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Preparation and recognition performance of cholic acid-imprinted material prepared with novel surface-imprinting technique

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ABSTRACT

Acrylamide (AM) was first graft-polymerized on the surface of crosslinked polyvinyl alcohol (CPVA) microspheres by initiating of cerium salt, and then the grafted polyacrylamide (PAM) was transformed to polyvinylamine (PVAm) via Hofmann degradation reaction, resulting in the grafted microspheres PVAm/CPVA. By adopting the novel surface molecular imprinting technique put forward by us, cholic acid molecule-imprinted material MIP-PVAm/CPVA was prepared with glutaraldehyde as crosslinking agent The binding character of MIP-PVAm/CPVA towards cholic acid molecules was studied in depth with both batch and column methods and using cholesterol as a contrast compound whose chemical structure is similar with cholic acid to a certain extent. The experimental results show that the surface-imprinted material MIP-PVAm/CPVA has excellent binding affinity and recognition selectivity for the template molecule, cholic acid. The selectivity coefficient of PVAm/CPVA microspheres (non-imprinting material) for cholic acid relative to cholesterol is only 1.314, displaying very poor recognition selectivity for cholic acid. However, after imprinting, the selectivity coefficient of MIP-PVAm/CPVA for cholic acid in respect to cholesterol is remarkably enhanced to 11.231, displaying the excellent recognition selectivity and binding affinity towards cholic acid molecules. Besides, MIP-PVAm/CPVA microspheres have fine desorption property, and by using a mixture of ethanol and NaOH aqueous solution as an eluent, the desorption ratios can reach 99.73% as the effluent amount gets up to 20 bed volumes (BV).

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1. Introduction

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 [1–4] 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, such as separation and pre-concentration, constructing sensors, chromatography stationary phases, pseudo-immunoassay, catalysis [5–12] and so on. Especially, in the separation and enrichment area, a new technique, molecularly imprinted solid-phase extraction, in which MIPs are used as high selective solid extraction sorbents, has attracted much attention [13–17].

The conventional method to prepare MIPs, entrapment way, has some disadvantages, such as time-consuming and complicated preparation process, less recognition sites inside matrices particles obtained via crushing and grinding the imprinted polymeric monolith, and greater diffuse barrier for the template molecules coming from thick matrices, leading to poor binding capacity and lower binding kinetic of MIPs towards the template molecules. In order to overcome these drawbacks, we have developed the surface-imprinting method, and put forward a novel surface molecular imprinting technique [18,19]: (1) Functional polymers are pre-grafted (in the manner of "grafting from" or "grafting to") on the surface of micron-sized silica gel particles and a thin layer (or a film) of the grafted polymer is formed on the surface of silica gel particles; (2) The adsorption of the grafted particles towards template molecules reaches saturation by right of intermolecular interaction; (3) Post-imprinting of template molecules is conduced towards the grafted polymers using special crosslinking agent which has two reactive end groups. After the removal of the template molecules, a mass of the imprinted caves capable of recognizing and re-binding the template molecules is left and distributed within this thin polymer layer, resulting in the imprinted material with high performance such as more accessible

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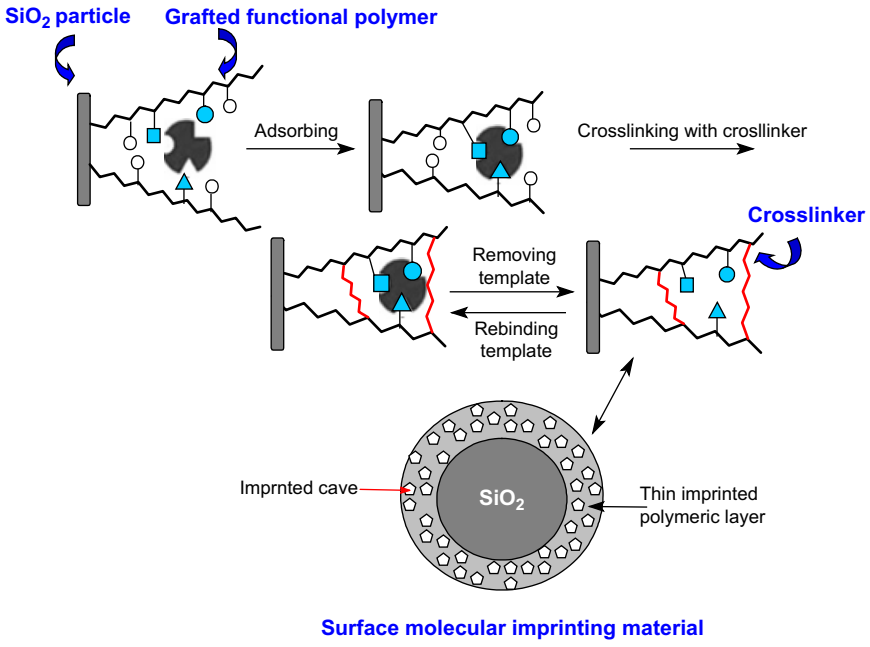
sites, fast mass transfer, and high recognition and binding ability for template molecules. This new surface-imprinting method is based on the interactions between the grafted functional polymers and template molecules rather than based on the interactions between the functional monomers and template molecules like as the conventional imprinting method, and this is a obvious difference between the two methods. The surface molecular imprinting process can be expressed schematically as follows.



In our previous investigation, the matrix particles used in the surface-imprinting technique were all silica gel particles, and it has some limitations. Under some given conditions, silica gel particles are not suitable probably. For example, in alkaline medium, silica gel particles cannot been used; for preparing those imprinting materials with biocompatibility or blood biocompatibility, silica gel particles are also inapplicable. In the present work, this new surface-imprinting technique is further developed, and we will use polymeric microspheres as matrix particles to conduct the surface-imprinting process.

Bile acids, the major metabolites of cholesterol, are a mixture of steroids, and play an important physiological role in the elimination of cholesterol from the body and in facilitating the absorption of dietary lipids and fat-soluble vitamins by formation of micelles [20,21]. The concentration of bile acids in body is related with hepatitis, gallstone and other diseases in liver. Therefore, the quantification of bile acids in body fluids (such as plasma and urine) is an important tool for the diagnosis of hepatobiliary diseases. On the other hand, bile acids also have pharmacological activity, and some components of bile acids have important therapeutic applications for treating some diseases, such as primary biliary cirrhosis and cholesterol gallstone [22,23]. Thus, analysis of these bile acids may be useful for monitoring bile acid therapy in such diseases. Obviously, for the concentration determination of bile acid components, it is important and useful to prepare solid-phase extraction sorbents with high selectivity for bile acid components.

Cholic acid as well as its derivatives is the main component of bile acids. In this work, polyvinylamine (PVAm)-grafted crosslinked polyvinyl alcohol (CPVA) microspheres, PVAm/CPVA, were used as matrix particles, and the surface-imprinting process was performed using cholic acid as template, and a cholic acid molecule-imprinted material, MIP-PVAm/PVA, was prepared. The results of the binding experiments indicate: (1) The surface-imprinting process put forward by us can be effectively carried out on the surface of polymeric microspheres; (2) By right of the interaction between the grafted PVAm and cholic acid molecules, hydrogen bond and electrostatic interactions, the molecular imprinting material MIP-PVAm/PVA has high recognition selectivity and binding affinity towards cholic acid molecules. It can be expected that by using polymeric microspheres, the application range of the new surface-imprinting technique will be extended greatly, and especially, in medical and pharmaceutical fields, to use polymeric microspheres as matrix particles of surface imprinting is promising and significant.

