Preparation of Amino-Reserved Magnetic Chitosan Microsphere and Its Application in Adsorbing Endotoxin

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ABSTRACT: In this article, we present a novel amino-reserved magnetic chitosan microsphere (ARMCM) prepared from chitosan and Fe_3O_4 through inverse suspension crosslinking by formaldehyde and epichlorohydrin. The density of ARMCM was 1.47 g/mL, and the amino content was 2.44 mmol/g. The measurements data indicated that the saturation magnetization (σ_s) of ARMCM was 23.2 emu/g. The adsorption results showed that these magnetic chitosan microspheres had good adsorption capacity on endotoxin: the maximum absorption capacity was about 1792.1EU/g in 40 min; the endotoxin adsorption efficiency in the protein solution was close to 80% and what is more, protein adsorption efficiency was only 23.2 and 2.0%, respectively, for acidic and basic proteins. It is expected that this ARMCM will have a feasible and useful application in adsorbing endotoxin from biological products. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: biomaterials; ion exchangers; magnetic polymers; separation techniques

INTRODUCTION

The applications of magnetic polymer microspheres became a new topic of research since the successful synthesis of Dynabeads by Professor Ugelstad (Norway).[1] Magnetic polymer microsphere indeed has its strength because it can be benefit from both components: magnetic particles and polymer. The magnetic particles make the rapid and facile separation possible in an external magnetic field; due to the functional groups on the polymer (such as —NH₂, —OH, and —COOH), the microspheres also can be combined with bioactivators such as enzyme, oligonucleotide, and antibody.[2–4] Thus, the magnetic polymer microspheres have great potential applied value in biological areas. Chitosan (CS) is a polyaminosaccharide, normally obtained from crustacean shells, insects, molluscan organs, and fungi.[5] The excellent biological and chemical properties such as biodegradability, biocompatibility, and bioactivity, especially susceptibility to chemical modification due to its reactive amino and hydroxyl functional groups endow chitosan with significance.[6–8] In addition, chitosan is the second abundant natural copolymer of the world next to cellulose and appears economically attractive. Thus, it has been widely applied in many areas including agriculture, environment, pharmaceuticals, medicines, and food processing.[9–13]

Endotoxin is an integral part of the outer cell membrane of Gram-negative bacteria whose general structure is a polar heteropolysaccharide chain, covalently linked to a nonpolar lipid moiety (lipid A) as showed in Figure 1.[14] Endotoxin shows strong biological effects at very low concentration in blood system of human beings as well as animals, for example, during a bacterial infection or via intravenous application of a contaminated medicament.[15] Many separation methods are developed for removing endotoxin based on its physicochemical properties.[16–19] Chitosan, containing free amino groups, has been successfully applied in adsorbing endotoxin because the free amino on chitosan could combine with the phosphate groups on endotoxin in solution. However, the chitosan used for endotoxin adsorption was mostly made into membranes,[20] because most crosslinkers will take reaction with amino groups during the process of microsphere synthesis, and the applications are limited. In this study, a novel microsphere with magnetic particles and chitosan was prepared to adsorb endotoxin. The functional group was the original amino, which was protected during the synthesis process on the chitosan. And what is more, this microsphere can be rapidly and easily separated from sample solution simply by the application of an external magnetic field.

