The Preparation of Functionalized Crosslinked Macroporous Chitosan Microspheres and their Adsorption Properties for Bilirubin

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Summary: glutaraldehyde cross-linked macroporous chitosan microspheres (CS) were prepared by inverse phase suspension reaction with sugar as porogenic agent. The microspheres were modified with different reagents of 1, 6 hexanediamine (HDA) and low generation polyamidoamine (PAMAM) dendrimers including PAMAM G1.0, PAMAM G2.0, PAMAM G3.0. The content of amino groups on CS, CS-PAMAM G1.0, CS-PAMAM G2.0, CS-PAMAM G3.0, CS-HDA was 3.56, 5.10, 5.47, 6.47, 4.66 mmol/g, respectively. The bilirubin adsorption on the above five microspheres was carried out in 0.05M phosphate buffer solution (pH = 7.2–7.4) at 37 °C. The results indicated all the modified CS microspheres were better than unmodified CS microspheres for bilirubin adsorption. CS-HDA has the best adsorption property even if the content of the amino groups was not very high.

Keywords: bilirubin adsorption; chitosan microsphere; hexanediamine; polyamidoamine (PAMAM)

Introduction

At present, the available therapy for the gravis type hepatitis and hepatic failure patients is liver transplantation and ALSS[1–17] (artificial liver support system) including hemodialysis, hemofiltration, hemoperfusion, plasmadialysis, plasma exchange (plasmapheresis),[9] plasma perfusion, hemodiafiltration, molecular adsorbent recycling system (MARS),[1–4,8,10,11,15,16] Prometheus system[1,4,10] and bioartificial liver support system,[1,2,4,5,7,8,10,13,14] et al. So far, the most effective established treatment is liver transplantation.[8,9,14] However, gravis type hepatitis and hepatic failure patients still have a high mortality rate because of the worldwide shortage of organ donors.[11] Moreover, problems associated with liver transplantation such as immunological rejection may lead to failure of the graft and necessitate retransplantation. In contrast, the ALSS can be effectively used as a 'bridge' for patients to recovery by their own hepatocyte regeneration or to liver transplantation.

An ideal extracorporeal liver support system has to provide the main functions of the liver: detoxification, synthesis and regulation.[3,10] The detoxification function using adsorbents plays a key role among the three main functions whether in biological or 'non-biological' ALSS. It aims at removing water-soluble and/or lipophilic protein-bound toxins in the blood associated with liver failure, such as bilirubin, bile acids, ammonia, and nitric oxide, metabolites of aromatic amino acids and medium-chain fatty acids. Adsorbents play a key role in the detoxification of ALSS.

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Bilirubin is the metabolism product of the heme with a molecular weight of 584. Its chemical structure is shown in Scheme 1. There are two kinds of bilirubin in human body, i.e. free bilirubin and conjugated bilirubin. The former is lipophilic and the latter is water soluble. The normal concentration of bilirubin in human body is about 10 mg/L. But the concentration of bilirubin would be raised and lead to hyperbilirubinemia when the function of the liver was damaged. It would greatly destroy the organ of the brain and lead to hepatogenic encephalophathy. The investigation of the bilirubin adsorbents are the heat research topics for ALSS.

Since the late 1970s, many scientists have been engaged in developing various adsorbents for bilirubin removal.[18–39] The reported adsorbents were mainly made from natural polymers, synthetic polymers and amorphous materials. For example, the natural polymers are agar,[19] β-cyclodextrin,[20,21] polylysine membranes,[27] cross-linked chitosan resins,[6] et al. The synthetic polymers are cross-linked Poly(2-hydroxyethylmethacrylate) resins,[22,23] guanidine immobilized cross-linked polyvinyl alcohol gel,[24] bilirubin-imprinted polymer,[28–31,37,39] dye-affinity microbeads[25] or membrane,[26,29] polytetrafluoroethylene capillary,[32,35] PEI-GMA/AM/MBA terpolymer microbeads,[34] et al. The amorphous materials are activated charcoal,[18] carbon nanotube,[38] titania films[33] or particles,[36] et al.