2. Experimental section

2.1. Material and equipment

Crosslinked polyvinyl alcohol (CPVA) microspheres (180 μm in mean diameter) were self-prepared by suspension copolymerization of vinyl acetate as main monomer and divinyl benzene as crosslinker, followed by alcoholysis of the obtained crosslinked microspheres [24]. Acrylamide (AM, Beijing Chemical Reagent Company, China) was of analytical grade and was purified by recrystallization from acetone before use. Ammonium cerous sulfate (ACS Tientsin Bodi Chemical Engineering Ltd., Tientsin, China) was of analytical grade. Sodium hypochlorite (NaOCl, Tientsin Damao Chemical Reagent Plant, Tientsin, China) was in a form of aqueous solution with a concentration of 10% and was of analytical grade. Cholic acid (Tientsin Kaixiao Science and Technology Ltd., Tientsin, China) was of analytical grade and was received. Cholesterol (Chemical reagent Ltd. of National Medicine Group, Beijing, China) was of analytical grade. Glutaraldehyde (50% of aqueous solution, Tientsin Baishi Chemical Engineering Ltd., Tientsin, China) was of analytical grade. Other reagents were all commercial chemicals with analytical pure and purchased from Chinese companies.

The instruments used in this study were as follows: Unic-2602 UV spectrophotometer (Unic Company, Shanghai), Perkin-Elmer 1700 infrared spectrometer (Perkine-Elmer Company, USA), PHS-2 acidimeter (The Second Analytical Instrument Factory, Shanghai, China), TG16-WS high-speed centrifuge with desk type (Changsha Xiangyi Centrifuge Factory, Province Jiangsu, China) and THZ-92C constant temperature shaker equipped with gas bath (Boxun Medical Treatment Equipment Factory, Shanghai, China).

2.2. Preparing PVAm/CPVA microspheres and study on adsorption property for cholic acid

2.2.1. Preparation of grafted microspheres PAM/CPVA

0.6 g of CPVA microspheres, 1.9 g of AM and 50 mL of distilled water were added in turn into a four-necked flask equipped with a mechanical stirring, a condenser and a N_2 inlet. After the microspheres were soaked for 10 h so as to be fully swelled, 0.2 g of ACS and 0.5 mL of concentrated sulfuric acid were introduced, and the temperature was raised to 45 °C. The graft polymerization of AM was performed under N_2 atmosphere at the constant temperature of 45 °C for 6 h. By filtrating, the grafted microspheres, PAM/CPVA, were collected, soaked and washed with distilled water to remove a few of polyacrylamide physically attached to the microspheres, and dried under vacuum to constant weight. The grafting degree of PAM of the PAM/CPVA microspheres was determined with weighing method, and their chemical structure was characterized by infrared spectrum. The prepared and used PAM/CPVA microspheres in this study have a grafting degree of 27.13 g/100 g.

2.2.2. Preparation of functional microspheres of PVAm/CPVA

The functional microspheres of PVAm/CPVA were prepared via Hofmann degradation reaction of the grafted PAM. 0.5 g of PAM/CPVA microspheres was soaked in 10 mL of distilled water containing dimethyl sulphoxide for 10 h so as to be fully swelled. The mixture was added into a three-necked flask, followed by adding 15 mL of aqueous NaOH solution, whose concentration was 14% (wt%). The content in the flask was cooled to −3 °C in a cryohydrate bath, and 4.5 mL of aqueous NaOCl solution was added. The degradation reaction of the grafted PAM was carried out for 11 h with stirring. By filtrating, the product microspheres PVAm-CPVA were collected, washed repeatedly with distilled water and dried under vacuum to constant weight. The infrared spectrum of the microspheres PVAm/CPVA was determined with KBr pellet method and the chemical structure was characterized. The amination degree (mol/100 g) of PVAm/CPVA microspheres was determined with acid–base titration method and based on the number of VAm units on PVAm/CPVA. The used PVAm/CPVA microspheres in this study had an amination degree of 0.2 mol/100 g.

2.2.3. Examining adsorption property of PVAm/CPVA for cholic acid

The adsorption behavior of PVAm/CPVA for cholic acid was examined with batch method and column method, namely static and dynamic methods, respectively. The adsorption dynamics behaviors of PVAm/CPVA for cholic acid in ethanol and in a mixture of water and ethanol (V:V = 1:8) were first measured to determine the time in which the adsorption reached equilibrium (it was about 6 h). Based on the adsorption dynamics determination, the adsorption isotherms of PVAm/CPVA for cholic acid in the two mediums, ethanol and a mixed solvent, were determined at 35 °C, respectively. The adsorption experiments were conduced in a constant temperature shaker. Cholic acid concentration before and after adsorption was determined with UV spectrophotometry (at 211 nm). The equilibrium adsorption amount was calculated by Equation (1).

$$Q_e = \frac{V(C_0 - C_e)}{1000m} \quad (1)$$

where Q_e (mg/g) was the equilibrium adsorption amount of cholic acid; C_0 and C_e (mg/L) were the initial and final concentrations of cholic acid, respectively; V (mL) was the volume of the cholic acid solution; m (g) was the weight of PVAm/CPVA microspheres.

2.2.4. Examining effect of pH value on adsorption of PVAm/CPVA for cholic acid

The pH values of the cholic acid solutions of the mixed solvent of water and ethanol were adjusted with dilute solution of HCl and NaOH. The isothermal adsorption experiments of PVAm/CPVA for cholic acid were carried out in these solutions with different pH values to examine the effect of pH value on the adsorption capacity and to study the adsorption mechanism PVAm/CPVA towards cholic acid.

2.3. Preparation of MIP-PVAm/CPVA and study on its binding characteristic for cholic acid

2.3.1. Preparing cholic acid molecule-imprinted material MIP-PVAm/CPVA

1 g of PVAm/CPVA was added into 50 mL of cholic acid solution of the mixed solvent of ethanol and water with a concentration of 3.5 g/L, and the pH value was adjusted to 6 with dilute NaOH solution. The mixture was shaken on a constant temperature shaker for 6 h until PVAm/CPVA microspheres were fully swelled and the adsorption for cholic acid reached equilibrium. After filtrating, the PVAm/CPVA microspheres, which had adsorbed cholic acid in a saturation state, were dried under vacuum. A certain amount of PVAm/CPVA microspheres adsorbing cholic acid was placed in a mixed solution of water and ethanol (V:V = 1:8), in which cholic acid with a concentration of 3.5 g/L was contained. The pH value of the solution was adjusted to 6, and a certain amount of crosslinking agent glutaraldehyde (0.1 g of glutaraldehyde aqueous solution) was added. The crosslinking reaction was performed at 50 °C for 8 h. The resultant microspheres were filtered off, washed repeatedly with NaOH aqueous solution and ethanol to remove the template cholic acid, and finally, cholic acid molecule-imprinted material MIP-PVAm/CPVA was obtained. The infrared spectrum of MIP-PVAm/CPVA was determined.