MATERIALS AND METHODS

Materials

Chitosan ($M_W = 3.0 \times 10^6$; N-deacetylation, 80%) was purchased from Zhejiang Aoxing Biotechnology
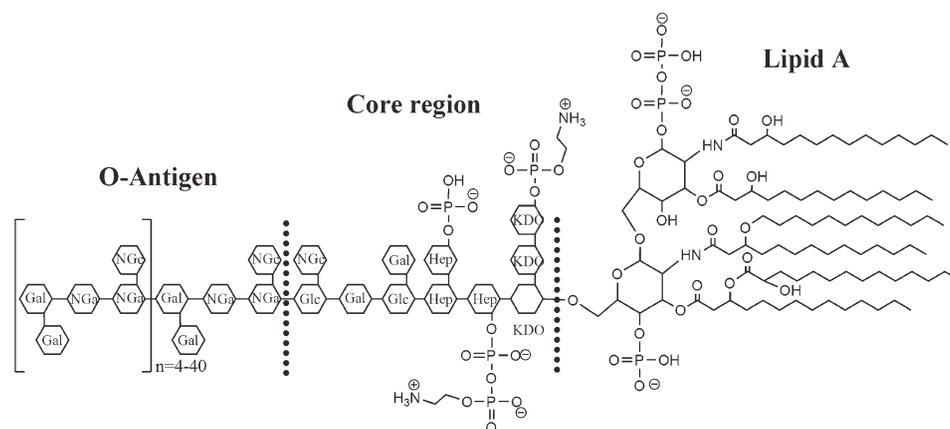
Figure 1 Schematic view of the chemical structure of endotoxin from *E.coli* O111. Hep, L-glycero-D-manno-heptose; Gal, galactose; Glc, glucose; KDO, 2-keto-3-deoxyoctonic acid; Nga, *N*-acetyl-galactosamine; NGc, *N*-acetyl-glucosamine.

(Zhejiang, China). Fe_3O_4 was purchased from Aladdin (Shanghai, China). Endotoxin standards, Limulus amoebocyte lysate (LAL) for endotoxin detection and endotoxin-free water were purchased from Xiamen Horseshoe Crab Reagent Manufactory (Xiamen, China). Bovine serum albumin (BSA) and lysozyme (LYZ) were purchased from Sangon (Shanghai, China). DEAE-Sepharose was purchased from GE Healthcare. Span-80 and Tween-80 were purchased from Wenzhou Qingming Chemical (Zhejiang, China). Other chemicals were of analytical grade.

Preparation of amino-reserved magnetic chitosan microsphere

Amino-reserved magnetic chitosan microsphere (ARMCM) were prepared by inverse suspension crosslinking as follows: a 3% (w/v) Chitosan solution was prepared by 3.0 g chitosan powder and 100 mL 2% aqueous acetic acid solution containing 1.5 g Fe_3O_4 particles. After ultrasonic dispersion for about 30 min, the mixture was poured slowly into the suspension medium, which was composed of 100 mL paraffin oil and five drops of emulsifier (Span-80). During these processes, the suspension medium was stirred with a mechanical stirrer at 400 rpm at 40°C. After 30 min, 6.0 mL formaldehyde solution (40% v/v) was added in the mixture solution, with continuous stirring for another 1 h. Then added 50 mL petroleum ether and poured off the upper organic phase, 100 mL water containing 5 drops hydrophilic emulsifier (Tween-80) was added instead and meanwhile, the pH value of the mixture was adjusted to 12–13 by 10% NaOH solution, the temperature was changed to 70°C. After stirring for 1 h, 4.0 mL epichlorohydrin as crosslinker was added and kept stirring for another 2 h. The products were filtered and washed consecutively with ethanol and distilled water several times. Magnetic chitosan microspheres (MCM) were then obtained.

Then the products were immersed in an excess amount of 0.5 mol/L HCl solution over night. After washing with distilled water, the products were immersed in 1.0 mol/L NaOH solution for another 4 h. At the end of the period, the microspheres were filtered and washed with distilled water until the pH value of filtrate reached neutral, which can be detected by phenolphthalein. The final products were named as ARMCM. The reaction was illustrated in Scheme 1.

Characterizations of the microspheres

Shape, size distribution, and magnetic property

Particle shape and size distribution of the ARMCM were observed and analyzed by scanning electron microscope (SEM; Philips, Netherlands) and zetasizer (Mastersizer 2000). The magnetization curve was recorded by physical property measurement system (PPMS; Quantum Design, America).

Density

The density was determined by pycnometer method and can be calculated on the following formula:

$$\rho = \frac{M_2 \rho_0}{M_1 + M_2 - M_3} \tag{1}$$

where M_1 is the weight of pycnometer filled with water (g), M_2 is the weight of wet sample after filtration (g), M_3 is the net weight of the whole after the sample added into the water filled pycnometer (g), and ρ_0 is the density of water.

