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Preparation and protein adsorption of porous dextran microspheres

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ABSTRACT

In present work, porous dextran microspheres with good morphology were synthesized by reversed-phase suspension polymerization. Dextran was used as raw material, epichlorohydrin (ECH) as crosslinker, and dimethyl ether of polyethylene glycol (DMPE) as porogen. And porous dextran microspheres were prepared by freezing–drying method. The morphology of the porous dextran microspheres was characterized by the scanning electronic microscope (SEM). The dry and hydrated densities, average pore volume, porosity, hydroxyl content and equilibrium water content were measured. Micropore structure was found on the dextran microspheres. With the increase of porogen amount, the dry density decreased, the hydrated density, the average pore volume, porosity and equilibrium water content initially increased and then decreased, while the hydroxyl content increased. Bovine serum albumin (BSA) was used as an adsorbate model to examine the adsorption behavior of the porous microspheres. The saturated adsorption capacities of these microspheres ranged from 59.1 mg/g to 138.9 mg/g while the amount of porogen increased from 10% to 50%.

1. Introduction

Recently, polysaccharide microspheres have got much attention because of their low toxicity, good biocompatibility and biodegradability, which are of interest for application in biomedical and pharmaceutical (Tatsuro, Toshifumi, Tomohiro, & Yuichi, 2004). Microspheres including agarose (Gustavsson, Axelsson, & Larson, 1998, 1999), polysucrose (Hou, Yang, Tang, et al., 2006; Hou, Yang, Huang, Wang, & Yao, 2006; Hou, Wang, Gao, & Yang, 2008), and chitosan (Fu et al., 2006; Kim, Kim, Yang, & Cho, 2004; Mi, Shyu, Chen, & Schoung, 1999) have been widely used as supporting materials for protein purification by affinity, ion exchange, and hydrophobic interaction chromatography. However, these hydrophilic microspheres were "gel-type" with low surface area and low porosity, which limited their applications.

Dextran hydrogels are interesting systems for the controlled release of proteins (Stenekes, Franssen, van Bommel, Crommelin, & Hennink, 1999). There are some preparation methods commonly used to prepare dextran hydrogels, including polymerization of metharyloyl groups attached to dextran in all-aqueous system (Hennink, Franssen, Dijk-Wolthuis, & Talsma, 1997), free radical polymerization of aqueous solutions of glycidyl methacrylate derivatized dextran (dex-GMA) (Hennink, Franssen, Dijk-Wolthuis, & Talsma, 1996; Stenekes, Franssen, van Bommel, Crommelin, & Hennink, 1997), and water-in-water emulsion technique (Franssen, Stenekes, & Hennink, 1999).

Vacuum freezing–drying has been introduced for preparing hollow or porous microspheres (Yin & Yates, 2008). Microspheres containing water were frozen and then dried under vacuum. Temperature-induced phase separation in the microspheres induced structured polymer phase domains to form. After being dried, the solvent-rich phase was removed, and then a hollow or porous structure was formed.

The aim of our work is to synthesize hydrophilic dextran porous microspheres by combining inverse phase suspension polymerization with freezing–drying method. In order to improve the adsorption efficiency of dextran microspheres, we chose adding porogen to increase the pore volume and porosity of the dextran microsphere. Here DMPE was chosen as porogen, and the advantages are as follows: Firstly, it does not react with ECH or dextran in this reaction process. Secondly, it is water-soluble and could form homogeneous phase with dextran and water. Due to the phase behavior and the phase separation kinetics of the solution system involving porogen, dextran and solvent, this nonreactive linear polymer porogen allows the porous structure formation by dissolving them in water. The chemical structures and morphologies of the microspheres were characterized. The average pore volume, porosity, hydroxyl content, and equilibrium water content of porous microspheres were investigated as well. Bovine serum albumin (BSA) was used as an adsorbate model to examine the adsorption behavior of the porous microspheres.

