins, lysozyme and BHb, are approximate and the adsorption selectivity of CPMAA-HEMA/NVP microspheres (non-imprinted microspheres) for lysozyme is absent; (2) the selectivity coefficient of MIP-HEMA/NVP for lysozyme with respect to BHb is remarkably enhanced, and gets up to 31.07, displaying a very high recognition selectivity of MIP-HEMA/NVP for lysozyme; (3) the relative selectivity coefficient of MIP-HEMA/NVP is 25.85, indicating a remarkable enhancement of the binding affinity and selectivity of the imprinting material MIP-HEMA/NVP for the template molecule in relation to non-imprinting material CPMAA-HEMA/NVP. The above facts again clearly reveal that MIP-HEMA/NVP has high recognition selectivity and binding affinity for the template, lysozyme.

The reason for the above facts can be explained as follows. Although BHb is also one of globular proteins like as lysozyme, its molecule size, shape and the functional groups contained in BHb molecule are different from that of lysozyme molecule: lysozyme is a small protein with a molecular weight of 14,000, whereas the molecular weight of BHb is 64,500, suggesting that BHb molecule is far larger than lysozyme molecule; lysozyme molecule is ellipsoidal, whereas BHb is a globular protein composed of four molecular subunits; the isoelectric points of BHb and lysozyme are 6.8 and 11, respectively, implying that the amino acid residues consisting of lysozyme molecule are different from that consisting of BHb molecule. Based on the above reasons, it can be concluded that the cavities imprinted by lysozyme molecules within the thin polymeric layer on the surfaces of MIP-HEMA/NVP microspheres are not matched with BHb molecules in size, shape and spatial arrangement of action sites, and the excellent recognition selectivity and high binding ability of MIP-HEMA/NVP for lysozyme come from a mass of the suited imprinted caves within the thin polymeric layer on the surfaces of MIP-HEMA/NVP microspheres.

3.4. *Elution property of MIP-PVAm/CPVA*

An aqueous $NaHCO_3$ solution of 0.2 M as eluent upstream passes through the column packed with MIP-HEMA/NVP adsorbing lysozyme in a saturated state. The dynamic desorption curve is given in Fig. 8. It can be seen from Fig. 9 that the desorption curve is cuspidal and without trailing formation, displaying a fine elution property of MIP-HEMA/NVP microspheres. By calculating, the desorption ratios in 62 BV and in 70 BV reach 96.86% and 98.86%, respectively. These greater bed volumes seems that the elution rate appears slower and the used amount of eluent is more as the adsorbed lysozyme is completely eluted. The possible reasons for this are as follows. On the one hand, the lysozyme adsorption amount on the MIP-HEMA/NVP column is greater leading to taking time for elution; on the other hand, lysozyme molecule is a protein macromolecule, and its volume is large and the interaction sites within the imprinted cave are more, resulting in slowly eluting. Both the two reasons lead to the slower elution rate and the more used amount of the eluent. But then, Fig. 8 displays that lysozyme molecules combined on MIP-HEMA/NVP microspheres can be completely eluted. This implies that the surface-imprinted

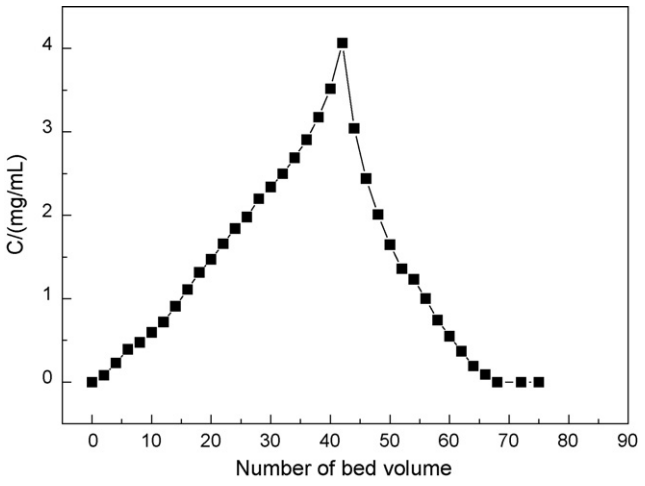


Fig. 8. Elution curve of lysozyme on MIP-HEMA/NVP column. Temperature: 20 °C.

material MIP-HEMA/NVP has an excellent eluting performance and it is easy to be recovered and reused.

4. Conclusions

In this paper, by designing a new surface-molecular imprinting method, lysozyme-surface-imprinted material MIP-HEMA/NVP with high performance was prepared with MMA as functional monomer and with crosslinked microspheres HEMA/NVP as matrix microspheres. The imprinted microspheres MIP-HEMA/NVP not only have high binding affinity and specific binding selectivity for the template protein, basic protein lysozyme, but also have biocompatibility probably owing to the biocompatibility of the matrix HEMA/NVP. Therefore, it can be expected with reason that by adopting the same method and using the same materials, the surface imprinting of low-density lipoproteins (LDL) should also be feasible, and the imprinted material to be obtained may be a very effective solid adsorbente for LDL apheresis in the hemoperfusion treatment. In a word, perhaps it is a promising route for enhancing the efficiency of blood purification to introduce the surface-imprinted materials into the hemoperfusion technique, and the new surface-molecular imprinting method designed in this investigation is more effective in preparing the surface-imprinted materials with high performance.

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