Amino content

The amino content (S, mmol/g) on the microsphere can be evaluated by acid-base titration method. About 0.05 g of sample was dispersed in 10.0 mL 10.0 mmol/L standard solution of hydrochloric acid for about 30 min, then accurately measured 8.0 mL
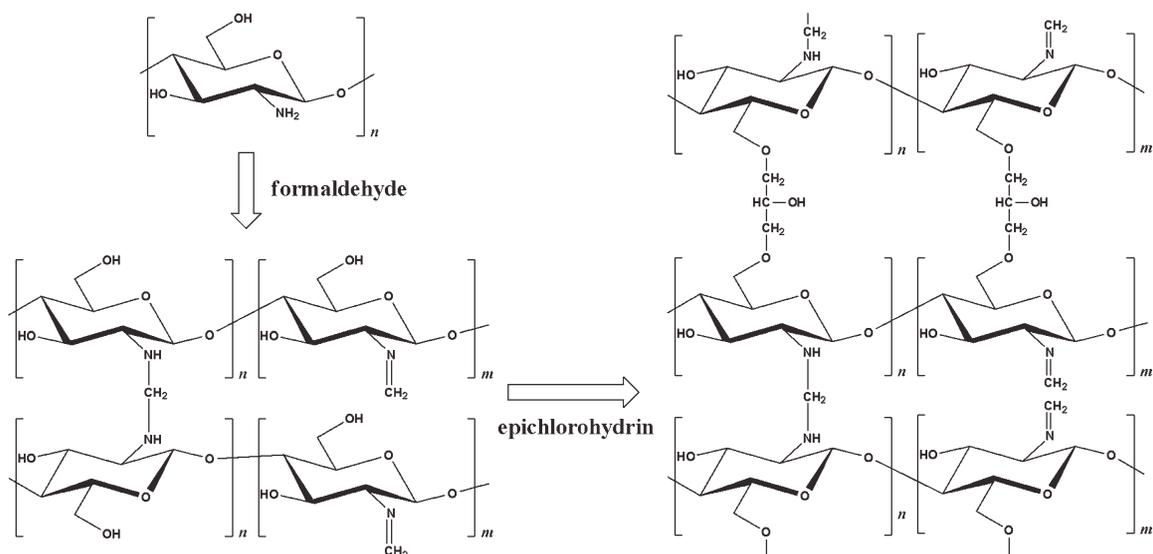
Scheme 1 Synthesis of ARMCM.

solution into a conical flask, added a little methyl orange as indicator. Titrated until end point with 10.0 mmol/L standard solution of sodium hydroxide. The amino content was calculated as follows:

$$S = \frac{10 \times 10 - 1.25 \times 10 \times \Delta V}{1000 \times W} \quad (2)$$

where ΔV is the volume of standard solution of NaOH used in the titration (mL); W is the weight of sample (g); for only 8.0 in 10 mL was measured for determination, the factor 1.25 should be multiplied.

Weight percentage of Fe_3O_4

The weight percentage of Fe_3O_4 can be measured like determination of ash content: first, place the crucibles in the muffle furnace at 550–600°C for 4 h, after cool for a period of time, weigh the crucibles; second, repeat the first step until the weight change are <0.3 mg; third, weigh 0.5 g dry microspheres into the crucible and record the weight to the nearest 0.1 mg, place the crucible in the muffle furnace and then, do as described in step one and step two, the weight percentage of residue remaining can be calculated as follows:

$$W_t\% = \frac{W_w - W_c}{W} \times 100\% \quad (3)$$

where W_w is the whole weight of crucible and the residue remaining (g); W_c is the weight of crucible (g); W is the weight of dry microspheres (g).

