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Synthesis and Characterization of Chitosan-Albumin Conjugates as pH-Sensitive Bioactive Hydrogels

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Abstract A new kind of biodegradable pH-sensitive drug delivery system was developed using chitosan-albumin conjugate hydrogel. Through changing the feeding modes of reactants, two types of hydrogels (Comb-type and Reticular-type) were synthesized by amidation reactions between 6-O-succinoylated N-phthaloyl chitosan and albumin. The structures, properties and morphologies of the hydrogels were characterized by NMR, FTIR, UV and SEM. And their water swelling capacity, drug loading and releasing properties under different pH values were also investigated. It was found that the comb-type hydrogels with looser space construction had better water swelling ratio (more than 400% of its original weight) than the reticular-type ones did (about 180% of its original weight). *In vitro* release experiments of Rifampicin showed that the hydrogels provided controlled release of the entrapped drug for more than 50 hours. The drug release rates of both types of hydrogels under acidic condition were lower than those under neutral or basic condition. The introduction of albumin not only improved the hydrophilicity of chitosan, but also provided the possibility of the carrier system combining other biologically active materials more easily to fulfill the delivery and therapy functions.

Keywords chitosan; albumin; hydrogel; pH sensitive; drug delivery

1 Introduction

Natural polymers such as polysaccharides and proteins are widely investigated and used in pharmaceutical fields because of their abundant sources, sterility, nontoxicity, and favorable biocompatibility and biodegradability [1, 2]. However, the use of protein materials was limited since their high sensitivity to temperature and pH, poor processability, immunogenicity, enzymatic hydrolysis and excretion response from liver and kidney. As for polysaccharides, the poor solubility in water or organic solvents is often happened. So the modification of these natural materials becomes important and necessary for their application in pharmaceutical fields.

Up to now, the research on protein and polysaccharide mainly focused on their own modification and deep processing [3-7], materials blending [8-10], the interactions between the two kinds [11-17], antibody carrier using protein [18] and so on. The conjugation between the two kinds of materials to be used as the bioactive vehicle or tissue engineering scaffold was seldom mentioned [19, 20], since the self cross-linking of proteins was often unavoidable. Accompany with the combination between polysaccharide and protein molecules [19], the abundance of functional groups, like amino and carboxyl groups on the surface of protein molecules, may lead to undesirable side-reactions which make the further purification and application difficult.

In this paper, controllable reactions between modified chitosan and bovine serum albumin (BSA) were successfully carried out, and two kinds of chitosan-albumin conjugated hydrogels were obtained by changing the reaction modes. The structures, water-swollen properties, pH sensitivities, drug loading and releasing behaviors of the hydrogels were also investigated. The introduction of protein not only improved the hydrophilicity to the carrier system, but also increased the size of the whole system to realize

long-term drug delivery. It is noteworthy that proteins provide much more functional groups to the polymers thereby endow with the possibility of combining other bioactive materials (including anticancer drugs, amino acid, peptide, cell active factor, targeting ligand, antibody, or antigen, etc.), which makes it easy to not only fulfill the delivery and therapy functions, but also extend its application in the scaffold field and edible packaging material field.

2 Experimental Part

2.1 Materials

BSA (MW = 66 KDa), *N, N*-dicyclohexylcarbodiimi -de (DCC) and *N*-hydroxysuccinimide (NHS) were from Sinopharm Chemical Reagent Co. Ltd., China. Chitosan (Mμ = 500 KDa; degree of deacetylation (DDA) = 90%) was obtained from Yuhuan Golden -shell Biochemical Co., Ltd., Zhejiang province, China. 4-Dimethylaminopyridine (DMAP) was from Chenghu Chemical Plant, Suzhou, China. Glutaraldehyde was from Tianjin Rongda Chemical Plant, China. *N, N*-dimethylformamide (DMF) was purified by distillation under reduced pressure after immersed with CaH_2 for more than two weeks. The water used in experiments was secondary distilled water. All other reagents were analytically pure and used without further purification.