2.3.2. Evaluating binding property

The binding behavior of MIP-PVAm/CPVA for cholic acid was examined also with batch method and column method, respectively. Based on the determination of the binding dynamics behavior of MIP-PVAm/CPVA for cholic acid (the equilibrium binding time also was 6 h), the combining isotherm was measured. Numbers of 40 mL of cholic acid solutions with different concentrations were taken and transferred into conical flasks. MIP-PVAm/CPVA microspheres with the same mass were added into these solutions, respectively. These mixtures were shaken on a constant temperature shaker at 35 °C, centrifuged after reaching binding equilibrium, and the equilibrium concentrations of cholic acid in the supernatants were determined with UV spectrophotometry, respectively. The equilibrium binding amounts of MIP-PVAm/CPVA towards cholic acid were calculated according to Eq. (1), and the binding isotherm was figured.

For the column method, the experimental procedures are explained as follows. A certain amount (0.42 g) of MIP-PVAm/CPVA microspheres was packed into a piece of glass pipe with an internal diameter of 0.8 cm, and the bed volume (BV) of the packed column was 2 mL. The cholic acid solution with a concentration of 0.5 g/L was allowed to gradually flow through the packed column at a rate

of five bed volumes per hour (5 BV/h) in countercurrent manner. The effluents with one bed volume (1 BV) interval were collected, and the cholic acid concentrations of these effluents were determined with spectrophotometry. The dynamic binding curve was plotted, and the leaking adsorption amount and saturated adsorption amount of cholic acid were calculated with the data of the concentrations and bed number of effluents, respectively.

2.3.3. Selectivity experiments

Cholesterol is one of steroid compounds like cholic acid, and its chemical structure is similar with cholic acid to a certain extent. In order to examine the recognition selectivity of MIP-PVAm/CPVA microspheres towards cholic acid, cholesterol was selected as a contrast substance in this study. The molecular structures of the two substances are schematically expressed in Scheme 1.

For the sake of comparing the binding selectivity of MIP-PVAm/CPVA for cholic acid, the static binding isotherm and dynamic binding curve of MIP-PVAm/CPVA as well as PVAm/CPVA for cholesterol were also determined with spectrophotometry (at 243 nm), separately.

In order to further show the binding specificity of MIP-PVAm/CPVA for cholic acid, the competitive adsorption of cholic acid with respect to cholesterol were studied. A binary mixed solution of cholesterol/cholic acid was prepared, and both the concentrations of the two substances in the mixed solution were the same, and were 3.5 g/L.

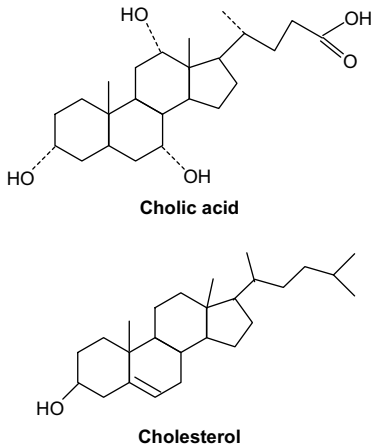
0.3 g of MIP-PVAm/CPVA was added into 30 mL of the above mixed solution, and the static adsorption experiments were performed. After binding equilibriums were reached, the concentrations of two substances in the supernatant were determined with spectrophotometry at 211 nm and at 243 nm, respectively. The distribution coefficient for each substance was calculated according to Eq. (2), and this equation was originated from Ref. [25].

$$K_d = \frac{Q_e}{C_e} \tag{2}$$

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

The selectivity coefficient of MIP-PVAm/CPVA for cholic acid with respect to the competition species, cholesterol, can be obtained from the equilibrium binding data according to Eq. (3)

$$k = \frac{K_d(\text{cholic acid})}{K_d(\text{cholesterol})} \tag{3}$$



Scheme 1. Schematic expression of chemical structures of cholic acid and cholesterol.

where k is the selectivity coefficient, and the value of k allows an estimation of selectivity of MIP-PVAm/CPVA for cholic acid. A relative selectivity coefficient k' is also defined as expressed in Eq. (4) [25], and the value of k' can reveal the enhanced extent of the adsorption affinity and selectivity of the imprinted material towards the template molecule with respect to the non-imprinted material.

$$k' = \frac{k_{\text{impr}}}{k_{\text{non-impr}}} \tag{4}$$

where k_{impr} is the selectivity coefficient of MIP-PVAm/CPVA for cholic acid with respect to the competition species, cholesterol, and $k_{\text{non-impr}}$ is the selectivity coefficient of PVAm/CPVA for cholic acid also respect to cholesterol.

2.4. Desorption experiment

A certain amount of MIP-PVAm/CPVA microspheres adsorbing cholic acid in a saturation state was packed into a piece of glass pipe with an internal diameter of 0.8 cm, and the bed volume (BV) of the packed column was 2 mL. A mixed solvent of ethanol and aqueous solution of NaOH (0.1 M) with a volume ratio of 8:2 as an eluent was allowed to gradually flow through the column at a rate of two bed volumes per hour (2 BV/h) in countercurrent manner. The effluents with one volume (1 BV) interval were collected, and the concentration of cholic acid was determined with spectrophotometry. The dynamic desorption curve was plotted, and elution property of MIP-PVAm/CPVA was evaluated.

3. Results and discussions

3.1. Graft polymerization and characterization of three kinds of microspheres

3.1.1. Effect of main factors on graft polymerization

3.1.1.1. Effect of H^+ concentration. The graft polymerization was carried out in an acidic medium. By fixed other reaction conditions, the graft polymerizations of AM on CPVA microspheres were performed with different concentrations of sulfuric acid, and the grafting degree of PAM on microspheres PAM/CPVA as a function of H^+ concentration is given in Fig. 1.

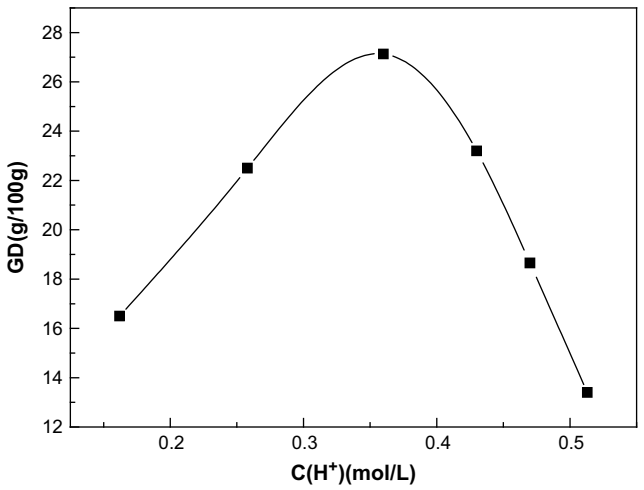


Fig. 1. Grafting degree of PAM on CPVA/PAM as a function of H^+ concentration Cerium salt conc.: 5.98×10^{-3} mol/L; reaction time: 6 h; reaction temperature: 45 °C; AM conc.: 0.535 mol/L.