Swelling ratio in water

Dry microspheres were immersed in an excess amount of distilled water for 1 h at room tempera-

ture. After removing the surface water with filter paper, the wet weight of microspheres was measured and the swelling ratio(SR) was calculated on the following formula:

$$SR = \frac{W_w}{W_d}, \quad (4)$$

where W_w is the wet weight of microspheres (g) and W_d is the dry weight of microspheres (g).

Adsorption experiments

Adsorption kinetics

ARMCM (25 mg) were added to the 73.7EU/mL endotoxin solution (2 mL), shook at room temperature, and determined the endotoxin content at 10, 20, 30, 40, and 50 min, respectively.

Determination of adsorption isotherm

Endotoxin solutions (2 mL) with six different concentrations were prepared and added 25 mg microspheres respectively. Shook at room temperature for 40 min and determined the endotoxin content.

Adsorption of endotoxin in protein solution

BSA and LZY were used as acidic protein and basic protein in the experiment. ARMCM (25 mg) were added to protein solution (2 mL, 2 mg BSA, and 18 EU endotoxin as acidic protein solution, 1 mg LZY and 23 EU endotoxin as basic protein solution), shook at room temperature for 40 min. The protein content was determined by Coomassie brilliant blue stain and spectrophotometry. The amount of adsorbed endotoxin was calculated from the
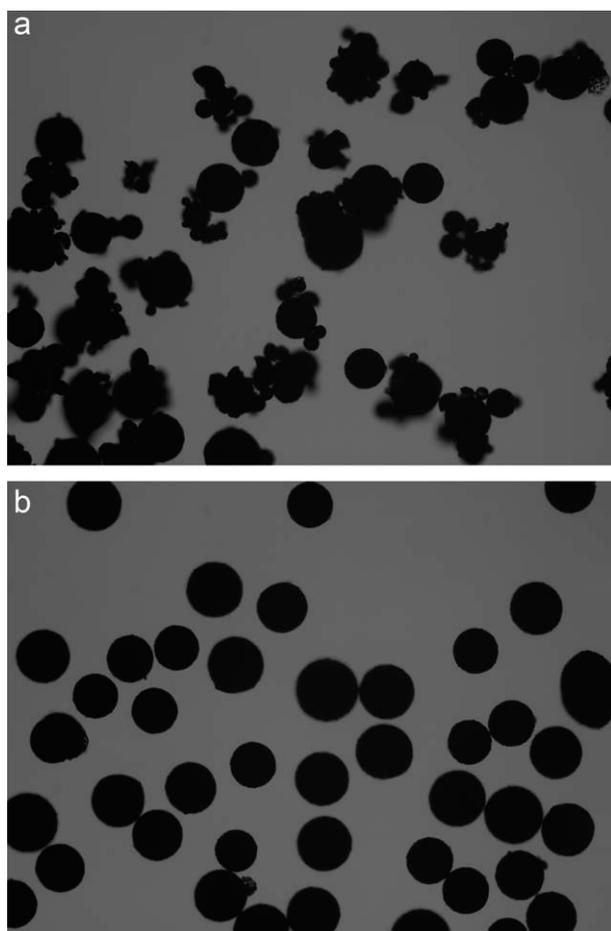
Figure 2 Optical micrographs of ARMCM.

differences between the contents of endotoxin before and after the adsorption. Another anion-exchanger DEAE-Sepharose was also used in contrast with ARMCM.

The adsorption capacity was calculated as follows:

$$Q_e = \frac{(C_0 - C_e)V}{W} \qquad (5)$$

where Q_e is the equilibrium adsorption capacity of the sample (EU/g); C_0 and C_e are the concentration of endotoxin before adsorption and after adsorption (EU/L); V is the volume of solution (mL); and W is the weight of sample (g).

The endotoxin content was determined using a quantitative, chromogenic LAL-assay (endpoint method). Samples were diluted appropriate factor by pyrogen-free water, and then added the LAL reagent following the instructions of the supplier. It is a chromogenic reaction and the endotoxin content could be calculated according to the standard curve and the absorbance value at 575 nm. All assays should be performed as described by the manufacturer's instructions.