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2. Experiments

2.1. Materials

Dextran (40,000, analar) was purchased from Sinopharm Chemical Reagents Company. Chlorobenzene (analar) and epichlorohydrin (analar) were purchased from Tianjin Suzhuang Chemical Regent Factory. Span 80 (analar) was purchased from Tianjin Guangfu Fine Chemical Research Institute. DMPE-2000 was purchased from Jiangsu Tianyin Chemical Regents Company. Sodium hydroxide (analar), pyridine (analar) and ethanol were purchased from Tianjin Jiangtian Chemical Regents Company. Acetic anhydride (analar) was purchased from Tianjin No. 1 Chemical Reagent Factory.

2.2. Preparation of porous dextran microspheres

A mixture of 5 g soluble dextran, 2 g DMPE, 10 g distilled, deionized water (DDW), 4 ml of 50% aqueous NaOH (w/w) and 6 ml of ECH in a 250 ml flask were added 50 ml of chlorobenzene containing 3 g Span 80 to make a W/O suspension system with mechanically stirring. This reversed suspension polymerization was aged at 70 °C for 2 h in a thermostated water bath. Then the temperature was increased to 90 °C for another 5 h. In the end, the microspheres were filtered, washed with ethanol to extract oil from the beads, and then washed by large amounts of DDW. At last, the microspheres were dried at −40 °C in vacuum oven for 24 h.

Procedures of preparing other dextran microspheres with different porogen amounts were similar to the above one. Table 1 shows the detailed experimental formula for preparing porous dextran microspheres.

2.3. Characterization of porous dextran microspheres

2.3.1. The morphology of porous microspheres

The morphologies of dried porous dextran microspheres were observed by Philips XL-30 scanning electron microscope (Philips, The Netherlands). Samples were sputter coated with a thin layer of gold to enhance the surface contrast and reduce the surface charging.

2.3.2. Dry and hydrated densities

Dry and hydrated densities of porous dextran microspheres were measured with a 10 ml pycnometer, using heptane and deionized water as steeps respectively.

2.3.3. Pore volume and porosity of microspheres

Porosity is an important parameter to describe the pore volume and the ability of adsorption of microspheres. The excess surface-adhered water on the microspheres was removed by blotting. The average pore volume V_p and porosity P_r were calculated according to Eqs. (1)–(3):

$$V_w = \frac{W_w - W_d}{\rho w} \tag{1}$$

$$V_p = \frac{V_w}{W_d} \tag{2}$$

$$P_r = \frac{V_w}{V} \tag{3}$$

where V_w was the volume of water in the wet microspheres, W_w was the wet weight of microspheres, W_d was the dried weight of microspheres, ρ_w was the density of water, and V was the volume of wet microspheres.

2.3.4. Hydroxyl content

Acid number was determined through the following procedures: 1.0 g of dried porous microspheres was introduced into ethanol (20 ml). The solution of NaOH (1.0 mol/L) was used to titrate the excess of acetic acid using a phenolphthalein solution as the indicator. Blank titration was performed in the same way to avoid systematic errors. The acid number was calculated according to:

$$acid\ number\ (\mathrm{mg/g}) = 40 \times (V - V_0) \times \frac{c}{m} \tag{4}$$

where V and V_0 were the volume of NaOH solution for the experimental and blank titration, respectively. c was the concentration of NaOH solution (mol/L) and m was the mass of sample (g).

Non-aqueous titration was employed to determine the hydroxyl content in porous dextran microspheres: a pyridine solution of acetic anhydride (20 ml, 10%, v/v) was added with 1.0 g of porous dextran microspheres at room temperature, and then raised to 115 °C to acetylate for 1 h. Afterwards, 5 ml of distilled water was added to the reaction system and the system was kept for another 30 min. The aqueous solution of NaOH (1.0 mol/L) was used to titrate the excess acetic acid using phenolphthalein solution as indicator. A blank titration was performed in the same way to avoid systematic errors. The hydroxyl content was calculated according to Eqs. (5) and (6):

$$hydroxyl\ number\ \ (\mathrm{mg/g}) = 40 \times (V - V_0) \times \frac{c}{m} + acid\ number \tag{5}$$

$$hydroxyl\ content\ \ (\mathrm{mmol/g}) = \frac{hydroxyl\ number}{N} \tag{6}$$

where V_2 and V_1 were the volume of NaOH solution for the experimental and blank titration, respectively. c was the concentration of NaOH solution (mol/L), m was the mass of the sample (g), and N was the mole mass of NaOH (g/mol).