It can be observed from Fig. 1 that the grafting degree of PAM first increases and then decreases with the increase of H^+ concentration, and the grafting degree has a maximum as H^+ concentration is 0.36 mol/L. This result reflects the mechanism [26,27] of the radical polymerization initiated by the redox initiating system constituted by cerium and reduction component (in the present system, it is the hydroxyl groups on CPVA microspheres). Ce^{4+} ion has strong hydrolysis property as indicated in Scheme 2, so the graft polymerization is conducted in an acidic medium to inhibit the hydrolysis process of Ce^{4+} ion [26,27]. In a certain range of H^+ concentration, a greater concentration of H^+ ion will restrain the hydrolysis process of Ce^{4+} ion effectively, so the graft polymerization will be accelerated resulting in the increase of the grafting degree of PAM with increasing H^+ concentration. However, H^+ ions will be produced during the initiating step [26,27] in which the primary free radicals are produced as shown in Scheme 2, and overmuch H^+ ions will inhibit the initiating step as well as chain propagation step, leading to the decrease of the grafting degree of PAM. Therefore, in the polymerization system initiated by cerium, the concentration of H^+ ion should be controlled to be suitable. For the present system, 0.36 mol/L is the fitting concentration of H^+ ion, and over this concentration the grafting degree of PAM will decrease instead of increasing.

3.1.1.2. Effect of cerium salt concentration. By fixed other reaction conditions, the graft polymerizations of AM on CPVA microspheres were performed with different concentrations of cerium salt ACS, and the grafting degree of PAM as a function of ACS concentration is shown in Fig. 2.

It can been observed in Fig. 3 that the grafting degree of PAM also exhibits a variation trend similar to the result seen above, namely, the grafting degree first increases and then decreases with the increase of ACS concentration, and the grafting degree has a maximum as ACS concentration is 5.98×10^{-3} mol/L. This experimental result can be explained reasonably with the mechanism of the radical polymerization initiated by cerium salt. For the radical polymerization process initiated by cerium salt, except two manners of chain termination, coupling termination and disproportionation termination, there is another termination manner [26,27], oxidation termination, and it is expressed in Scheme 3. For the graft polymerization of AM on the surfaces of CPVA microspheres, the oxidation termination is probably only termination manner. Furthermore, in the graft polymerization system, the primary free radicals are also inactivated probably due to the oxidation termination as shown in Scheme 3.

As the cerium salt concentration is lower, the number of the free radicals produced on CPVA microspheres will increase with increasing the cerium salt concentration, and the graft polymerization will be accelerated, leading to the increase of the grafting degree of PAM on PAM/CPVA microspheres. However, as the cerium salt concentration is overmuch, the oxidation termination process will be accelerated, especially the oxidation termination of the primary free radicals. The number of the active sites on CPVA microspheres will be reduced remarkably owing to the accelerated oxidation termination process, resulting in the inhibiting of the graft polymerization and the decreasing of the grafting degree of PAM. Therefore, there is a suitable cerium salt concentration in the graft polymerization system initiated by cerium salt. For the present system, the appropriate concentration of cerium salt is 5.98×10^{-3} mol/L.

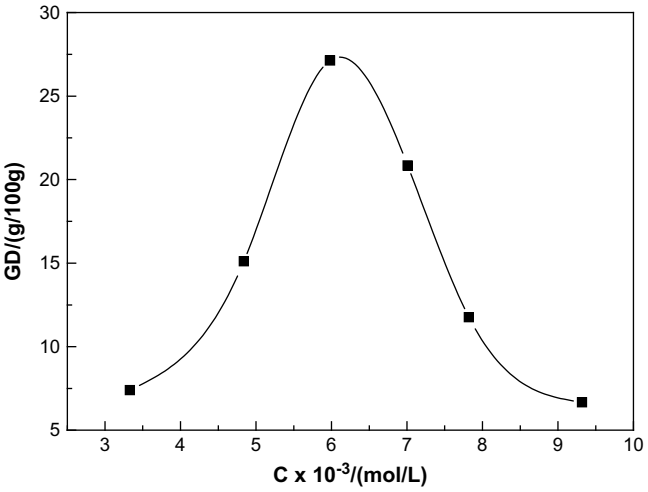


Fig. 2. Grafting degree of PAM on CPVA/PAM as a function of cerium salt concentration. Sulfuric acid conc.: 0.18 mol/L; reaction time: 6 h; reaction temperature: 45 °C; AM conc.: 0.535 mol/L.

3.1.2. FTIR spectra of three kinds of microspheres

FTIR spectra of the three kinds of microspheres, PAM/CPVA, PVAm/CPVA and MIP-PVAm/CPVA, were determined with KBr pellet method, and their FTIR spectra are shown in Fig. 3.

In the spectrum of PAM/CPVA, the stretching vibration of $-NH_2$ in amido groups appears at 3453 cm^{-1} (it overlaps partially with the vibration absorption of the hydroxyl groups of CPVA). The bands at 1698 cm^{-1} is attributed to the vibration absorption of carbonyl C=O in amido groups. The spectrum data indicate that the graft polymerization of AM on CPVA microspheres has occurred and PAM macromolecules have been grafted onto the surface of CPVA microspheres, resulting in the grafted microspheres PAM/CPVA.

In the spectrum of PVAm/CPVA, the band at 1698 cm^{-1} has been weakened greatly, and a new band at 1657 cm^{-1} has appeared. It

Hydrolysis of Ce^{4+} ions

$$Ce^{4+} + H_2O \rightleftharpoons Ce(OH)^{3+} + H^+$$

Formation of primary free radicals in initiation step

$$Ce^{4+} + ROH \longrightarrow \dot{R}OH + Ce^{3+} + H^+$$

Scheme 2. Two reaction steps related to H^+ ions in the graft polymerization process.

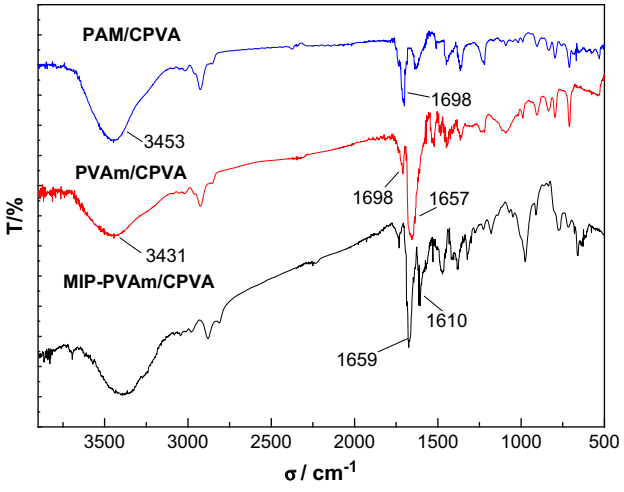
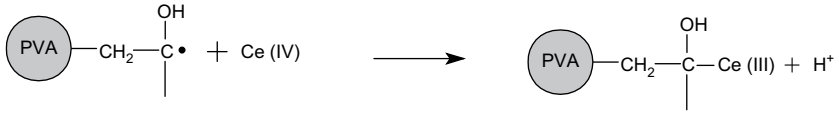
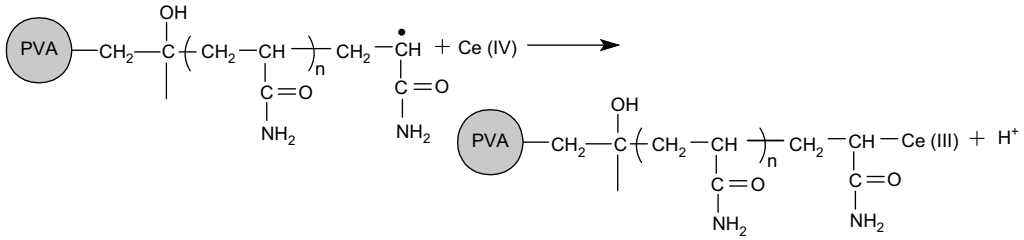


Fig. 3. FTIR spectra of three kinds of microspheres.