RESULTS AND DISCUSSION

Characterizations

Morphology and size distribution of the microspheres

Inverse suspension crosslinking is a common and useful method in preparing microspheres. During the process, the emulsifier is important to form water in oil (W/O) system to keep the microspheres steady and spherical, after crosslinking, it will be
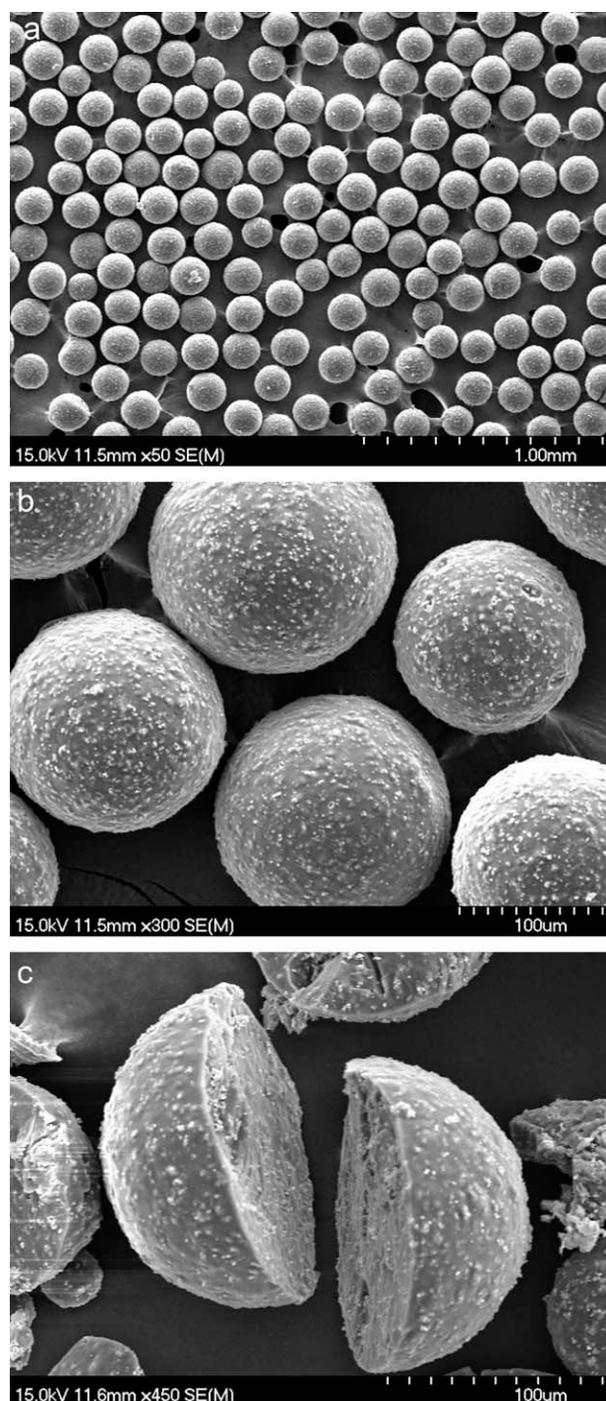
Figure 3 The SEM micrographs of ARMCM.
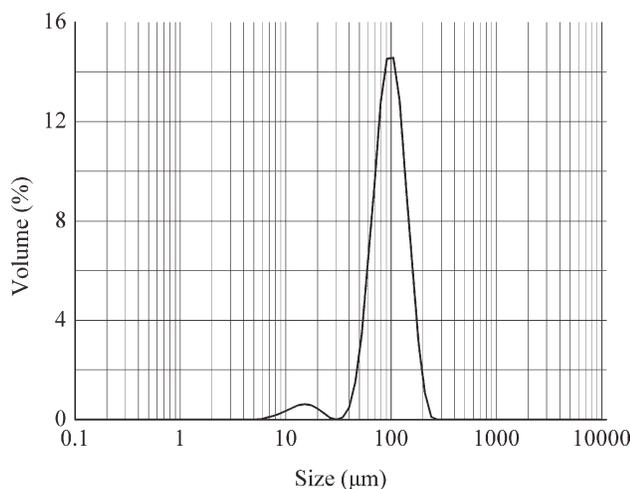
Figure 4 Particle size distribution of ARMCM.

