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Genipin-crosslinked hydrophobical chitosan microspheres and their interactions with bovine serum albumin

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ABSTRACT

In this work chitosan microspheres have been prepared by ion precipitation using sodium sulfate as precipitant, and crosslinked with natural crosslinker genipin. Then the microspheres were hydrophobically modified by covalent and ion interaction grafting of stearic acid and sodium stearate respectively. Results indicated that the there were still some amount of $-NH_2$ groups on the genipin-crosslinked chitosan microspheres. And the contact angles for hydrophobical graft microspheres were increased greatly from 27° to 104°, which indicates stearic chains have been grafted on the surface of the microspheres even only by a simple graft process based on ion interaction. The interactions of protein with the hydrophobically modified chitosan microspheres were investigated by surface absorption method using bovine serum albumin as a model protein. And results indicated that adsorption efficiency for bovine serum albumin of the fatty chain grafted chitosan microspheres was higher than that of the prestine one distinctly, due to the additional hydrophobic interaction. And the adsorbed protein could well-off be desorbed under a general condition.

1. Introduction

Protein drug delivery material has become an investigation highlight with the great development of recombinantly expressed therapeutic proteins in the pharmaceutical industry in recent years (Frokjaer & Otzen, 2005; Wender, Verma, Paxton, & Pillow, 2008). The research is mainly focused on the exploitation of high performance materials. The related topics including drug protein carrying modes, the morphology control of the carrying materials, improving the oral bioavailability, increasing the transfection rate of crossing biomembrane barriers and designing of drug targeted properties (Balthasa et al., 2005; Borges, Borchard, Verhoef, Sousa, & Junginger, 2005; Kim et al., 2005; Ravi Kumar, Bakowsky, & Lehr, 2004; Takeuchi et al., 2005).

Chitosan, a polysaccharide derived particularly from crustacean chitin, is composed of glucosamine and N-acetylated glucosamine (2-acetylamino-2-deoxy-D-glucopyranose) units linked by 1-4 glycosidic bonds. Properties such as biodegradability, low toxicity and good biocompatibility make it very suitable for use in biomedical and pharmaceutical formulations (Guo, Xia, Hao, Song, & Zhang, 2004; Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001). In recent years, it has arisen as a promising alternative for improving the transport of biomacromolecules such as peptide and protein delivery vectors (Thanou, Verhoef, & Junginger, 2001).

The protein loading manner has always been a key problem for chitosan based protein delivery materials. Generally, entrapment mode is a much often used method, which the drug was introduced during the chitosan sphere forming process (Dass, Contreras, Dunstan, & Choong, 2007; Ding, Huang, Li, & Liu, 2007; Ge, Chen, Xie, & Zhang, 2007). The merit of this method is that the preparation is simple and convenient to manipulate. However the disadvantages are also obvious. First, the forming conditions of chitosan sphere should be corresponding to the dissolving conditions of proteins. However, there are various dissolving conditions for different kinds of protein drugs. It is very difficult for them to correspond to each other in most situations. In other words, the particle formation condition must be match with the protein loading condition. Second, chitosan spheres formed by onefold ion precipitation are unstable in structure under acid environmental condition. Sometimes further crosslinking is needed to meet the application demands, but the crosslinking process will certainly affect the stability and bioactivity of the loaded protein.

Thus in this work chitosan spheres with specific size have been prepared using ion precipitation method. Then the spheres were crosslinked using a naturally occurring crosslinking agent genipin in order to get a stable structure. Genipin is manufactured from geniposide, a glucoside, by using b-glucosidase, and it is much safer as a chitosan crosslinker for biomedical use than other chem-

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ical crosslinking agents, such as glutaraldehyde, tripolyphosphate, ethylene glycol, diglycidyl ether and diisocyanate (Muzzarelli, 2009). Herein the loading amount of protein is important for the sphere surface adsorbing loading mode. Previous work indicated that hydrophobic modification of chitosan could improve the adsorbing capacity of protein on the material (Höhne, Frenzel, Heppe, & Simon, 2007; Kwon et al., 2003; Liu, Chena, et al., 2005; Liu, Chen, & Park, 2005; Parra-Barraza et al., 2005; Tangpasuthadol, Pongchaisirikul, & Hovena, 2003). Therefore the surface hydrophobic modification for genipin-crosslinked chitosan sphere was carried out in order to improve their interactions with protein so as to further increase the adsorption amount of protein. Comparing to the imbed loading system in which the particle must embed protein during the particle formation process, the preparation of chitosan microspheres with specific size and morphology becomes easier and the spheres can be crosslinked further in different ways to obtain microspheres with stable structure. The protein loading procedure based on surface adsorbing of microspheres can also be operated very easily.

Therefore, the major goal of this work is to study the effect of interaction improvement of protein with the surface hydrophobic modified chitosan spheres on adsorption capacity for chitosan spheres. For this purpose, the modified materials were characterized using FTIR, TG, TEM or SEM and Zeta-potential, etc. Protein adsorption and desorption profiles on the modified surface were obtained using a spectrophotometer. The modified chitosan based materials of this work has a promising application in construction of fully natural and biodegradable protein delivery system or protein separation materials.