Oxidation termination for the primary radicals



Oxidation termination for the propagation chains



Scheme 3. Schematic illustration of oxidation termination.

should be ascribed to the deformation vibration absorption of amino groups. The stretching vibration absorption of amino groups locates at 3431 cm^{-1}. The spectrum changes imply that Hofmann degradation reaction of the grafted PAM has been produced, and the functional microspheres PVAm/CPVA have been formed.

In the spectrum of MIP-PVAm/CPVA, a new band at 1610 cm^{-1} has appeared obviously, and it should be ascribed to the vibration absorption of N=C bond, which is formed in the Schiff base reaction of crosslinking process. This fact suggested that the crosslinking and imprinting process have been successfully carried out.

3.2. Adsorption property and mechanism of PVAm/CPVA towards cholic acid

3.2.1. Adsorption property PVAm/CPVA towards cholic acid in two medium

Cholic acid is soluble in ethanol, but is nearly non-soluble in water. So a mixed solvent of ethanol and water (V:V = 1:8) was prepared to examine the adsorption property of PVAm/CPVA in the medium containing water. Fig. 4 gives the adsorption isotherms of PVAm/CPVA towards cholic acid in ethanol and the mixed medium.

It can be observed that in ethanol, PVAm/CPVA has certain adsorption ability for cholic acid. In ethanol, the carboxyl groups of cholic acid molecules do not dissociate nearly, whereas the nitrogen atoms of the amino groups of the grafted PVAm also will not be protonized. Therefore, hydrogen bond interaction between the amino groups of the grafted PVAm and the carboxyl groups as well as hydroxyl groups of cholic acid molecules should be responsible for the adsorption. Many studies show that the hydrogen bonding between organic compounds and absorbents in ethanol always is suffered from competition of the hydrogen bond interaction between the solvent ethanol molecules and the absorbents. Therefore, it can be considered that the adsorption capacity of PVAm/CPVA for cholic acid in ethanol is the net result of competition adsorption, or else the adsorption capacity of cholic acid onto PVAm/CPVA should be higher.

Fig. 4 displays that in the mixture of ethanol and water, the adsorption capacity of PVAm/CPVA for cholic acid is obviously greater than that in ethanol. It can be affirmed that in the mixture of ethanol and water, the hydrogen bond interaction between cholic acid molecules and the absorbent PVAm/CPVA will be restrained more severely owing to the competition adsorption of the solvent water molecules. However, in the mixed solvent, PVAm/CPVA exhibits stronger adsorption ability for cholic acid, suggesting that except for hydrogen bond interaction, there is other interaction between cholic acid and PVAm/CPVA in the mixed solvent. This acting force just is electrostatic interaction. In the medium containing water, the carboxyl groups of cholic acid molecules will dissociate partially and produce negative ions of carboxyl groups, while the nitrogen atoms of amino groups of the grafted PVAm will be protonized and produce positive ions. In this way, stronger electrostatic interaction between the negative ions of carboxyl groups and positive protonized amino groups will be produced, leading to greater adsorption capacity of PVAm/CPVA towards cholic acid in the mixed medium than that in ethanol. Therefore, the adsorption driving force of PVAm/CPVA towards cholic acid in the mixture of ethanol and water should be the synergy of electrostatic interaction and hydrogen bonding. Besides, it can be seen from the shape of the adsorption isotherms in Fig. 1 that the adsorptions of PVAm/CPVA towards cholic acid in two mediums fit with Langmuir model, and are typical monolayer adsorption. According to the reciprocal form of Langmuir equation ($1/Q_e = (1/kQ_m) \times 1/C_e + 1/Q_m$), two fine straight lines were obtained, and the

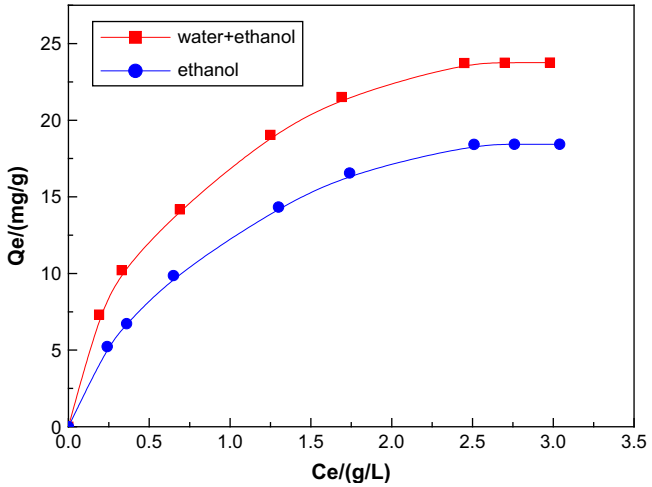


Fig. 4. Adsorption isotherms of PVAm/CPVA for cholic acid in two mediums. Temperature: 35 °C; pH = 6.

correlation coefficients were 0.9976 (in the mixed solvents) and 0.9983 (in ethanol), respectively. The values of Langmuir constant k were 2.27 and 1.16, respectively. The Langmuir constants again show that the adsorption ability of PVAm/CPVA for cholic acid in the mixture of ethanol and water is obviously greater than that in ethanol.

3.2.2. Further discussion of adsorption mechanism of PVAm/CPVA towards cholic acid in medium containing water

Fig. 5 gives the adsorption isotherms of PVAm/CPVA for cholic acid in the mixture of ethanol and water with different pH values. In order to more clearly display the effect of pH value on the adsorption capacity, the saturated adsorption amount as a function of pH value can be figured using the data in Fig. 5, and shown in Fig. 6.

The following facts can be seen from Fig. 6. The adsorption capacity of PVAm/CPVA for cholic acid is small as pH value is lower; then the adsorption capacity increases rapidly with the increase of pH value; the adsorption capacity attains a maximum as pH reaches a value of 6; subsequently, the adsorption capacity turns to decreasing with the increase of pH value. The above experimental facts reflect the adsorption mechanism of PVAm/CPVA towards cholic acid. As pH value is lower, the carboxyl groups of cholic acid nearly do not dissociate. So there is no electrostatic interaction between PVAm/CPVA and cholic acid molecules, leading to very small adsorption capacity only caused by hydrogen bond interaction, in despite of the high protonation of the nitrogen atoms of amino groups of PVAm/CPVA. The dissociation degree of the carboxyl groups of cholic acid increases with the enhancement of pH value, resulting in strengthening electrostatic interaction. By the driving of electrostatic interaction, a mass of cholic acid molecules will be closed to the surface of PVAm/CPVA microspheres and will be adsorbed. Moreover, the formation of hydrogen bond interaction between cholic acid and PVAm/CPVA will be accelerated due to the approach of cholic acid molecules to the surface of PVAm/CPVA microspheres. Simultaneously, along with the continuous increase of pH value, the protonized degree of the nitrogen atoms of the amino groups of PVAm/CPVA is decreased, and this is disadvantageous to the electrostatic interaction between PVAm/CPVA and cholic acid molecules. As pH $<$ 6, this negative affect may do not emerge, whereas after pH $>$ 6, this weakening action for the electrostatic interaction may exhibit obviously, resulting in the decline of the adsorption capacity with the increase of pH value.