2.2 Instrumentation

^{1}H-NMR spectrums were recorded on the Bruker AVANCE DRX 400 or 300 spectrometers in DMSO-d_6. Fourier transform infrared (FTIR) spectrums were measured with a Bruker Vertex 70 spectrometer on KBr pellets. The morphology of the hydrogels was observed by a field emission scanning electron microscope (SEM) (JXA-840). The ultraviolet spectrums were measured by Shimadzu UV 2450 UV-Vis spectrophotometer.

2.3 Preparation of Chitosan-BSA hydrogel

2.3.1 Chitosan modification

Modified chitosan was synthesized through two steps according to the reported method: [9, 20, 21]

2.3.1.1 *N*-phthaloyl chitosan (PHCS)

Briefly, 5.0g of chitosan was dispersed in 100 mL DMF and stirred overnight. About 15.0 g of phthalic anhydride (3 equiv) was added, and the mixture was stirred at 120℃ for 12 h under nitrogen atmosphere. Then the reaction mixture was poured into ice water. The crude product was filtered, washed completely with ethanol and diethyl ether, and then vacuum-dried over Silica gel to give 7.3 g PHCS (yield: 90%). The degree of substitution was about 1.0.

^{1}H-NMR (δ, DMSO-d_6, ppm): 1.7(CH_3 in acetamide), 3.3-5.0(pyranose), 7.4-7.7(arom, phth). FTIR (KBr): 3600-3100(O-H, pyranose), 2980-2830(C-H, pyranose), 1777 and 1716(s, C=O, imide), 1196-970(C-O, pyranose), 722 cm[-1](arom, phth).

2.3.1.2 6-O-succinoylation of *N*-phthaloyl chitosan (PHCSSA)

N-Phthaloyl chitosan (3.0 g, 11.2 mmol pyranose) was dissolved in dried DMF (60mL) with stirring. Succinic anhydride (5.6 g, 5 equiv) was added, the reaction was carried out at 100-110 ℃ under nitrogen atmosphere for 24 h. Then the reaction mixture was poured into ice water. The crude product was filtered, washed completely with ethanol to remove unreacted succinic anhydride, finally it was washed with ethyl ether and vacuum-dried to give 3.0 g 6-O-succinoylated N-phthaloyl chitosan (PHCSSA) (yield: 73.5%), the degree of substitution of 6-O-succinoylated was about 1.0 ,which was determined by ^{1}H-NMR.

^{1}H-NMR (δ, DMSO-d_6, ppm): 2.3-2.4 (methylene of succinic group), 3.3-5.0(pyranose), 7.4-7.7(arom, phth), 12.2 (COOH of succinic group). FTIR (KBr): 3600-3100(O-H, pyranose and COOH), 2980-2830 (C-H, pyranose), 1777 and 1716(s, C=O, imide and ester group), 1470 (C-H, methylene of succinic group), 1196-970(C-O, pyranose), 722 cm[-1](arom, phth).

2.3.2 Preparation of comb-type chitosan-albumin hydrogel

PHCSSA (0.352 g, 1 mmol pyranose), NHS (5.8 mg, 0.05mmol), DCC (15.3 mg, 0.05 mmol) and DMAP (1 mg, 0.01 mmol) and dried DMF (30mL) were added to a dried reaction bottle. The reaction mixture was stirred under nitrogen atmosphere at 0 ℃ for one hour and then at room temperature for 24 hours. After reaction, the by-product dicyclohexylurea (DCU) was filtered, and 5% carboxyl activated PHCSSA solution was obtained. 10 mL of the above 5% carboxyl activated PHCSSA solution was added dropwise into the aqueous solution of BSA (0.99 g, 15 μmol, 40 mL) with stirring. The reaction mixture was kept stirring at room temperature for 3-4 days. After that the solvent was removed under vacuum at 60 ℃, and the pale yellow solid obtained was immersed and washed with water, then freeze-dried (0.62 g, yield 50%).

2.3.3 Preparation of reticular-type chitosan-albumin hydrogel

BSA (0.02 g, 0.3 μmol) was dissolved into 10 ml secondary distilled water. The BSA solution was added dropwise into 20 mL of the 5% carboxyl activated PHCSSA solution mentioned above (0.12 g, 0.3 mmol pyranose) with stirring. The reaction mixture was kept stirring at room temperature for 3-4 days. After removing the solvent, pale yellow film-like solid was obtained. The crude product was immersed and washed with water, then freeze-dried (0.135 g, yield 78%).