Figure 5 Magnetization curve of ARMCM.

removed. However, we found that in the process of removing emulsifier, some microspheres will bond together, especially using saponification to resolve the emulsifier. Thus, after crosslinking, we took one more step to add hydrophilic emulsifier to form W/O/W system to increase the stability and dispersibility of the microspheres in the mixture. Then made saponification reaction to remove the emulsifier. As can be seen in Figure 2, the result also showed the improvement of the additional step, there was no bonding at all.

The SEM micrographs were given in Figure 3. The microspheres had spherical shape and there were some Fe_3O_4 particles on the surface from Figure 3(a,b). Figure 3(c) showed the section of the microspheres, Fe_3O_4 particles were uniformly distributed in the microspheres and macropores were not seen. Figure 3(a) also showed that the mean diameter of the microsphere was 100–150 μm, which corresponded with the size distribution in Figure 4.

Magnetic property

Figure 5 showed the magnetization curve of the microspheres recorded by PPMS. The saturation magnetization (σ_s) of the MCM was about 23.2 emu/g, which was 26.2% of the pure magnetite particles. This was quite close to the result of 28.4% of Fe_3O_4 weight percentage described below and this suggested that chitosan would hardly endow negative effect on magnetic property. Figure 5 also indicated that the remanence of ARMCM is 3.1 emu/g, which is lower than that of Fe_3O_4 particles. This remanence is so low that magnetic interactions among the particles is very weak and negligible, so ARMCM can be used in biomedical and bioengineering fields.

Other physicochemical properties

The last step of preparation in the HCl solution over night was to deoxidate the schiff base formed by —NH_2 and formaldehyde. So that the —NH_2 can be used as a functional group to adsorb endotoxin. However, after dealing with HCl solution, not only the amino content changed, but also the density, swelling ratio and weight percentage of Fe_3O_4. The physicochemical properties of the microspheres before (MCM) and after (ARMCM) dealing with HCl solution were given in (Table I). HCl would also take reaction with Fe_3O_4 as we all know. Thus, the weight percentage of Fe_3O_4 would reduce to 28.4% and the density decreased from 1.68 to 1.47 g/mL consequently. The amino content was up to 2.44 mmol/g after dealing with HCl solution, get rid of the 28.4% of Fe_3O_4, the amino content on chitosan was 3.41 mmol/g, which means the reserved rate can reach 69.5% (the amino content on the original chitosan was 4.90 mmol/g determined by the same method). Amino is a hydrophilic group, obviously, with the increase of amino content, the swelling ratio would rise to 2.80 after dealing with HCl.

Evaluation for endotoxin adsorption

Adsorption kinetics

Figure 6 showed the adsorption capacity of endotoxin at different time intervals when the ARMCM

TABLE I
Physicochemical Properties of ARMCM

	Amino content (mmol/g)	Density (g/mL)	Swelling ratio (g/g)	Fe_3O_4 content (%)
MCM	1.71	1.68	2.30	29.7
ARMCM	2.44	1.47	2.80	28.4
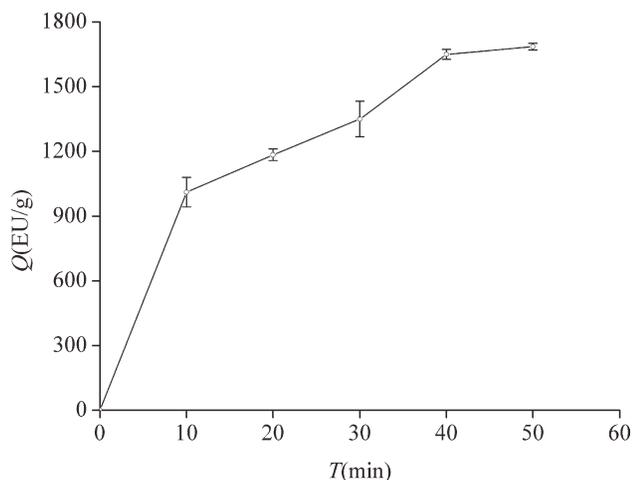
Figure 6 Relationship between adsorption capacity (Q) and adsorption time (T).