2.3.5. Equilibrium water content

The swelling behavior of the porous dextran microspheres was determined by monitoring the equilibrium water content. The

Table 1
Dry and hydroxyl densities of porous dextran microspheres prepared with different porogen amounts.

Microspheres	Porogen amount (%)	Crosslinker amount (%)	Dry density (g/ml)	Hydrated density (g/ml)
A1	10	50	1.412 ± 0.003	0.959 ± 0.007
A2	15	50	1.400 ± 0.002	0.989 ± 0.010
A3	20	50	1.369 ± 0.004	1.000 ± 0.005
A4	25	50	1.318 ± 0.003	1.004 ± 0.006
A5	30	50	1.316 ± 0.003	1.010 ± 0.009
A6	35	50	1.277 ± 0.003	1.004 ± 0.007
A7	40	50	1.263 ± 0.002	1.002 ± 0.006
A8	45	50	1.254 ± 0.003	1.000 ± 0.007
A9	50	50	1.243 ± 0.004	0.994 ± 0.006

Preparation conditions: W/O ratio (v/v) = 1:5; volume of DDW = 10 ml; temperature = 70 ± 2 °C; stirring speed = 240 ± 10 rpm; porogen amount = porogen/volume of DDW; crosslinker amount = crosslinker/volume of DDW.

equilibrium water content X (%) was to calculated according to Eq. (7):

$$X\ (\%) = \frac{m_2 - m_3}{m_2 - m_1} \times 100 \quad (7)$$

where m_1 was the weight of empty container, m_2 was the weight of wet sample microspheres and container, m_3 was the total weight of dry sample microspheres and container.

2.3.6. Protein adsorption

Bovine serum albumin (BSA) was used as a model protein to test the adsorption behavior of porous dextran microspheres. All adsorption experiments were conducted at 25 °C in 0.01 mol/L Tris–HCl buffer solution. Typically, 0.05 g of porous microspheres was added into 5.0 ml of BSA solution (0.25 mg/ml) for 24 h in a shaking incubator (pH 7.5). A series of the adsorption experiments were carried out with different concentrations (ranging from 0.25 to 2.5 mg/ml) under the same conditions. The BSA standard curve was determined firstly. After centrifugation, the optical density at 280 nm of the supernatant solutions was recorded, and the amount of adsorbed protein was calculated by mass balance according to Eq. (8):

$$Q\ (\mathrm{mg/g}) = (C_0 - C_1) \times \frac{V}{W} \quad (8)$$

where C_0 was the concentration of BSA in the liquid phase before adsorption (mg/ml), C_1 was the concentration of BSA in the liquid phase after adsorption (mg/ml), V was the volume of solvent (ml) and W is the mass of microspheres (g).

3. Results and discussion

3.1. Preparation of porous microspheres

The reaction of dextran and ECH follows the Villiamson Synthesis and nucleophilic substitution under alkaline solution at high temperature in this experiment. Under the alkaline condition, the reaction took place with increasing temperature, dextran and ECH reacted continually to form crosslinked structure due to the multiple active hydroxyl groups of dextran.

The formation process of dextran microspheres was shown in Fig. 1. DMPE was added as porogen, the suspended droplets of dextran and ECH in chlorobenzene were stabilized by Span 80. The phase-separation occurred in the suspended droplets during the process of crosslinking and solidifying. DMPE did not take part in the crosslinking reaction of dextran and ECH in alkaline solution. At last, the microspheres consisting of solid dextran and DMPE were obtained and DMPE was removed by washing. During the process of freeze-drying, water in the microsphere was frozen and sublimated under vacuum, then porous microspheres were obtained.