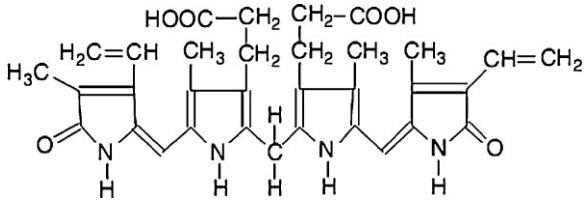
The adsorbents used in blood purification must be safe to bodies, possess stable chemical property, good biocompatibility, large adsorption capacity and high mechanical strength. So far, the satisfying clinical effect of used adsorbents has not been developed. Poor blood compatibility and low adsorption capacity are the main problems of the present used adsorbents in clinical. So in this paper, the biocompatible natural polymer chitosan was used as materials, 1, 6 hexanediamine (HDA) and low generation polyamidoamine (PAMAM) dendrimers as amination reagent, to prepare a series of functionalized cross-linked macroporous chitosan microspheres with relatively high mechanical strength. Their adsorption properties for bilirubin in 0.05M phosphate buffer solution (pH = 7.2–7.4) were studied.

Experimental Part

Materials

Bilirubin was provided by Haihe Hospital, Tianjin, China, medical-grade. Chitosan was purchased from Jinke biochemical corporation, Zhejiang, China, medical-grade, with 85% (w/w) degree of deacetylation and a molecular weight of $10.6^{*}10^{4}$. Sugar was bought from the super market, Tianjin, China, containing 97.95% (w/w) sucrose. PAMAM G1.0, PAMAM G2.0, PAMAM G3.0 were prepared with ethylenediamine and methylacrylate according to the method reported previously.[40] Half generations were ester terminated (Gn.5) and full generations were amine terminated (Gn.0). All other chemicals were of analytical grade and purchased from chemical reagent sixth factory, Tianjin, China.

Bilirubin solution (100 mg/l) was prepared by dissolving 5 mg of bilirubin into 3 ml of 0.1M sodium hydroxide aqueous solution and diluting to 50ml with 0.05M



Scheme 1.
Chemical structure of bilirubin.

phosphate buffer solution (pH = 7.2–7.4) before use in a dark room. All solutions were stored in amber glass vials wrapped with aluminum foil and placed in the dark to prevent light-initiated bilirubin oxidation. Bilirubin solution (pH = 7.2–7.4, 37 °C) was used in our experiments, unless otherwise stated.

Scanning Electron Microscope (SEM) S-3500N (HITACHI) and Fourier Transform Infrared Spectroscopy (FT-IR) FTS 6000 (Bio-Rad) were used to characterize the structure of functionalized crosslinked macroporous chitosan microsphere. The refractor index of the eluate for removing the porogenic agent in microspheres was determined with Abbe Refractometer (SYSBERY). Bilirubin adsorption experiments were carried out in a constant temperature immersion oscillator WE-1. The concentration of bilirubin was determined by a UV-2450 spectrophotometer (SHIMADZU).

Preparation of Functionalized Crosslinked Macroporous Chitosan Microspheres

Preparation of Chitosan Microspheres

100 ml mixtures of carbon tetrachloride and liquid paraffin (v/v = 1:1) containing 0.3ml of span-80 were added to a 250 ml three-neck flask with electric stirring. 50 ml mixtures of chitosan acetic acid (2%, v/v) aqueous solution (5%, w/w) and sucrose aqueous solution (50%, w/w) (v/v = 3:1) were added to the reaction with a stirring speed of 80 rpm for 40 minutes. The chitosan aqueous solution was dispersed into uniform beads with appropriate size. 0.6 ml of formaldehyde aqueous solution (37%, w/w) was dropwise added to the reaction. After 1.5 h 1.3 ml of glutaraldehyde aqueous solution (50%, w/w) were added dropwise to the reaction. Changing the amount of glutaraldehyde can get different cross-linked chitosan microspheres. After 1.5 h of gelation, sodium hydroxide aqueous solution (5%, w/w) was added dropwise to adjust pH value of the reaction to slightly alkaline. The reaction temperature was raised to 40 °C keeping for 3h, and then raised to 60 °C for 12 h of solidification. The product was leached and washed to neutual with deionized water and extracted with petroleum ether and ethanol for 8h, respectively, to remove liquid paraffinn and span-80 on the surface of chitosan microspheres, respectively. Chitosan microspheres were immersed and washed repeatedly with 70 °C hot water to remove the porogenic agent of sugar in microspheres until the refractive index of the eluate was similar to deionized water. CS microspheres were removed the formaldehyde protection with 0.5M HCl at room temperature for 24 h to release more amino groups. Finally microspheres was dried under the vacuum at 40 °C.