dispersed in the solution. With the time going on, the adsorption capacity increased and the equilibrium time was about 40 min. The adsorption capacity at initial concentration of 73.7 EU/mL was 1649.7 EU/g, which was similar with the result of the adsorption isotherm in Figure 7.

Adsorption isotherm

The result of ARMCM adsorbing in different concentration of endotoxin solution was given in Figure 7. As we know, higher concentration is favorable to increase the adsorption capacity. However, in Figure 7, when the initial concentration exceeds about 30EU/mL, the adsorption capacity tends to the same value, and this is the maximum adsorption capacity. The Langmuir plot ($C_e/Q_e = C_e/Q_m + 1/kQ_m$) was also showed in Figure 8. The adsorption isotherm was quite fit the Langmuir model ($y = 0.000558x +$
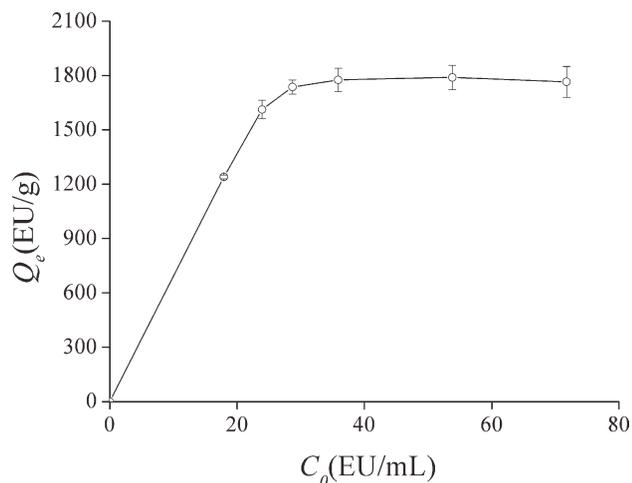
Figure 7 Relationship between adsorption capacity (Q_e) and initial concentration (C_0).
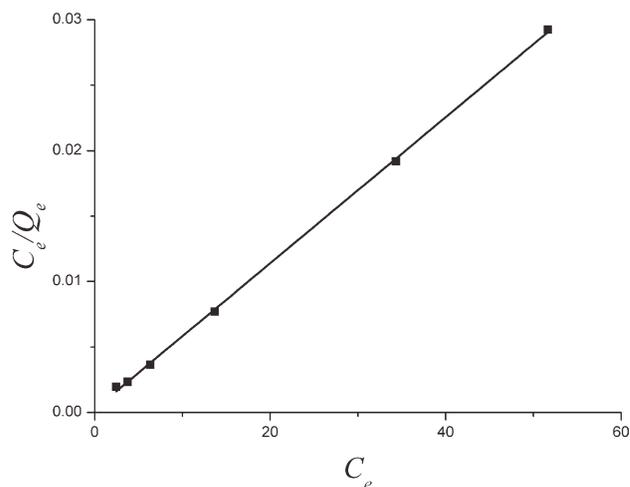
Figure 8 The Langmuir fitted curve.

0.00025), the R^2 was 0.999 and the maximum adsorption capacity (Q_m) was 1792.1 EU/g. The Langmuir model means this was monolayer adsorption and each functional group adsorb only one endotoxin molecule. The maximum adsorption capacity would be attained when every functional group had adsorbed endotoxin molecule. But there is another situation, because the adsorbent prepared in this article was not macroporous, the endotoxin molecule may not enter into the inside of the adsorbent. In another words, many functional groups inside the adsorbent did not get the full used. So, although the maximum adsorption capacity was determined, many methods can be used to increase this value.