3.2. Morphology of microspheres

Fig. 2 shows the SEM images of dried porous dextran microspheres with different porogen amounts. It showed that when the porogen amount was 20%, the surface was rough with round cavities and the bead was porous, the pore size was about 1 μm. When the porogen amount reached 30%, the pore size was about 4 μm. It showed that the pore size increased with the increase of the porogen amount, this may be because the DMPE was difficult to be washed when porogen amount was small, while it would become easy when porogen amount increased. And with the increase of porogen amount, the pore increased. This may be because more porogen occupied larger space and when porogen were washed, larger pore was left. This figure also indicted that freezing–drying could maintain the structure of the microspheres.

3.3. Pore volume of porous dextran microspheres

Fig. 3 shows the effects of porogen amount on pore volume of porous dextran microspheres. It was showed that the average pore volume of porous dextran microspheres increased from 15.2 ml/g to 55.9 ml/g with the amount of porogen increasing from 10% to 35%, and when the amount of porogen increased from 35% to 50%, the average pore volume of microspheres decreased a little. Less porogen amount would yield small pore volume and pore volume would increase with increasing porogen amount. However, at some high amount, more porogen molecules would be embedded in the crosslinked network, which resulted in the decrease of average pore volume.

3.4. Porosity of porous dextran microspheres

Fig. 4 shows the effects of porogen amount on porosity of porous dextran microspheres. It was showed that the porosity of porous dextran microspheres increased with the increase of porogen amount. The porosity increased from 85.3% to 98.9% while the porogen amount increased from 10% to 35%. But when the porogen amount increased from 35% to 50%, the average volume of microspheres also decreased a little. This might be because more porogen molecules would be embedded in the crosslinked network, resulting in the decrease of porosity. Besides, too many porogen would destroy the structure of the microsphere, which may also induce a lower porosity.

3.5. Dry and hydrated densities

Dry and hydrated densities of porous dextran microspheres in different synthesis conditions are also listed in Table 1. It was shown that the dry densities of microspheres decreased with the increase of porogen amount, and the hydrated densities increased initially and then decreased, were similar with the density of water. According to Figs. 3 and 4, they showed that the average pore volume and porosity increased with the porogen amount increasing. Therefore,

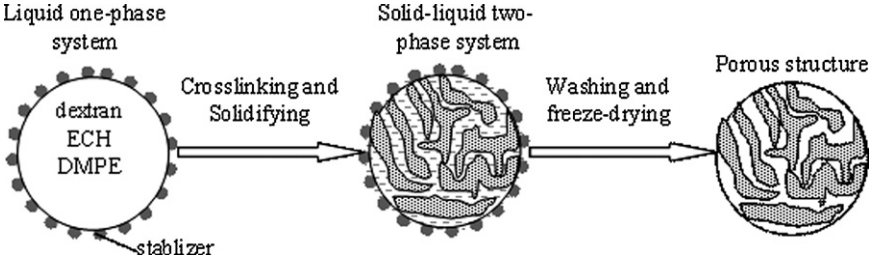

Fig. 1. The scheme of generating pores of dextran microspheres.