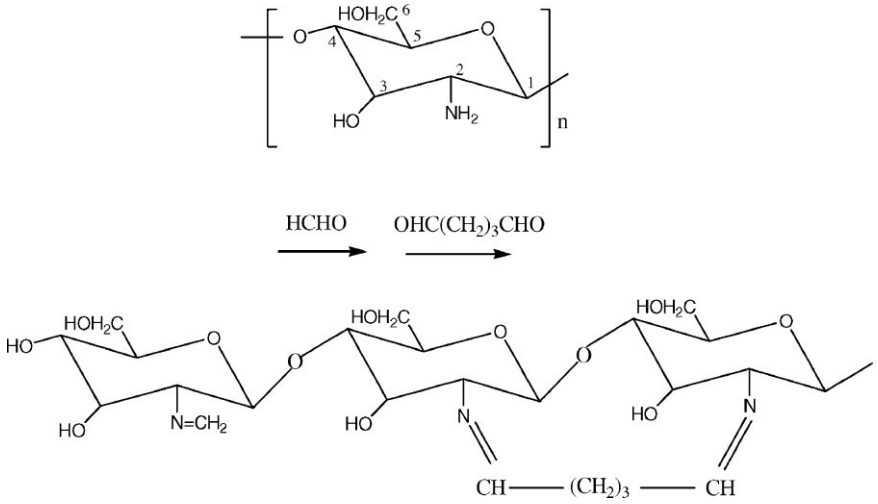
Modification of Chitosan Microspheres with Low Generations of PAMAM Dendrimers

Preparation of CS-G1.0 Microspheres

10 g of water-swollen CS microspheres (not removing the formaldehyde protection) and 50ml of epichlorohydrin were added to a three-neck flask with electronic stirring at 60 °C for 0.5 h. 30 ml of perchloric acid was dropwise added to the flask carefully. After 12 h, the product was leached and washed with ethanol and water repeatedly to neutral. Chloride hydroxypropyl chitosan microspheres (HPCS) were obtained. 8 g water-swollen HPCS and 100ml of PAMAM-G1.0 aqueous solution (40%, w/w) were added to a flask with electronic stirring and reacted at 60 °C for 24 h to prepare CS-G1.0 microspheres. The product was leached and washed to neutral with water. Finally CS-G1.0 microspheres were removed the formaldehyde protection with 0.5M HCl at room temperature for 24 h to release more amino groups.

Preparation of CS-G2.0 and CS-G3.0 Microspheres

It was difficult to prepare CS-G2.0 and CS-G3.0 microspheres using PAMAM-G2.0



Scheme 2.
Preparation of glutaraldehyde cross-linked macroporous chitosan (CS) microspheres.

and PAMAM-G3.0 directly reacted with CS microspheres in the same way. Therefore, in this paper according to PAMAM divergent synthesis methods,[40] CS-G1.0 microspheres alternately reacted with methyl acrylate to prepare CS-G1.5 and then reacted with ethylenediamine to prepare CS-G2.0. CS-G3.0 was prepared in the same method based on the CS-G2.0.

Preparation of CS-HDA Microspheres

CS-HDA was prepared using HPCS reacted with 1, 6-hexanediamine (HDA) in the method of CS-G1.0 preparation.

Characterization of Microspheres

Determination of the Content of Amino Groups on Microspheres

The content of amino groups on microspheres was detected by titration with HCl standard solution according to the method described in The National Standard GB 5760-86 of People's Republic of China (Determination of exchange capacity of anion-exchange resin method).