2. Experimental

2.1. Materials

Chitosan with an average viscosity molecular weight of 200 kDa and 87% degree of deacetylation was obtained from Yuhuan Marine Biochemistry Co., Ltd. (China). Genipin was purchased from Shanghai Seebio Biotechnology, Inc (Shanghai, China). Sodium sulfate was purchased from Tianjin Special Reagent Factory (China). Stearic acid (SA) was purchased from Shanghai Chemical Reagent Co. Ltd. (China). Sodium stearate (SS) was obtained from Tianjin Guangfu Fine Chemical Research Institute (China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Shanghai Aladdin Reagent Co. Ltd. (China). Dimethylsulfoxide (DMSO) and acetic acid was obtained from Tianjin No. 3 Chemical Reagent Factory (China). Bovine serum albumin (BSA) was supported by Beijing Dingguo Biology Co. Ltd. (China) and the protein solutions were prepared using 0.2 M sodium dihydrogen phosphate and sodium hydrogen phosphate buffer (pH 7.4). All reagents and solvents were analytical grade.

2.2. Preparation of chitosan microspheres

Chitosan (0.25%, w/v) was dissolved in an aqueous solution of acetic acid (2%, v/v). The solution was gently stirred for 24 h and then was filtered. The chitosan solution (100 mL) was added into a 200 mL beaker with a high speed dispersion machine. A solution of sodium sulfate (25%, w/w) was added dropwise (4 mL min^{-1}) into the beaker under stirring of 15,000 rpm at 60 °C. After the addition of sodium sulfate, the stirring speed was lowered to 200 rpm at ambient temperature for another 1 h to stabilize the microspheres. Then the microsphere suspension was centrifuged at 6000 rpm for 10 min and was washed fourth with deionized water. The sample of chitosan microspheres was named as CM.

Genipin was added to the microsphere suspension in deionized water at the final concentration of 0.1% (w/v) in a three-necked flask equipped with a mechanical stirrer, and allowed to crosslink for 24 h at 40 °C. The crosslinked microsphere suspension was centrifuged at 6000 rpm for 10 min and the microspheres were washed third using deionized water to remove the unreacted genipin. The final sample of genipin crosslinked chitosan microspheres was named CMG.

2.3. Synthesis of stearic acid modified microspheres (CMG-SA)

Stearic acid grafted chitosan microspheres was synthesized via the reaction of carboxyl groups of SA with amine groups of chitosan in the presence of EDC as reported in previous research (Tangpasuthadol et al., 2003).

Briefly, stearic acid, EDC and NHS were dissolved in 30 mL DMSO under mechanic stirring with 300 rpm at 40 °C. The wet CMG (2 g, after 9000 rpm for 10 min centrifugation) was dispersed in DMSO (20 mL) by sonicate treatment (Kunshan Ultrasonic Instrument Co., Ltd., China) at room temperature for 3 min, and then added into the SA mix solution. The reaction was continued for 24 h under stirring. After the reaction, the suspension was centrifuged at 6000 rpm for 10 min, the precipitate was further washed with 50 mL DMSO twice to remove the unreacted SA. After this process, the precipitate was dispersed in 40 mL deionized water, and then dialyzed using dialysis membrane (MWCO: 10 kDa, Beijing Newprobe Bioteachnology Co., Ltd., China) against deionized water for 72 h with successive exchange of fresh deionized water in order to remove other water-soluble by-products. The dialyzed product was lyophilized.

Two kinds of SA-grafted microspheres were prepared. The one of with lower SA amount was named CMG-SA-L and the higher one named CMG-SA-H. The preparation formulations of two samples were shown.

2.4. Preparation of sodium stearate modified chitosan microspheres (CMG-SS)

Sodium stearate (SS) was dissolved in the deionized water with a concentration of 1.5 mg/mL for the preparation of the SS-grafted microspheres in a three-necked flask under mechanic stirring with 600 rpm at 80 °C for 10 min. Then it was cooled down to 60 °C, and 40 mL of suspension of CMG particles (50 mg/mL) was dropwise-added to the SS solution and stirred for 5 min. And then the CMG-SS microspheres were washed eighth using 60 °C deionized water to remove the unreacted SS. The microspheres were isolated, frozen in liquid nitrogen, and lyophilized. The sample was named as CMG-SS.

2.5. Protein binding test

Different kinds of chitosan microspheres were immersed in phosphate-buffered solution (PBS) solution at pH 7.4 for 2 h prior to adsorption. Protein solutions were freshly prepared by dissolving BSA in PBS at pH 7.4 to give final concentrations of 0, 0.5, 0.7, 1.0, 1.2 and 1.5 mg/mL. The microspheres were incubated in a 7 mL centrifugal tube, containing 5 mL protein solution at 37 °C. BSA-loaded microspheres were separated from the solution by centrifugation at 10,000 rpm at room temperature for 10 min. Supernatant from the centrifugation was decanted carefully and the protein content in the supernatant was analyzed with UV–Vis spectrophotometer (Shimadzu; Model: UV-2550 Spectrophotometer) at 594 nm using the Bradford protein assay. Triplicate samples were analyzed at each time interval. The BSA adsorption capacity (AC) of microspheres and association efficiency (AE) were calculated by using