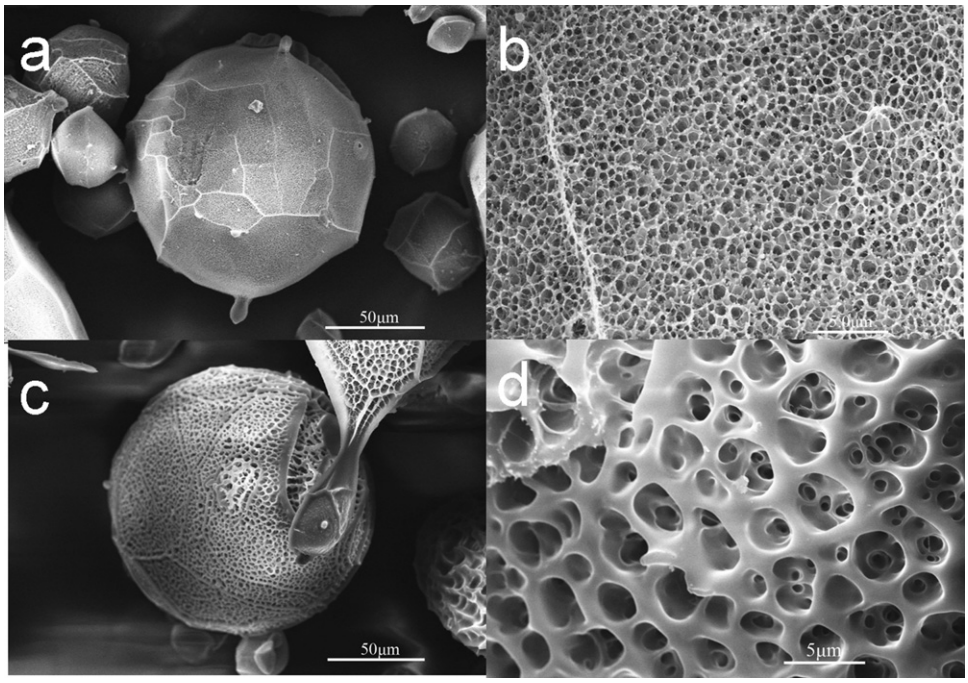

Fig. 2. SEM photographs of dried porous dextran microspheres with different porogen amounts. (a) A3 (×500); (b) A3 (×5000); (c) A5 (×500); (d) A5 (×5000).

the dry density also decreased. As for wet density, when porogen amount was small, it could not form connected pores and there would still be pores in the microspheres after microspheres were immersed into water. Thus, its hydrated density was lower than the density of water, when porogen was reached a certain amount, the microspheres could fully absorb water and the hydrated density was similar to the density of water.

3.6. Hydroxyl content

To determine the loading of hydroxyl groups, the dextran microspheres were acetylated by reacting with an excess of acetic anhydride. The excess acetic anhydride was then converted to acetic acid and titrated by standardized NaOH solution. Fig. 5 shows the effects of porogen amount on hydroxyl content of porous dextran microspheres. The loading of hydroxyl groups of microspheres increased from 17.1 to 23.7 mmol/g with increase of porogen amount. During the polymerization of dextran and ECH, each ECH molecule could consume two hydroxyl groups of dextran and generate a new one, which leaded to the decrease of hydroxyl groups. However, porogen molecules decreased the opportunity of the reaction between the soluble dextran and ECH in the droplets, leading to accessibility of active hydroxyl groups on the microspheres.

3.7. Equilibrium water content

Fig. 6 shows the dependence of equilibrium water contents in microspheres on the porogen amounts added in the preparation of microspheres. The results indicated that equilibrium water

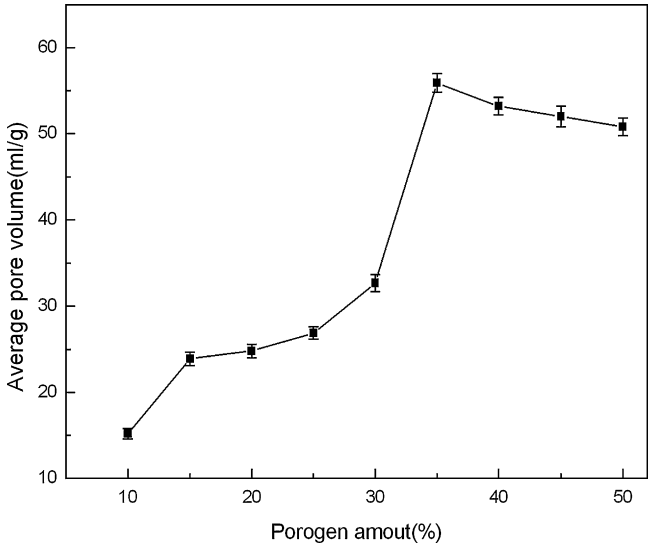

Fig. 3. Effects of porogen amount on average pore volume of dried porous dextran microspheres (A1–A9).

