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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

Eqs. (1) and (2), respectively:

$$AC = \frac{A - B}{C} \quad (1)$$

$$AE = \frac{A - B}{A} \times 100 \quad (2)$$

where A = total amount of BSA added in solution; B = total amount of BSA in supernatant after centrifugation; and C = weight of the microspheres measured after freeze-drying.

2.6. Protein desorption test

Different chitosan microspheres, 20 mg for each were transferred to a 7 mL centrifugal tube with 5 mL PBS. The sealed tube was placed in a table concentrator with temperature at 37 °C. At each time point, the tube was centrifuged at 10,000 rpm for 10 min and 100 μL of supernatant was withdrawn from the tube. The protein concentration was measured using Bradford agent. The samples in the 5 mL tube were replenished with 100 μL fresh PBS solution at 37 °C. Triplicate samples were analyzed. The total mass of desorbed protein M_i at time i was calculated from Eq.:

$$M_i = C_i V + \sum C_{i-1} V_s$$

where C_i is the concentration of protein in desorption solution at time i, V is the total volume of desorption solution and V_s is the sample volume.

2.7. Instrumental characterization

IR spectra were recorded with a Bio-Rad FTS 135 Fourier-transform infrared (FTIR) spectrometer in the range of 4000–500 cm^{-1} using KBr pellets.

The morphologies of the different kinds of chitosan microspheres were observed using scanning electron microscope (Hitachi S-3500N, Japan) and transmission electron microscope (Technai G2 20-S-TWIN).

The contact angles were measured by a JC2000C Contact Angle Meter. All the samples were coated onto the cover glasses. The thickness of the coated microspheres was about 0.5 mm.

Zeta-potential, size and size distribution of chitosan microspheres were checked on a zeta-potential analyzer (Zeta Plus, Brookhaven Instrument Co.). All samples were sonicated for 10 min in deionized water at 100 W with sonifier before every investigation.

Thermogravimetric (TG) measurements of lyophilized samples (CMG, CMG-SA-L, CMG-SA-H and CMG-SS) were performed in nitrogen atmosphere from 25 to 800 °C at a heating rate of 10.0 K/min using NETZSCH TG 209 (Germany).

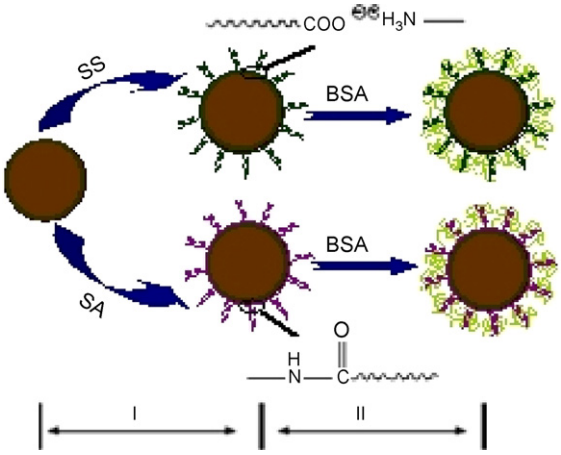
3. Results and discussion

3.1. Preparation of modified chitosan microspheres

In this work, genipin-crosslinked chitosan microspheres have been treated with sodium stearate solution to achieve fatty chain hydrophobic modified chitosan spheres, which is a more convenient method based the ion interaction between sodium stearate and amine groups of chitosan than the covalent grafting of stearic acid on the chitosan microsphere with the effect of EDC and NHS. The fabrication process was illustrated in Scheme 1.

3.1.1. FTIR analysis

In the spectrum of CMG (Fig. 1B), two characteristic peaks at around 1624 and 1521 cm^{-1} were assigned to C–N stretching of amide band and vibration of N–H bending of chitosan respectively.



Scheme 1. Schematic representation of preparing procedures for hydrophobically modified genipin-crosslinked chitosan microspheres.