Adsorption in protein solution

Theoretically speaking, the adsorption mechanism of ARMCM was ion exchange by ─$NH_3^+OH^-$ because endotoxin bears negative charges. Thus, the acidic proteins that also have negative charges in the water might compete with endotoxin adsorbing onto the
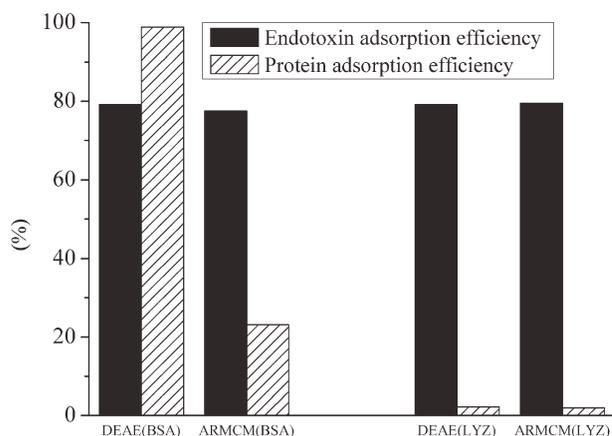
Figure 9 Adsorption efficiency in protein solutions.
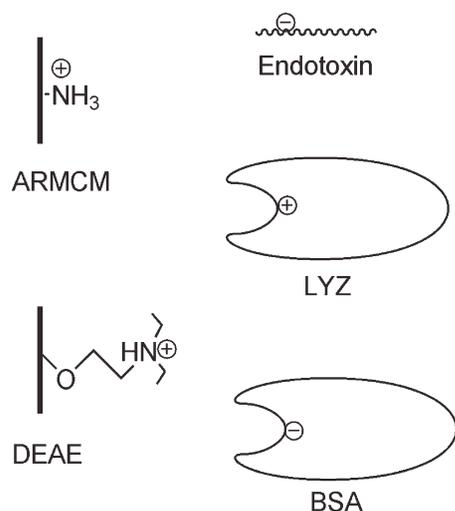
Figure 10 Structures of the adsorbents and biomolecules.

adsorbent. However, the results of adsorption in protein solution (Fig. 9) indicated that ARMCM had the similar endotoxin adsorption efficiency (the ratio of adsorbed and initial amount) of about 80% with anion-exchange agent DEAE-Sepharose. The protein adsorption efficiency of ARMCM was 23.2% in the acidic protein solution, which was much lower than that of DEAE-Sepharose (98.9%). It was expected and this result may caused by the steric hindrance because $-NH_3^+OH^-$ was an original group on the matrix and there was no space between the group and the sugar unit, on the contrast, DEAE-Sepharose had a arm between the functional group and the matrix, which was shown in Figure 10. Therefore, proteins whose molecules were much bigger than endotoxin were more difficult to be adsorbed to ARMCM attributed to their space structures. In the basic protein (bears positive charges in water) solution, both ARMCM and DEAE-Sepharose adsorbed few proteins, the adsorption efficiency was 2.0 and 2.2% because the proteins and the adsorbent both took positive charges and there were no interattraction and adsorption. These proved that the adsorption mechanism of ARMCM was ion exchange like DEAE-Sepharose and ARMCM had more advantages in removing endotoxin in acidic protein solution compared with DEAE-Sepharose.

CONCLUSIONS

A novel ARMCM was prepared by inverse suspension crosslinking and the reserved rate of amino used as the functional group was 69.5%. The morphological and other physicochemical analysis also indicated that ARMCM can be good used as an ion exchange agent for endotoxin adsorption. The adsorption equilibrium time was about 40 min and the maximum adsorption capacity was about 1792.1 EU/g. The results of its application in adsorbing in protein solution proved that ARMCM had a good endotoxin adsorption efficiency of about 80% and a good protein recovery, no matter in acidic protein solution or in basic ones. What's more, the imported magnetic material made more conveniences during the separation phase of the adsorbent. In a word, ARMCM prepared in the article had potential application value in adsorbing endotoxin from biological products.

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