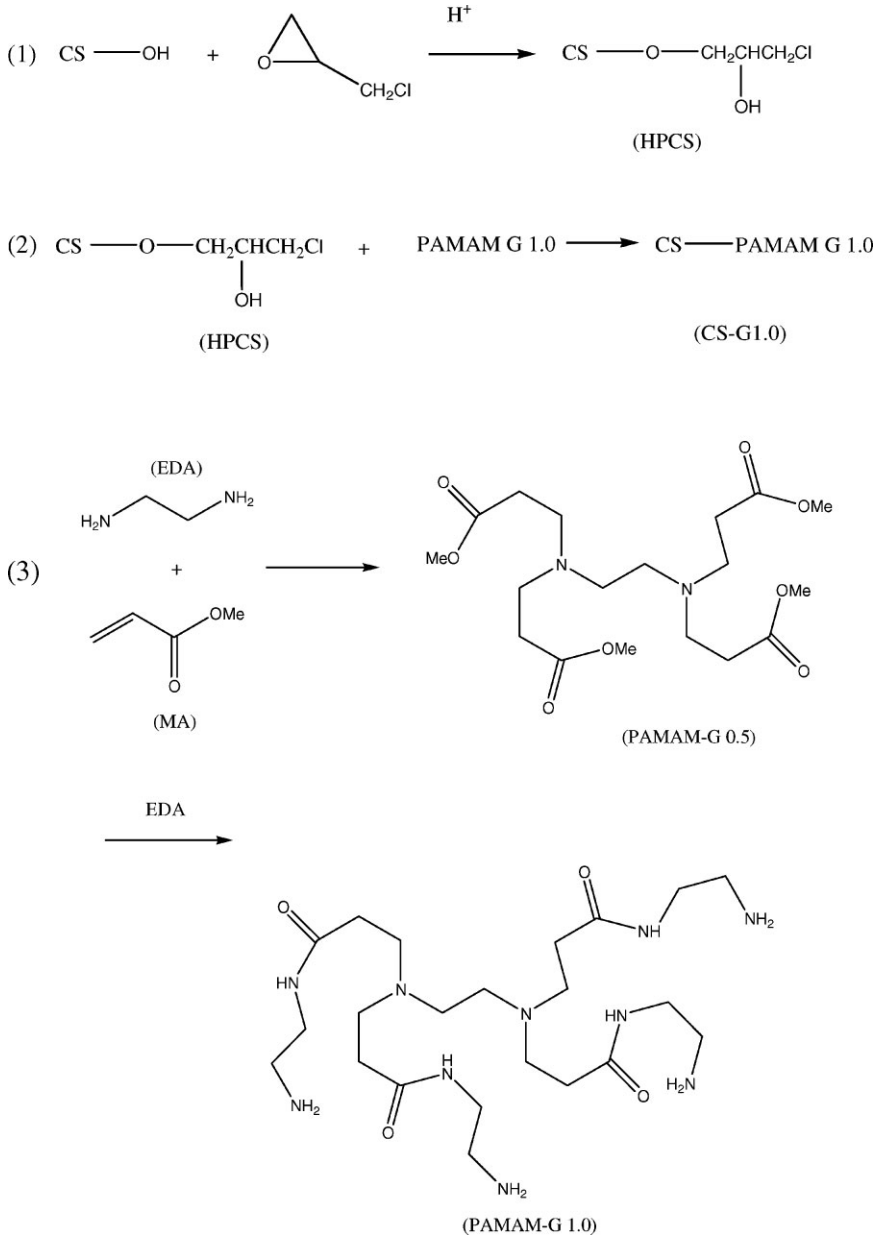
Table 1 showed the amino content of all microspheres. The order of the amino content was: CS-G3.0 > CS-G2.0 > CS-G1.0 > CS-HDA > CS-G2.5≈CS-G1.5 > CS. All amino contents of modified microspheres were higher than unmodified CS microspheres. All amino contents of full generations were obviously higher than that of half generations. It can prove the success for the preparation of PAMAM series.

FT-IR Analysis

Fig. 1 showed the FT-IR spectrum of (a) HPCS and CS-Gn.0 microspheres and (b) CS-G1.0 and CS-Gn.5 microspheres. It was found that C-Cl of HPCS absorption peaks appeared at 625 cm^{-1} in Fig. 1 (a), which proved the success of hydroxypropyl chlorination. Characteristic absorption peaks of C=O in amido groups of all CS-G1.5-3.0 microspheres appeared near 1630 cm^{-1} in Fig. 1 (a). Strong characteristic absorption peaks of C=O in ester groups of CS-G1.5 and CS-G2.5 microspheres appeared near 1728 cm^{-1} in Fig. 1 (b). It proved that CS-G1.5 and CS-G2.5 microspheres basically transformed into CS-G2.0 and CS-G3.0 microspheres.