By this token, in the mixture of ethanol and water with pH value of 6, there is strong interaction between PVAm/CPVA microspheres and cholic acid molecules, which originates from the synergism of hydrogen bond and electrostatic interaction. This lays a firm groundwork for the next surface molecular imprinting process. Since there is strong intermolecular interaction between PVAm/CPVA microspheres and cholic acid molecules, it is possible and feasible to imprint cholic acid towards the grafted PVAm on the surface of PVAm/CPVA microspheres.

3.3. Preparing processes of MIP-PVAm/CPVA microspheres

There is a great deal of hydroxyl groups on the surfaces of the crosslinked polyvinyl alcohol microspheres (CPVA). According to the redox initiation mechanism of cerium salt [26,27], the complex reaction between Ce^{4+} ions and the hydroxyl groups on CPVA will first occur, forming a complex [C]. Subsequently, an oxidation process of a single-electron transfer will produce and the complex [C] was disproportionated soon, forming free radicals on the carbon atoms bearing the hydroxyl groups. So the graft polymerization of AM on microspheres CPVA is initiated, resulting in the grafted microspheres PAM/CPVA. The Hofmann degradation reaction of the grafted PAM macromolecules on PAM/CPVA is conducted using sodium hypochlorite (NaOCl) as reaction reagent in the presence of excessive NaOH, and the grafted PAM is transformed into the grafted polyvinylamine (PVAm), resulting in the formation of the microspheres PVAm/CPVA.

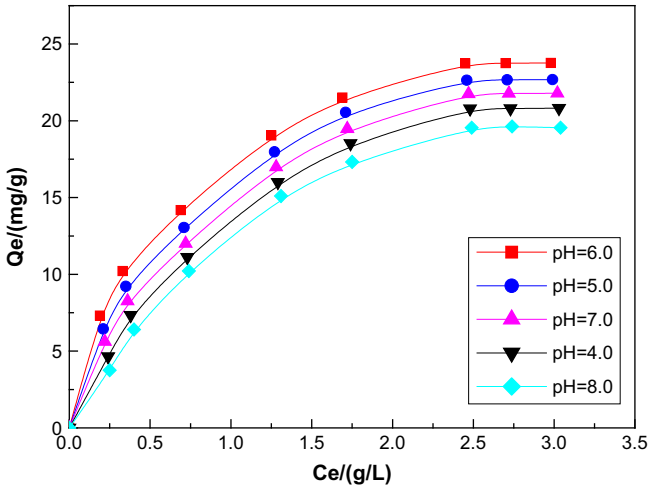


Fig. 5. Adsorption isotherms of PVAm/CPVA for cholic acid in mixed medium with different pH values. Temperature: 35 °C.

As the adsorption of cholic acid on PVAm/CPVA microspheres reached saturation, the crosslinking agent glutaraldehyde was added. The Schiff base reaction between the primary amine groups of the grafted PVAm and glutaraldehyde will be carried out favorably, leading to the crosslinking of PVAm macromolecules. As a result, cholic acid molecules were enveloped in the crosslinking networks, and the imprinting of cholic acid was realized. As the template molecules were washed away, large numbers of cholic acid molecule-imprinted caves remained within the thin polymer layer on the surface of PVAm/CPVA microspheres, thereupon, the cholic acid-imprinted material MIP-PVAm/CPVA was obtained. The total preparation processes of MIP-PVAm/CPVA are schematically expressed in Scheme 4.

3.4. Binding characteristic of MIP-PVAm/CPVA for cholic acid

3.4.1. Binding isotherms and dynamics binding curves

The adsorption experiments in the batch method were first performed, and Figs. 7 and 8 give the adsorption isotherms of

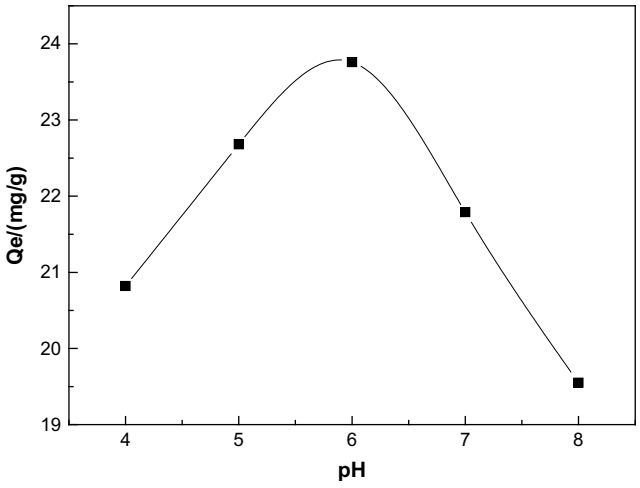
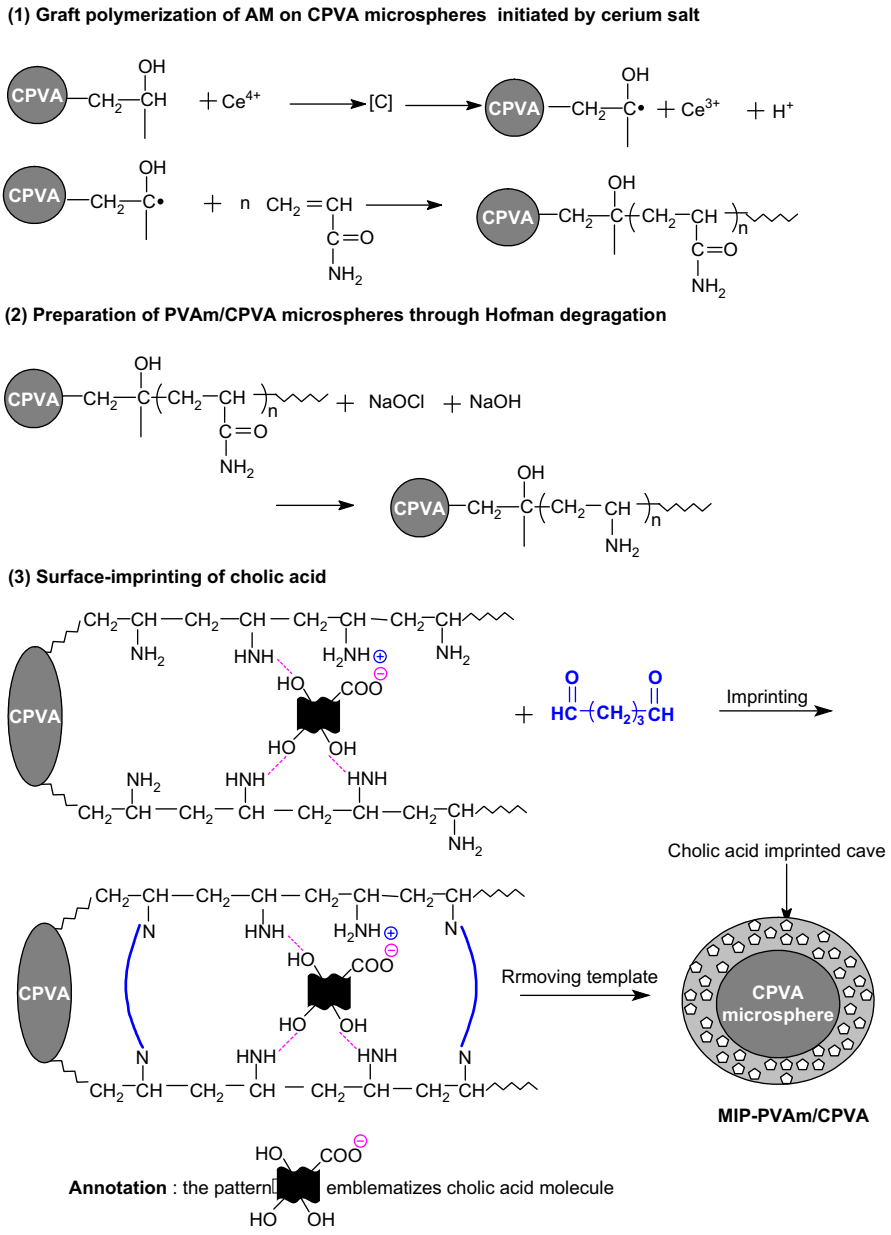