It could be inferred that the chitosan microspheres still have many amino groups after genipin crosslinking. Compared with CMG, the spectra of CMG-SA-L (Fig. 1C) and CMG-SA-H (Fig. 1D) showed that the amide C–N absorption peak was shifted to the higher region at 1636 cm^{-1}. Meanwhile, the peak of the vibration of N–H bending was also shifted to the higher region at 1537 cm^{-1}. These results confirmed that stearic acid had been associated to the $-NH_2$ of CMG. In Fig. 1E, the peaks of the vibration of CH_3- and $-CH_2-$ at 2849 and 2917 cm^{-1} looked sharp in shape due to large numbers of methyl and methylene groups of sodium stearate. And two new strong peaks appeared at 1467 and 1714 cm^{-1}, which were assigned to the COO^- and C=O stretching vibration. It indicated that many carboxylic anions (COO^-) from sodium stearate and $-NH_3^+$ from chitosan were held together by electrostatic forces. In addition, though the same hydrophobic chain was grafted on the chitosan microspheres by two different types of grafting ways, the different grafting ways would affect the interaction between graft chains and chitosan backbone on the surface of chitosan microspheres, which also would affect the distribution of grafted chains on the surface. As seen from the results of Fig. 1, it seems that the hydrophobic chains for ion effect grafting way were connected with chitosan backbone with a more unwound way than that of covalent grating. Thus its structure information was differentiated easy in IR measurement.

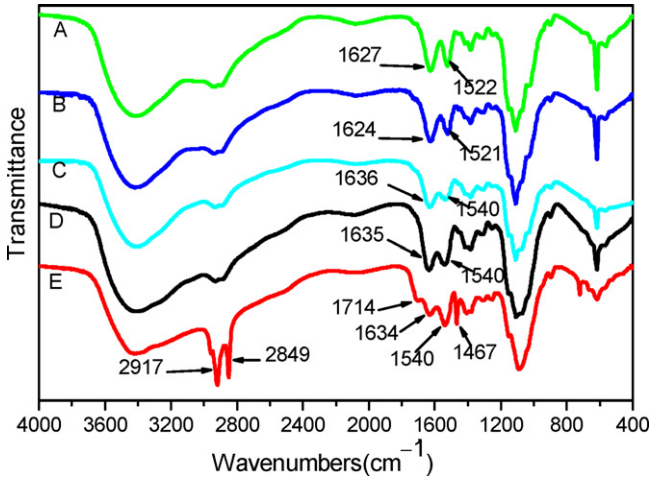


Fig. 1. Fourier transform infrared spectroscopy spectra of ion-crosslinked chitosan microspheres CM (A); genipin-crosslinked chitosan microspheres CMG (B); lower and higher amount of stearic acid-grafted microspheres CMG-SA-L (C) and CMG-SA-H (D); sodium stearate-grafted microspheres CMG-SS (E).