SEM Analysis

Fig. 2 showed the SEM photo of CS microspheres. It was found that CS microspheres were macroporous and had good



Scheme 3.
Synthetic route of CS-G1.0 microspheres.

spherical shape. The uniform particle size showed small dispersion. There was no conglutination between CS microspheres. It proved right to choose span-80 as dispersant and appropriate added amount.

Bilirubin Adsorption Experiments on Microspheres

0.1 g of dried microspheres (100–200 mesh) was added to amber glass vials wrapped with aluminum foil and immersed to be

Table 1.
The amino content on microspheres (100–200 mesh).

Amino content mmol/g	CS	CS-G1.0	CS-G2.0	CS-G3.0	CS-G1.5	CS-G2.5	CS-HDA
	3.56	5.10	5.45	6.47	3.65	3.71	4.66

fully swelled with 5 ml of 0.05M phosphate buffer solution (pH = 7.2–7.4) at room temperature in the dark for 24 h. 10 ml of 100 mg/l bilirubin solution freshly prepared was added to the above vials to carry out the adsorption experiments at 37 °C in a constant temperature immersion oscillator. There was a certain amount of attenuation because of the instability of bilirubin solution, so it is essential to use the initial bilirubin solution as a blank reference. The concentration of bilirubin solution was detected with a UV-2450 spectrophotometer at 438 nm. The adsorption result was shown in Fig. 3.

In Fig. 3, it was seen that adsorption equilibrium was achieved in about 2 h on microspheres except for CS-microspheres

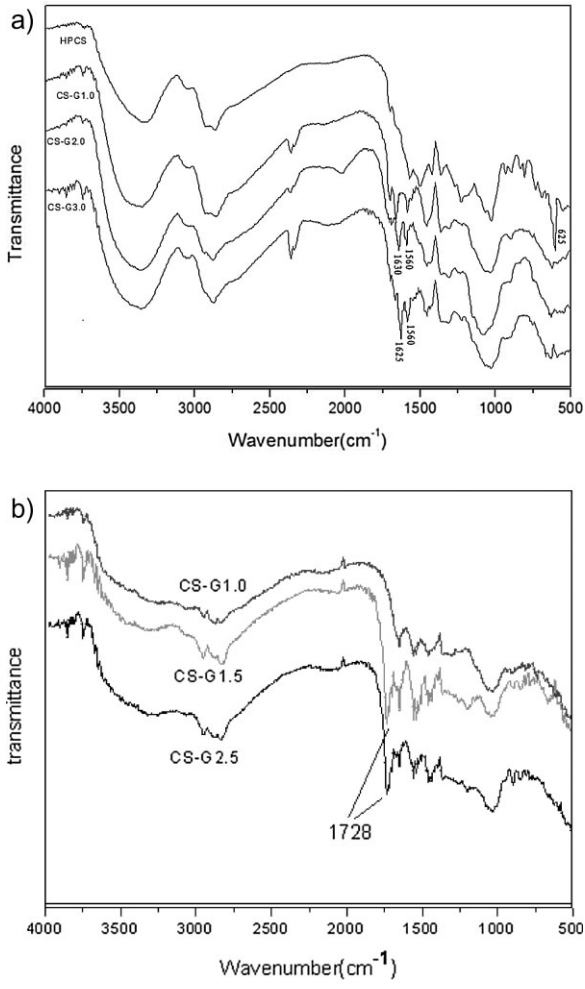


Figure 1.
The FT-IR spectrum of (a) HPCS and CS-Gn.0 microspheres and (b) CS-G1.0 and CS-Gn.5 microspheres.

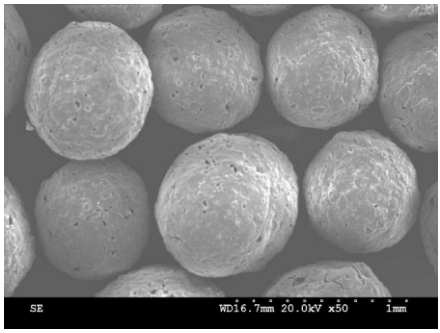


Figure 2.
The SEM photo of CS microspheres.