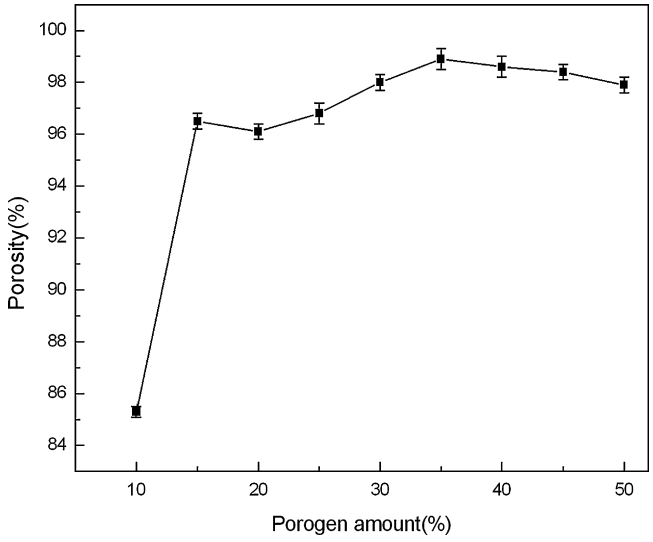

Fig. 4. Effects of porogen amount on porosity of dried porous dextran microspheres (A1–A9).

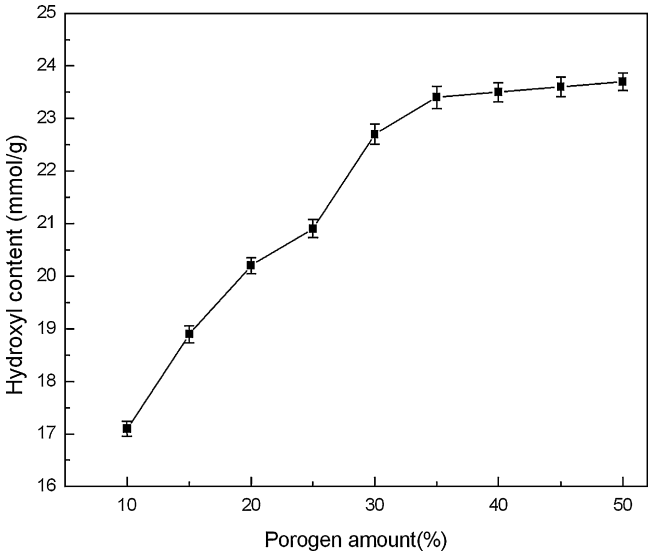

Fig. 5. Effects of porogen amount on hydroxyl content of porous dextran microspheres (A1–A9).

contents of microspheres increased with the increase of porogen amount. The average pore volumes, porosities and hydroxyl content of the microspheres increased with increasing porogen amounts. Therefore, the equilibrium water contents of microspheres increased, and equilibrium water contents showed a sharp increase when porogen amount increased from 10% to 15%, and a little decrease when porogen amount increased from 35% to 50%.

When porogen amount was 10%, there were only a few pores in the microspheres and when porogen amount was 15%, it would form larger holes which could connect with the surface of the microspheres. With the increase of porogen amount, the equilibrium water content increased, when porogen amount reached about 35%, the pores in microspheres were maximized. A larger amount of porogen would destroy the structure of microspheres, which affected the equilibrium water contents of dextran microspheres.

The overall mechanical strength of microspheres was low and their swelling properties were poor, which might be due to the presence of the porous structure and the unfully cross-linked

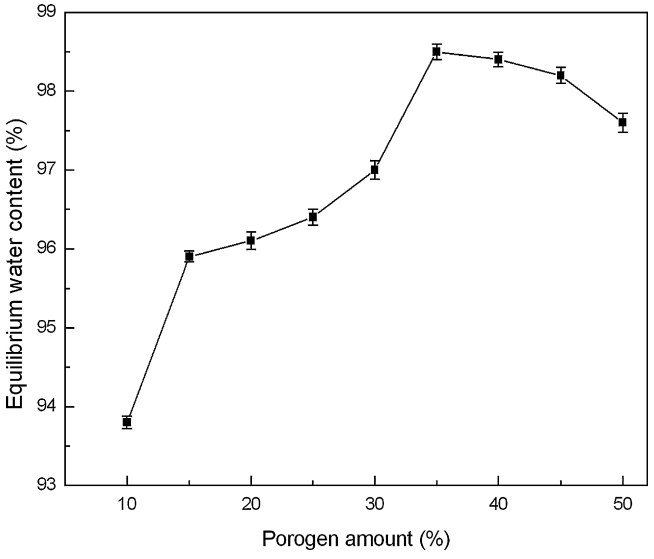

Fig. 6. Effects of porogen amount on equilibrium water content of porous dextran microspheres.