2.4 Performance testing of the chitosan-albumin hydrogels

2.4.1 Measurement of equilibrium swelling ratio of hydrogels

50 mg dried hydrogel sample was weighed, then it was incubated in 50 fold excess of water at room temperature for 3-5 days. After that, water was poured off, and the water on the surface of the hydrogel was absorbed with filter papers. Then the obtained swollen hydrogel obtained was weighed.

2.4.2 Drug loading and releasing of the hydrogels

0.1 g hydrogel and 10% fold of rifampicin were immersed into 50-fold excess of water and kept for one day. Then the UV spectrum of supernatant was measured to calculate the drug loading efficiency. The rifampicin-loaded chitosan-albumin hydrogel was then taken out and dried with filter papers.

The obtained drug-loaded hydrogel samples obtained were immersed into the buffer solutions with different pH value as 2, 7 and 10, respectively. At the designed time points, 2 mL of the solution was withdrawn for the UV measurement, and 2 mL of the buffer solution was replenished to the releasing system.

3 Results and Discussion

3.1 Modification of chitosan and preparation of chitosan-albumin hydrogels

In the reactions between polymers and proteins, two basic problems should be paid attention to. One is the solubility, the other is the denaturation of protein. Nearly all the modifications and operation procedures were designed to solve these two questions. Because chitosan can not dissolve in neutral water, the modification of it is necessary. After protecting of the amino groups on chitosan with phthalic anhydride, it could dissolve in some strong polar organic solvent (like DMF, DMSO, etc.). Then in order to react with the amino groups on BSA, carboxyl groups were introduced onto PHCS by reacting with succinic anhydride, and the resulting PHCSSA could dissolve in strong polar organic solvent like DMF and DMSO too. The modifications of chitosan decreased the operation difficulty of the reaction between chitosan and albumin.

For BSA, a kind of natural protein, the denaturation caused by organic solvents should always be avoided. Fortunately, DMF is one of the sustainable organic solvent for BSA. Although the solubility of BSA in DMF is poor, DMF and water is mutually soluble. We dissolved BSA into water and let it reacted with modified chitosan which was dissolved in DMF. In addition, because of the abundance of functional groups especially carboxyl and amino groups on the surface of BSA, the activation of functional groups on the surface of BSA should be avoided when site specific reactions were not taken. Based on the above considerations, the amino groups on chitosan were protected by phthalic anhydride to get PHCS and carboxyl groups were introduced onto the side chains of PHCS to get PHCSSA (see Scheme 1), then the carboxyl groups on PHCSSA were partially activated by NHS and reacted with the amino groups on the surface of BSA. Through these designations, the side reactions were reduced by a great degree.

Because of the abundance of functional groups on both PHCSSA and the surface of BSA, different types of copolymers could be obtained by controlling reaction modes and the addition sequence of the two reactants. For example, when the solution of PHCSSA was added dropwise into the aqueous solution of BSA, the comb-type structure would be formed, because a single PHCSSA chain had the chance to react with multiple BSA molecules. Otherwise, when the BSA solution was added dropwise into the PHCSSA solution, many activated carboxyl groups on PHCSSA would like to react with the amino groups on the surface of a single BSA molecule, which would cause the formation of reticular-type (or cross-linked) structure (Figure 1). In fact, after more than 3 days' reaction, chitosan-albumin hydrogels would be obtained effectively no matter what reaction mode was taken, and the yields were all above 80%. Self cross-linked BSA hydrogels as negative control were also synthesized using glutaraldehyde as cross-linking agent.