Fig. 6. Effect of pH value on adsorption capacity. Temperature: 35 °C.



Scheme 4. Schematic expression of preparing process of MIP-PVAm/CPVA.

PVAm/CPVA (non-imprinting material) and the binding isotherms of MIP-PVAm/CPVA (imprinting material) for cholic acid and cholesterol, respectively. It is obvious that the adsorption isotherms of PVAm/CPVA towards cholic acid and cholesterol as well as the binding isotherm of MIP-PVAm/CPVA towards cholic acid still fit with Langmuir model, indicating monolayer adsorption. For Fig. 7, according to the reciprocal form of Langmuir equation, two fine straight lines were obtained, and the correlation coefficients were 0.9976 (for cholic acid) and 0.9998 (for cholesterol), respectively, and the values of Langmuir constant k were 2.27 and 1.70, respectively. The values of Langmuir constant suggested that the adsorption ability of PVAm/CPVA for cholic acid is closed to that for cholesterol to a certain extent.

It can be seen from Fig. 7 that the adsorption capacities of PVAm/CPVA for cholic acid and cholesterol are similar and the adsorption capacity difference is not notable as indicated by the above Langmuir constants, namely, PVAm/CPVA microspheres have no obvious binding selectivity for cholic acid. However, after imprinting cholic acid, a remarkable difference of the binding capacity of MIP-PVAm/CPVA for the two substances is displayed, as shown in Fig. 8. The maximum binding amount of cholic acid is 22.38 mg/g, whereas for cholesterol, the corresponding binding amount is only 0.67 mg/g. This fact clearly demonstrates that the cholic acid-imprinted material MIP-PVAm/CPVA has excellent binding affinity and selectivity for the template cholic acid. This result shows that the novel surface molecular imprinting technique conducted on the surface of polymeric microspheres is also feasible and successful like as on the surface of silica gel particles. In addition, For Fig. 8, according to the reciprocal form of Langmuir equation, a fine straight line (for cholic acid) was also obtained, and the correlation coefficients were 0.9984, and the Langmuir constant k was 3.25. The greater value of the Langmuir constant displays well the excellent binding affinity of the imprinted material MIP-PVAm/CPVA for cholic acid.

In order to further study the binding characteristic of MIP-PVAm/CPVA for cholic acid, the adsorption experiments in the

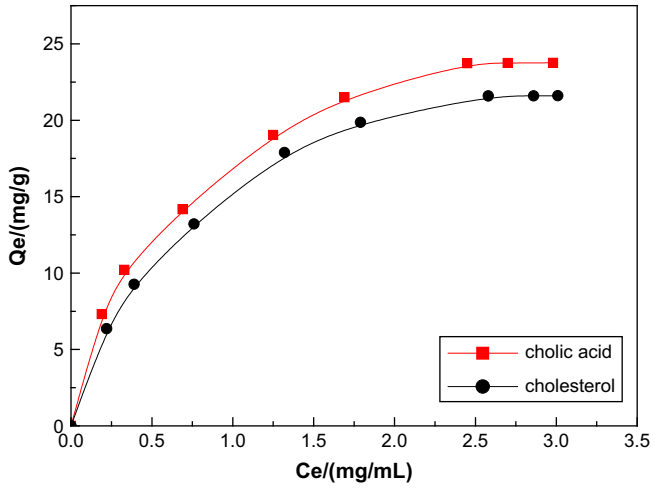


Fig. 7. Adsorption isotherms of PVAm/CPVA for cholic acid and cholesterol. Temperature: 35 °C; pH = 6.

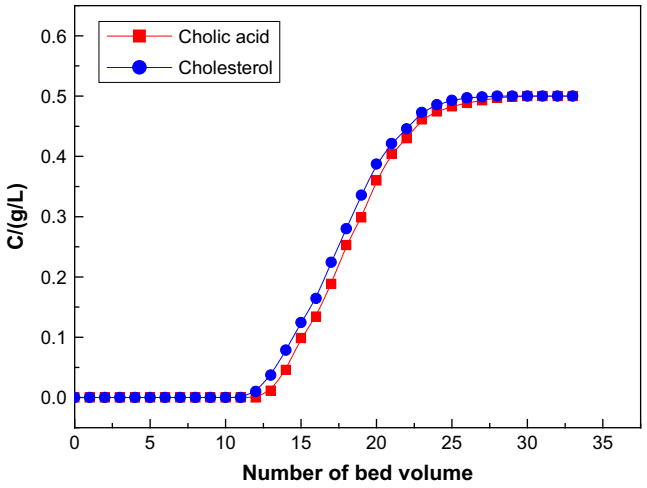


Fig. 9. Dynamic adsorption curves of PVAm/CPVA for cholic acid and cholesterol. BV: 2 mL; temperature: 20 °C; initial concentration: 0.5 g/L; flow rate: 5 BV/h.

column method were also performed. Figs. 9 and 10 display the dynamic adsorption curve of PVAm/CPVA and the dynamic binding curve of MIP-PVAm/CPVA for cholic acid and cholesterol, respectively.

It can be observed from Fig. 9 that as the solutions of cholic acid and cholesterol with the same concentration flow upstream through the column packed with PVAm/CPVA microspheres, respectively, the leaking volumes are approximately identical and are about 13 BV and 11 BV, respectively. This fact again shows that PVAm/CPVA has no adsorption selectivity for cholic acid. However, it is observed in Fig. 10 that the dynamic binding curve of MIP-PVAm/CPVA for cholic acid is obviously different from that for cholesterol. The leaking volume of cholic acid is 18 BV and far greater than that of cholesterol (only about 2 BV). By calculating, the leaking and saturated adsorption amounts of cholic acid are 20.23 mg/g and 25.26 mg/g, respectively, whereas for cholesterol, they are only about 1.19 mg/g and 1.56 mg/g, respectively. Obviously, MIP-PVAm/CPVA nearly does not recognize and bind cholesterol molecules. In contrast, it has excellent recognition selectivity for cholic acid, and it will be further discussed below.