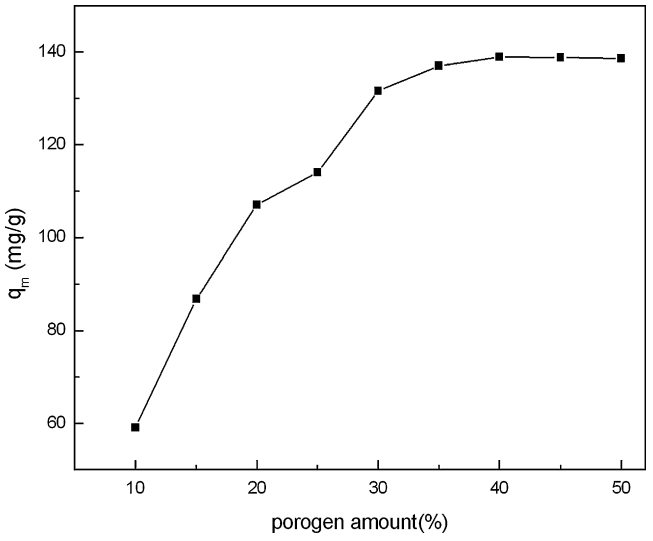

Fig. 7. Effect of porogen amount on saturated adsorption capacity of porous dextran microspheres.

structure. So the crosslinking density of dextran microspheres increased when the amount of crosslinking agent increased. The absorption capacity of free water gradually increased and the equilibrium moisture content also increased.

3.8. Protein adsorption

The adsorption behavior of the porous dextran microspheres for BSA can be described by the Langmuir equation:

$$q = \frac{q_m c}{K_d + c} \tag{9}$$

where c (mg/ml) was the equilibrium concentration of BSA in bulk solution, q (mg/g) was the adsorption quantity of microspheres, q_m was the saturation capacity, and K_d was the dissociation constant. The parameters in the Langmuir equation were estimated by fitting the equation to the experimental results using the least-square regression (Zhou, Xue, Bai, & Sun, 2002).

The adsorption capacities of microspheres behaved different trends showed in Fig. 7 as porogen amounts increased. The trend of the curve of saturation capacity to porogen amount was similar to that of the hydroxyl content to porogen amount. When the average pore volume and porosity increased with increasing porogen amount, the microspheres with the larger pore volumes and porosities were favorable for protein adsorption. And the hydroxyl group of microspheres also played an important role in the adsorption of protein. Due to their hydrophilicity and the formation hydrogen bonds between microsphere and protein, the trends of adsorption capacities of microspheres were similar to that of hydroxyl contents. The results demonstrated that the hydroxyl contents and porosities of porous microspheres were both important for protein adsorption of the porous beads.

4. Conclusions

A set of novel porous dextran microspheres were prepared by the inverse suspension polymerization and freezing–drying method, using soluble dextran as raw material, epichlorohydrin (ECH) as crosslinker and dimethyl ether of polyethylene glycol as porogen. The beads have spherical shapes and porous structures.

When porogen amount was 10%, there was no pore on the surface of the microsphere, with the increase of porogen amount, connected pores formed, the dry densities decreased, the average

pore volume, porosity, and the equilibrium water content increased initially and then decreased, the hydrated densities increased. The saturated adsorption capacities of the porous microspheres for bovine serum albumin as model ranged from 59.1 mg/g to 138.9 mg/g. But it was not that the more the porogen amount added, the better characterization the porous microspheres gained. When porogen amount exceeded 35%, due to the excess DMPE, it would hinder the crosslink process of ECH and dextran, many characterizations, such as pore volume, porosity, and equilibrium water content of porous dextran microspheres, would decrease.

Acknowledgements

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