3.2 Water absorbability of the hydrogels

Swelling ratio or water absorption rate is an important index to characterize the performance of hydrogel. The reason that we introduced albumin molecules was to endow hedrogels with larger water retaining capacity. The results showed that when the hydrogels were immersed into the aqueous solution, all the two types of chitosan-albumin hydrogels could absorb more amount of water than their original weight, but the hydrogel formed by self-cross-linked BSA only had the water absorption ratio of 63% (Fig. 2). It is implied that although BSA is hydrophilic, the increase of water absorption by BSA itself was quite limited. The space structures of chitosan-albumin copolymers may play a crucial role in the boost of water absorption. This point was exhibited more clearly in the case of different kinds of chitosan-albumin hydrogels with different structures. The water absorption ratio of the comb-type copolymer hydrogel could reach more than 400%; on the other hand, the water absorption ratio of the reticular-type copolymer hydrogel was only about 180%. It is noteworthy that the content of hydrophilic constituent was different. In the comb-type copolymer hydrogel, plenty of albumin molecules existed on the side chain of every single chitosan molecule, while in the case of reticular-type hydrogel, the albumin molecules were used as cross-linking agents with very limited concentration. The hydrophilic character of albumin for the reticular-type hydrogel was not as significant as that for the comb-type hydrogel. What's more, the interaction between polymer chains of the comb-type copolymers was exhibited as physical entanglement, making the hydrogel a looser packing structure, which allowed

more water to get in. While in the case of reticular-type hydrogel, the chitosan chains were cross-linked by covalent binding through BSA. This tightly packed structure could not only shield the hydrophilic property of the hydrogel, but also limit the chain movement of the hydrogel system, which made it difficult to swell more water.

The effect between hydrogel structure and water adsorption was further confirmed using SEM measurement. Figure 3 clearly showed that the comb-type hydrogel had a rod-like structure with large space between each other. On the contrast, the reticular-type hydrogel exhibited the dense plate or film morphology with small space. The BSA self cross-linked hydrogel also had a rod-like structure, however, the rods were thicker and the interspace between the rods were smaller than that of the comb-type ones.

3.3 Drug loading and release of the hydrogels

One important application of hydrogels is drug loading and release. The drug loading and release behaviors of different kinds of chitosan-albumin hydrogels were investigated using rifampicin as the model drug, because rifampicin has an ultraviolet (UV) characteristic adsorption in 477 nm, which could be easily detected and calculated.

Firstly, all kinds of hydrogels were immersed into the determined amount of rifampicin solution for a certain time until the swelling became balanced. After removing the hydrogels, the remained rifampicin solutions were detected again using UV. We know that the common swelling behavior only decreases the volume of the outer water phase, but leaves the drug concentration unchanged. However in our experiments, the concentrations of rifampicin varied largely after hydrogels being immersed, which is implied that drug absorption occurred during the swelling process. It is well known that albumin can transport various nutritive and metabolic materials, and can carry such small molecules as drugs. As the results indicated, the self cross-linked BSA had an absorption rate up to 62 mg/g, and the average absorption of the chitosan-albumin hydrogels was about 75 mg/g (Fig. 4). The results strongly showed that, when the hydrogels swelled water, the specific absorption of rifampicin onto BSA molecules made great contribution to the high efficiency of drug loading of the chitosan-albumin hydrogels. This important character makes protein material a potential candidate as a high-dose drug carrier.

All kinds of hydrogels had initial fast release of rifampicin when they were immersed into PBS solution of pH 7.0 (Fig. 5.). The rifampicin cumulated release amount of comb-type hydrogel reached to 400 μg after just 4 hours releasing. After that, the rifampicin was kept releasing slowly for 50h, and the cumulated release amount could reach to about 700 μg. Although the reticular-type hydrogels and self cross-linked BSA had different albumin concentra-

tions, the cumulated release amount of both hydrogels was very limited and could not exceed 200 μg, which may be due to the tight packing structure of the hydrogels. It is implied that, in the system of chitosan-albumin conjugate hydrogel, the drug release behavior may not only depend on albumin content but also depend on the space structure of hydrogel.