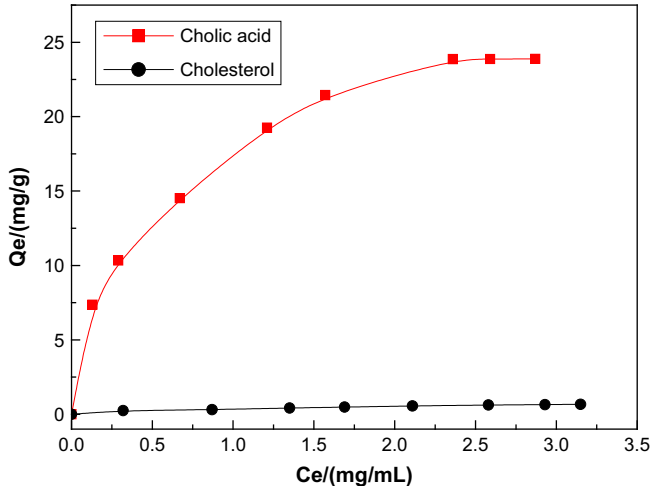


Fig. 8. Binding isotherms of MIP-PVAm/CPVA for cholic acid and cholesterol. Temperature: 35 °C; pH = 6.

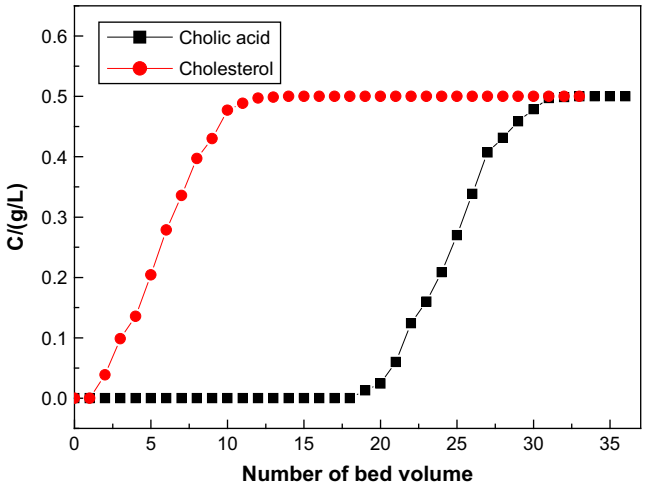


Fig. 10. Dynamic binding curves of MIP-PVAm/CPVA for cholic acid and cholesterol. BV: 2 mL; temperature: 20 °C; initial concentration: 0.5 g/L; flow rate: 5 BV/h.

3.4.2. *Recognition selectivity of MIP-PMAA/SiO_2 for creatinine*

Competitive adsorption experiments on MIP-PVAm/CPVA were conduced in a binary solution of cholic acid and cholesterol. In Table 1, the data of the distribution coefficients K_d, selectivity coefficients k and relative selectivity coefficients k' are summarized.

From the data in Table 1, the following facts can be found: (1) The selectivity coefficient of the non-imprinting material, PVAm/CPVA, for cholic acid in relation to cholesterol is very low, and is only 1.314. This implies that the adsorption abilities of PVAm/CPVA for the two substances, cholic acid and cholesterol, are approximate and selectivity-absent; (2) The selectivity coefficient of MIP-PVAm/CPVA for cholic acid with respect to cholesterol is remarkably enhanced, and gets up to 11.231, displaying a high recognition selectivity MIP-PVAm/CPVA for cholic acid; (3) The relative

Table 1
Distribution coefficient and selectivity coefficient data.

Adsorb material	PVAm/CPVA		MIP-PVAm/CPVA	
	Cholic acid	Cholesterol	Cholic acid	Cholesterol
K_d (mL/g)	10.647	8.103	4.256	1.27
k	1.314		11.231	
k'			8.547	

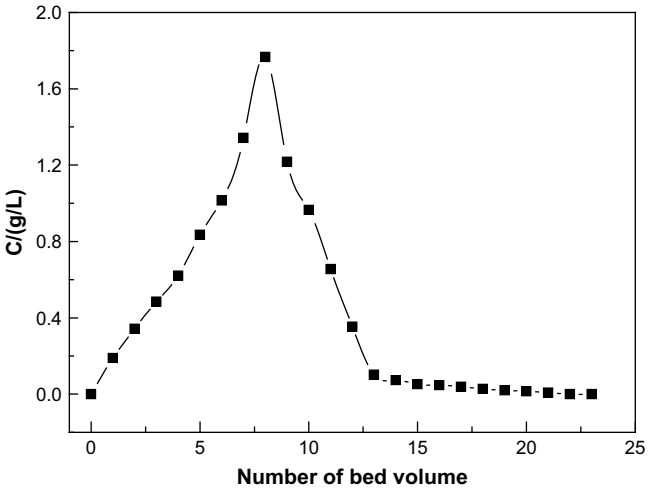


Fig. 11. Elution curve of cholic acid on MIP-PVAm/CPVA column. Temperature: 20 °C.

selectivity coefficients of MIP-PVAm/CPVA is 8.547, indicating a remarkable enhancement of the adsorption affinity and selectivity of the imprinting material MIP-PVAm/CPVA for the template molecule in relation to non-imprinting material PVAm/CPVA. The above facts clearly reveal that MIP-PVAm/CPVA has high recognition selectivity and binding affinity for the template cholic acid.

The reason for the above facts can be explained as follows. Although cholesterol is also one of steroid compounds like as cholic acid, the functional groups contained in its molecule are different from that in cholic acid molecule as shown in Scheme 1, so that the cavities imprinted by cholic acid molecules within the thin layer of PVAm on the surface of MIP-PVAm/CPVA are not suited to cholesterol molecules in size, shape and spatial arrangement of action sites. Obviously, the excellent recognition selectivity and high binding ability of MIP-PVAm/CPVA for cholic acid come from a mass of the suited imprinted caves within the thin polymeric layer on the surface of PVAm/CPVA microspheres.

3.5. Elution property of MIP-PVAm/CPVA

A mixed solvent of ethanol and NaOH aqueous solution (in a volume ratio of 8:2) was used as the eluent. The eluent upstream passes through the column packed with MIP-PVAm/CPVA adsorbing cholic acid in a saturated state. The dynamic desorption curve is given in Fig. 11. It can be seen in Fig. 11 that the desorption curve is cuspidal and without trailing formation. By calculating, the desorption ratios in 13 BV and in 20 BV reach 97.20% and 99.73%, respectively. These desorption data indicate that cholic acid molecules combined on MIP-PVAm/CPVA microspheres are easy to be desorbed or eluted, namely, MIP-PVAm/CPVA microspheres have excellent eluting property. This implies that the surface-imprinted material MIP-PVAm/CPVA is easy to be recovered and reused.

4. Conclusions

In this paper, cholic acid-imprinted material MIP-PVAm/CPVA with high performance has been prepared with the novel surface molecular imprinting technique, and it is more important that the surface-imprinting process was successfully performed on the surface of polymeric microspheres. This is a further development of this new surface-imprinting technique. Henceforth, this surface-imprinting technique not only can be conducted on the surfaces of inorganic particles, but also can be allowed to be conducted on the surfaces of various polymeric microspheres. As long as there are stronger intermolecular interactions between the functional macromolecules grafted onto the surfaces of the two kinds of matrix particles and template molecules, the surface molecular imprinting process can be favorably carried out by employing special crosslinker, in whose molecule there are two reactive end groups. By employing this surface-imprinting technique, various surface-imprinting materials with high performance will be obtained. Thus, the application range of this new surface molecular imprinting technique is extended greatly.

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