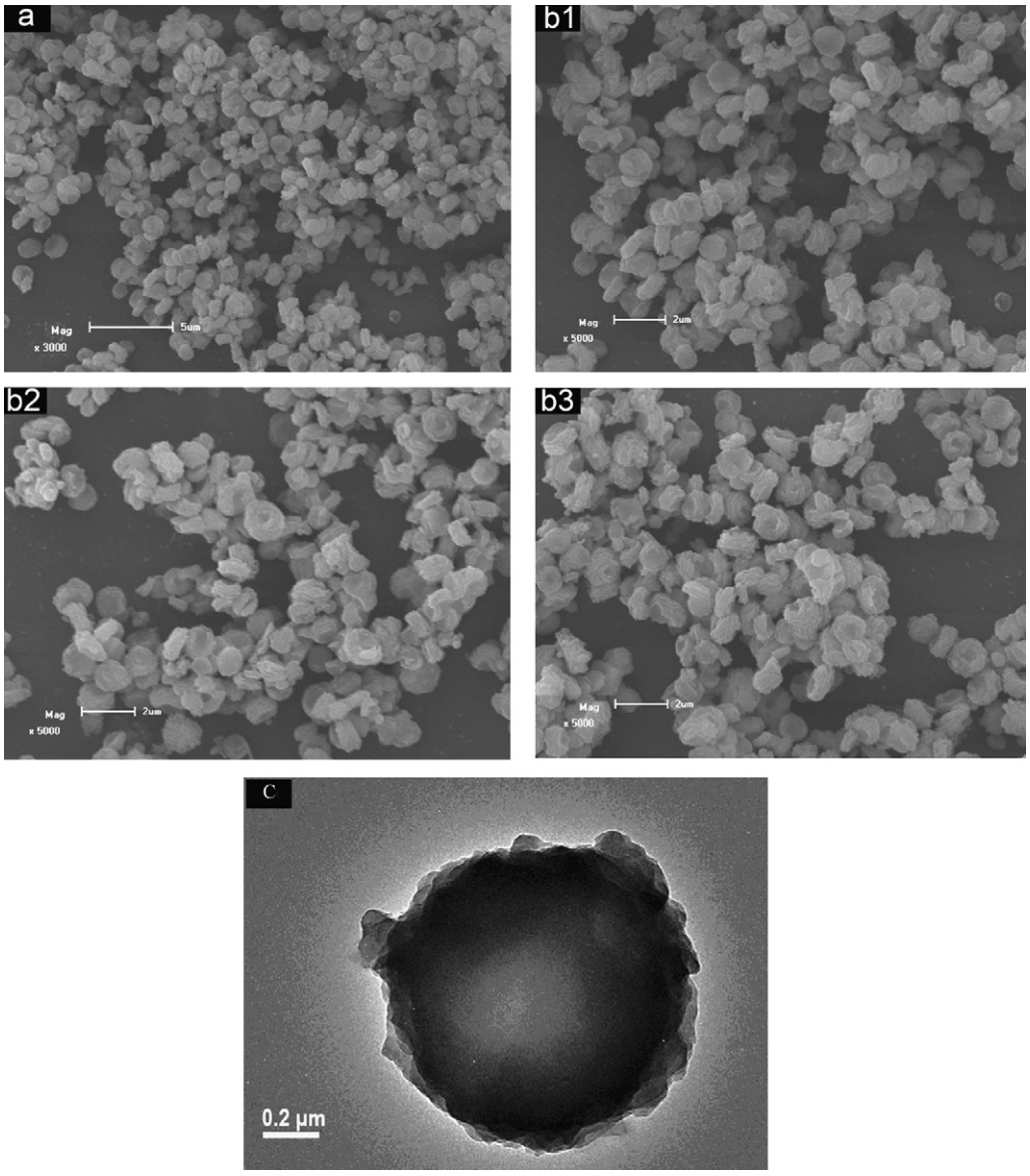


Fig. 2. SEM images of genipin-crosslinked chitosan microspheres CMG (a); the hydrophobic modification by stearic acid of CMG-SA-L (b1), CMG-SA-H (b2), sodium stearate of CMG-SS (b3); TEM image of CMG(c).

3.1.2. SEM and TEM

TEM microphotograph of the genipin crosslinked chitosan microsphere (CMG) is shown in Fig. 2c. It can be observed from Fig. 2c that, the microsphere has a relative regular spherical shape and a rough surface, which is favorable as a surface absorption carrier. SEM microphotograph of CMG in Fig. 2a. shows that the microspheres were uniform in size. The size of CMG in Fig. 2a as being observed in dynamic laser scattering method depicted in Table 1 was approximately 1.1 μm.

Fig. 2b1, b2 and b3 depicted the hydrophobic modified chitosan microspheres with lower and higher amount of stearic acid and sodium stearate respectively. All of these three microphotographs demonstrated that the surface modifications of microspheres have not obvious effect on the morphology of chitosan spheres.

3.1.3. Surface charge measurements

Data in Table 1 show that the average zeta potential values of pristine chitosan microspheres (CM) and genipin-crosslinked chitosan microspheres (CMG) were +22.35 ± 0.51 mV and +15.01 ± 0.36 mV. The stearic acid-grafted microspheres (CMG-SA-L) and (CMG-SA-H) were +11.03 ± 1.63 mV and +7.79 ± 1.33 mV. And the sodium stearate-grafted microspheres (CMG-SS) was +4.93 ± 0.49 mV. The positive zeta potential values for CM (non-crosslinked) and CMG (crosslinked) could be explained by the fact that most of the amine groups of chitosan are positively charged in deionized water, because the pK_a of the amine groups of chitosan is 6.3 (Risbud, Hardikar, Bhat, & Bhonde, 2000). The zeta potential value for CMG was lower than that of CM, owing to the

Table 1
Average zeta potential values, sizes and contact angles of CM, CMG, CMG-SA-L, CMG-SA-H and CMG-SS microspheres.

Formulation	Size (μm)	Zeta potential (mV)	Contact angle (°)
CM	1.05	+22.35 ± 0.51	-
CMG	1.08	+15.01 ± 0.36	0
CMG-SA-L	1.28	+11.03 ± 1.63	27
CMG-SA-H	1.39	+7.79 ± 1.33	79
CMG-SS	1.43	+4.93 ± 0.49	104

Notes: the formula for CMG-SA-L: SA 0.09 g, EDC 0.06 g, NHS 0.04 g; for CMG-SA-H: SA 0.57 g, EDC 0.38 g, NHS 0.23 g.