Since there were many carboxyl groups on the modified chitosan, and also the steric configuration of BSA varied greatly with pH value, the chitosan-albumin hydrogels is expected to possess certain pH sensitivity. In order to verify that, the rifampicin-loaded hydrogels were immersed into buffer solutions of different pH values to investigate the release behavior. The result shows that, no matter under what pH value, the comb-type hydrogel had the much faster release rate and much higher release amount of rifampicin than the reticular-type hydrogel and self cross-linked BSA (Fig. 6.). For each hydrogel, the initial release behavior of rifampicin under different pH values were basically the same, and after the initial stage, the pH value dependence of drug release clearly exhibited. All the hydrogels were inclined to release more rifampicin under neutral or basic condition. We considered that the rifampicin absorbed by BSA molecules was first released, because all the three kinds of hydrogels had familiar initial release behavior. At sustained release stage, the pH value dependence of drug release behavior might relate to the variation of space structures of albumin and carboxylated chitosan (PHCSSA) under different pH values. BSA has an isoelectric point (PI) of 4.2, with negative charge on the surface under neutral and basic conditions. When BSA was under acidic condition, the negative charge was neutralized, resulting in the loss of the stability of its space structure. Because of that, BSA was inclined to form more tight structure, which limited the release of the swollen rifampincin. At the same time, PHCSSA with many carboxyl groups was also apt to form more constrictive structure under acidic conditions, resulting in the hampered release of rifampicin.

4 Conclusions

Different kinds of chitosan-albumin hydrogels with comb-type or reticular-type structures were prepared. The comb-type copolymer hydrogel had better water swelling capacity and looser space structure than the reticular-type one. Those hydrogel systems could effectively absorb and load drug molecules, and the drug release rate and amount under acidic condition was significantly lower than that under neutral or basic condition, exhibiting pH sensitivity. Among them, the comb-type copolymer hydrogel had the best loading and release efficiency, which depended on its suitable albumin content and looser space structure.

The investigation of chitosan-albumin hydrogel was not only the fundamental research of the synthesis technology of carrier material, but also the application research of the bioactive polymer in biomedical field.

The aims of these kinds of materials at the urgent demand of clinical applications, such as sustained-release anticancer drugs, hemostasis materials for internal and external injury, gene carrier, enriching materials for antibody and antigen, would definitely benefit economic and social progress.
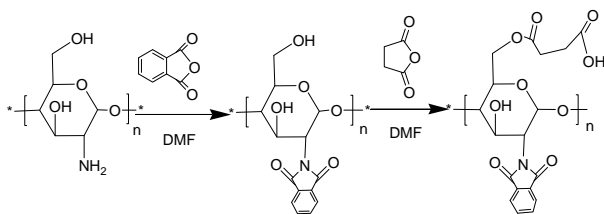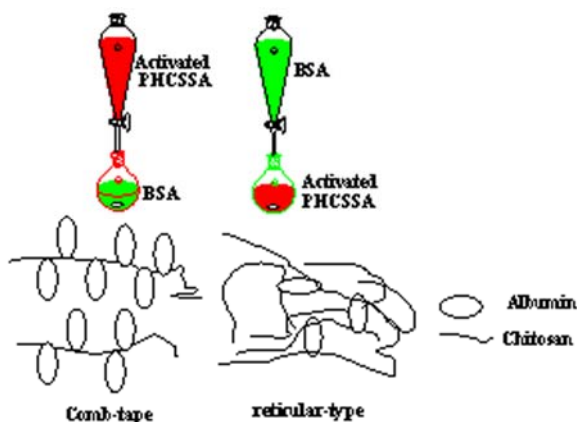

Scheme 1. Synthesis of PHCSSA

Fig.1 Schematic diagram of the synthesis methods and structures of the chitosan-albumin hydrogels
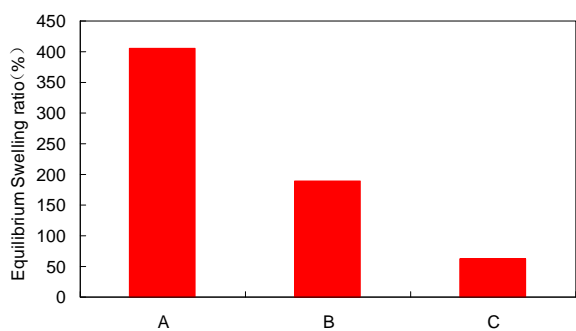

Fig. 2. Equilibrium swelling ratio of different structure of hydrogels

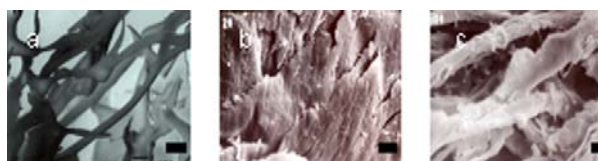A, comb-type；B, reticular-type；C, self cross-linked BSA.