which was about 4h. The adsorption order was: CS-HDA > CS-G1.0 > CS-G2.0 > CS-G3.0 > CS. Compared with the order of amino content: CS-G3.0> CS-G2.0> CS-G1.0> CS-HDA > CS, it can be drawn that high amino content of microspheres was not necessarily a good adsorption for bilirubin. CS-HDA has a good adsorption property though the content of the amino group was not very high even lower than that of any other CS-Gn.0 microspheres. The reason maybe there was proper carbon chain hydrophobic space in CS-HDA because of the free bilirubin being lipophilic. It was seemed that the role of high hydrophobic property was greater than that of high amino content for bilirubin adsorption. Whether hydrophobic property or amino content of CS-HDA, CS-G1.0, CS-G2.0 and CS-G3.0 microspheres was higher than that of CS microspheres, So all the modified CS microspheres were better than unmodified CS microspheres for bilirubin adsorption. For CS-Gn microspheres, in the adsorption process of higher generation products, higher steric hindrance made bilirubin hard to diffuse into the interior of dendrimer microspheres and to be absorbed. So it led to the result of the adsorption order: CS-G1.0 > CS-G2.0 > CS-G3.0. This phenomenon was similar to that in ref.[41] to some extent. Adsorption capacity of high generation products decreased due to the steric hindrance. But in ref.[41] the adsorption order for noble metal ions was: SiO_2-G1.0 < SiO_2-G2.0 < SiO_2-G3.0 > SiO_2-G4.0, their corresponding amino content was 1.91, 1.87, 1.83 and 2.09, respectively. Comparison with the amino content on microspheres in Table 1 showed that the rate of increase of amino content on CS-Gn.0 was obviously higher than that in ref.[41]. This would lead to earlier appearance of steric hindrance in much lower generations.

Adsorption conditions: bilirubin initial concentration: 100 mg/l; bilirubin solution volume: 10 ml; microsphere weight: 0.1 g; pH: 7.2–7.4; phosphate buffer concentration:

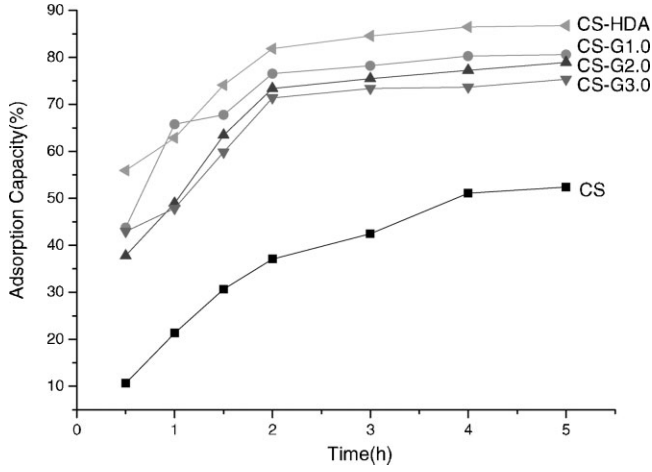


Figure 3.
bilirubin adsorption dynamic curves on microspheres.

0.05 M; temperature: 37 °C; detected wave length: 438nm; Size of microspheres: 100–200 mesh.

Conclusion

In this paper, biocompatible natural polymer chitosan was used as materials to prepare a series of functionalized cross-linked macroporous microspheres: CS-HDA, CS-G1.0, CS-G2.0 and CS-G3.0 microspheres by inverse phase suspension reaction with sugar as porogenic agent. The content of amino groups on CS, CS-G1.0, CS-G2.0, CS-G3.0, CS-HAD microspheres was 3.56, 5.10, 5.47, 6.47, 4.66 mmol/g, respectively. Their adsorption properties for bilirubin in 0.05M phosphate buffer solution (pH = 7.2–7.4) were studied at 37 °C. All the modified CS microspheres were better than unmodified CS microspheres for bilirubin adsorption. CS-HDA has a good adsorption property though the content of the amino groups was not very high. The reason maybe there was proper carbon chain hydrophobic space in CS-HDA. If the increase of hydrophobic property and amino content was combined organically, it would greatly improve adsorption properties of absorbents for bilirubin. These biocompatible adsorption microspheres are promising for adsorbing bilirubin through ALSS technique. In this paper studies for bilirubin adsorption were carried out in phosphate buffer solution (pH = 7.2-7.4), studies in human blood would be performed in the future to evaluate the value in the practical use.

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