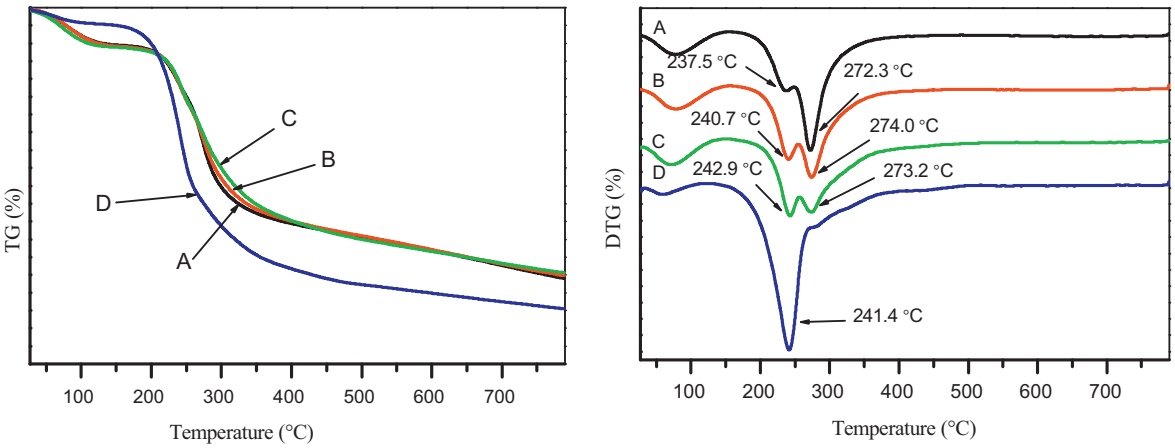


Fig. 3. TG and DTG thermograms of CMG (A); CMG-SA-L (B); CMG-SA-H (C); CMG-SS (D).

reaction between genipin and part of the amine groups of chitosan. But the potential value of CMG was still positive, which could be explained by that many amine groups of chitosan were still existed on the surface of the CMG, which was correlated well with what was shown in FTIR spectra of Fig. 1. Thus, it is possible for the next step of hydrophobic modification on the surface of the CMG. The zeta potential values for the CMG-SA-L, CMG-SA-H and CMG-SS were all lower than that of the CMG. This could be explained by that the hydrophobic moisties were grafted to the amine groups of chitosan. Meanwhile, it noted that ion effect grafting was also as successful as the covalent grafting.

3.1.4. TG

The TG and the corresponding DTG curves of CMG, CMG-SA-L, CMG-SA-H and CMG-SS are shown in Fig. 3 respectively.

The weight loss curves of CMG, CMG-SA-L, CMG-SA-H and CMG-SS were shown in two stages. The first thermal event occurs in the temperature range of 25–140 °C, where all samples present a mass loss ranging from 5% to 11%. This is attributed to the evaporation of adsorbing water, whose content is a function of the morphology and crystallinity of the materials. In the second stage, the temperatures of reaching a maximum rate of weight loss for samples CMG, CMG-SA-L, CMG-SA-H and CMG-SS are all in 237–242 °C, which indicates that the hydrophobic modification did not affect the thermal stabilities of the samples obviously.

3.1.5. Particle surface hydrophobicity measurements

The different chemical modifications could all increased the hydrophobicity of the microsphere surface (as seen in Table 1). The contact angle of genipin cross-linked chitosan microspheres (CMG) was near 0°, which indicated the hydrophilic surface of CMG microsphere. The contact angles of CMG-SA-L and CMG-SA-H microspheres were 27° and 79° respectively. And the contact angle for sodium stearate-grafted chitosan microspheres (CMG-SS) was 104°. Followed by the increases of the contact angles, it can be deduced that there were more and more fatty chains grafted onto the microspheres. It was consistent with the previous results of zeta potentials.

3.2. Protein adsorption

In alkaline medium, the amino groups at chitosan were in the form of -NH_2. Therefore, there were no charges on the surfaces of chitosan spheres. At this condition the electrostatic force was in a shielded state, there are only weak hydrogen bond and van der waals' force between the surface of chitosan microsphere and BSA. However, after hydrophobically modified by SA and SS for the chitosan microspheres, the hydrophobic residues on the surface of spheres could increase the interaction between the material and BSA.

Table 2
Adsorption capacities of the different chitosan microspheres (error bar, ±SD, $n = 3$).