Fig.3. SEM pictures of hydrogels (Magnification times x1000, bar =10 µm)

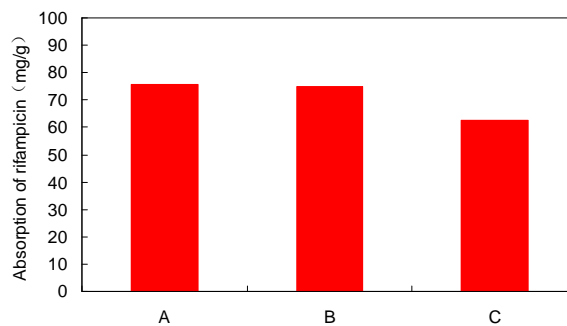a, comb-type; b, reticular-type; c, self cross-linked BSA.

Fig.4. The rifampicin absorption ratios of chitsan-albumin hydrogels

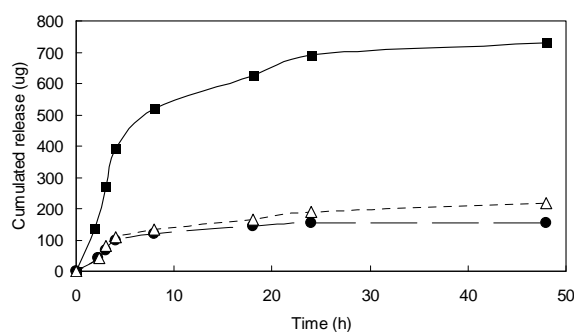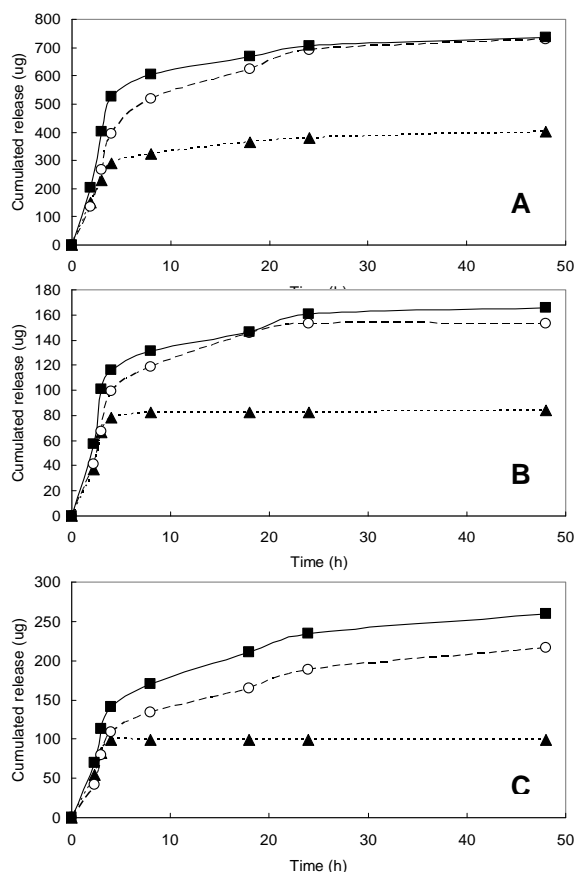A, comb-type；B, reticular-type；C, self cross-linked BSA.

Fig.5. The releasing curves of rifampicin from hydrogels with different structures under pH 7.

(■, comb-type; ●, reticular-type; △, self cross-linked BSA)

**Fig.6. The drug release curves of different kinds of hy-
drogels under different pH values.**

A, comb-type；B, reticular-type；C, self cross-linked BSA.

(■, pH 10.0;○, pH 7.0; ▲, pH 2.0)

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