Samples	Protein adsorption amount				
	0.5 mg mL^{-1}	0.7 mg mL^{-1}	1.0 mg mL^{-1}	1.2 mg mL^{-1}	1.5 mg mL^{-1}
CMG	4.5 ± 0.3	14.8 ± 0.5	90.3 ± 2.1	104.8 ± 3.3	124.0 ± 3.3
CMG-SA-L	9.8 ± 0.8	21.4 ± 1.1	96.3 ± 2.4	107.2 ± 2.5	126.0 ± 2.9
CMS-SA-H	20.3 ± 1.4	44.8 ± 1.7	111.3 ± 3.0	129.8 ± 3.1	131.4 ± 3.5
CMG-SS	12.2 ± 0.9	27.8 ± 1.0	102.3 ± 3.1	110.6 ± 2.7	127.2 ± 3.2

Table 2 shows that the BSA adsorption amount was enhanced dramatically from 4.5 mg/g (for CMG) to 20.3 mg/g (for CMG-SA-H) when the initial BSA concentration was low at 0.5 mg/mL, and was also enhanced from 90.3 mg/g (for CMG) to 111.3 mg/g when the initial BSA concentration was 1.0 mg/mL, from which we could infer that the hydrophobic residues could enhance the loading capacity obviously especially for the low initial BSA concentrations.

The association efficiency was different at different initial BSA concentrations, as depicted in Fig. 4. When the initial BSA concentration was 1.0 mg/mL, the maximum value of association efficiency about CMG-SA-H was 44.53%. The amount of protein adsorption onto the sphere surface was dependent on the employed BSA concentration evidently. When the concentration was lower than

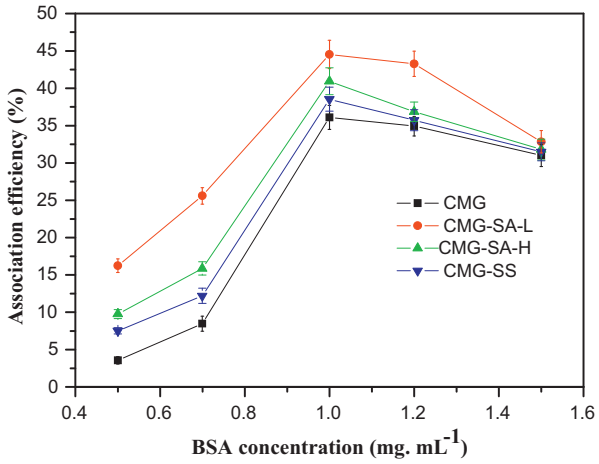


Fig. 4. Association efficiencies of different chitosan microspheres (error bar, ±SD, $n = 3$).

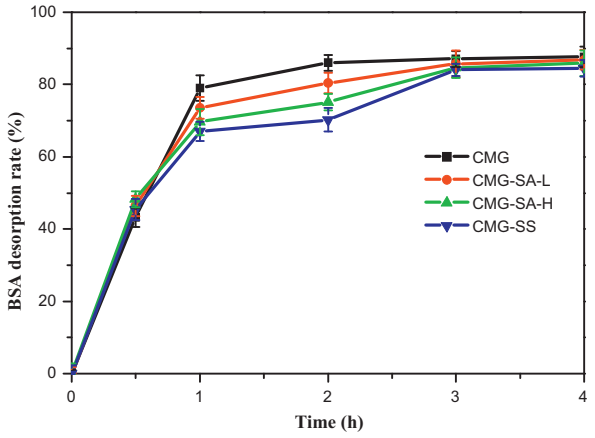


Fig. 5. Desorption profiles of BSA from different microspheres in pH 7.4 PBS at 37 °C (error bar, ±SD, $n = 3$).

1.0 mg/mL, a higher initial concentration will led to a higher loading efficacy and a higher association efficiency. But when the concentration was higher than 1.0 mg/mL, a higher initial concentration will led to a higher adsorption efficacy and lower association efficiency.

3.3. Protein desorption

The desorption profiles of the uncoated microspheres in 4 h are shown in Fig. 5. The desorption rate of BSA was rapid, indicating a severe burst desorption. Only in 1 h, nearly 80% of BSA on CMG had been desorbed. The desorption rates of CMG and various modified CMGs have not obvious different in 0.5 h. And it could be observed that the desorption rates of CMG-SA-L, CMG-SA-H and CMG-SS were somewhat lower than that of CMG during 1–2 h. The microspheres hydrophobically modified by stearic acid and sodium stearate could alleviate the burst desorption evidently at some extent. These results suggested that the desorption rate was affected by the interactive force between adsorbed-BSA and the surface of microspheres.

4. Conclusion

In this work, we have explored a feasible preparation procedure for fully natural chitosan based microspheres. The present results demonstrate that the hydrophobic fatty chains could be introduced onto the surface of genipin-crosslinked chitosan microspheres by an ion interaction grafting of sodium stearate. And the hydrophobic fatty chains would act as the hydrophobic interaction sites between protein and the material, which can successfully increase the BSA adsorption amount. The genipin-crosslinked hydrophobical chitosan microspheres would be a promising material for the protein delivery vectors.

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