Amphiphilic Self-assembled Nanoparticles Composed of Chitosan and Ursolic Acid for Protein Delivery on the Skin

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ABSTRACT

Nano-sized polymeric amphiphilic micelle was prepared using the hydrophilic polysaccharide, oligo-chitosan, and hydrophobic side chains, ursolic acid which assists the skin penetration, because the amphiphilic polysaccharide nanoparticles have been widely investigated as the carriers for active agents such as small molecules, proteins, peptides, and nucleic acids due to many advantages in protection, transport and delivery of active agents. The bovine serum albumin was used as the model agents for the encapsulation into the polymeric particles. The particles showed the pH-sensitive in the size and protein entrapment. At pH-3, the nanoparticle size increased due to the amino-groups of chitosan chain. In addition, the protein entrapment also increased with particle size. To enhance the stability of nanoparticles, the nanoparticle surface consisting of chitosan was cross-linked with glutaaldehyde. Thus chitosan-ursolic acid can be useful as carriers for active agents.

Keywords: amphiphilic, self-assemble, nanoparticle, protein delivery

1 INTRODUCTION

Several promising studies have been reported for the protein delivery such as cytokines using nano-sized particles [1], which have detrimental effect on the loading the protein without localization and releasing it without deactivation because the proteins have relatively long chain length in comparison with other active materials. To design the protein delivery system using the nanoparticles, the surface of nanoparticle should have the high free volume in the medium for freely moving the protein. Thus, hydrogel type, cross-linked water-soluble polymer, is suitable for the surface of the particles. In addition, the hydrophobic groups should be attached on the water-soluble polymer to prepare the amphiphilic block which can be self-assembled to form micelles in a selective solvent that was a precipitant for one of the copolymer components and a good solvent for the other component. The core-shell-type polymeric nanosphere systems consisted of a hydrophobic inner core and a hydrophilic outer shell [2]. Hydrophilic group, chitosan has biocompatibility, biodegrability, antibacterial properties and remarkable affinity to proteins, it has been found to increase applications in areas such as hematology, immunology, wound healing, drug delivery, and cosmetics [3-4]. In particular, the amino group, which is rare in polysaccharides, of chitosan has influenced on the pH-responsive behavior, because pH-sensitive hydrogels usually contain either acid or basic pendent groups in the network [5]. Note that the protein delivery is relative with the particle size which is easily controlled by changing the pH of medium. Ursolic acid was used as a hydrophobic group of the nanoparticle. Several pharmacological effects, such as, anti-tumor, hepatoprotective, anti-inflammatory, anti-ulcer, antimicrobial, anti-hyperlipidemic and antiviral, can be attributed to ursolic acid. In particular, its anti-inflammatory, anti-tumor, and antimicrobial properties are pertinent to the cosmetic industry.

Thus, the nano-sized polymeric micelle was prepared using the hydrophilic polysaccharide, chitosan and hydrophobic side chains, ursolic. The bovine serum albumin was used as the model agents for the encapsulation into the polymeric particles.

2 EXPERIMENTAL

2.1 Materials

Oligo chitosan (M_n = 1,600 determined by the supplier) was purchased from Bioland Co. Ltd.(Ansan-si, Korea) and used after dissolved in aqueous solution and filtered using a glass filter. Ursolic acid was purchased from Sigma Chemicals. 1-Ethyl-(3-3-dimethylaminopropyl) carbondiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) were purchased from Sigma Chemicals. Tetrahydrofuran (THF, Duksan Pure Chemicals, Seoul, Korea) was used as purchased without any further purification. As a model drug, bovine serum albumin (BSA) was purchased from Sigma Chemicals. Bicinchoninic acid assay (BCA) kit was purchased from Sigma Chemicals. Water was first treated with a reverse osmosis system (Sambo Glove, Ansan, Korea) and further purified with a Milli-Q Plus system (Water, Millipore, Billerica, MA, USA). Other chemicals were reagent grade and used without any further purification.

2.2 Synthesis of Amphiphilic Copolymer

Chitosan and ursolic acid were simultaneously dissolved in THF with 1.5 wt% concentration at room temperature. EDC and NHS were added to the solution to form amide bonds between the amino groups of chitosan and the carboxyl groups of ursolic acid. The solution had a chitosan/ursolic acid molar ratios of 1:1 (see Table 1), and chitosan/EDC/NHS molar ratio of 1:1:1 with reference to the chitosan amino group. The mixed solution was continuously stirred overnight at room temperature. After precipitation with deionized water and centrifuge, the precipitant was dialyzed using the cellulose tube (molecular weight cut-off: 12,000, Sigma) in water for four days, and then freeze-dried.

Sample code	Molar ratio	
	Chitosan	Ursolic acid
CsU-1	1	1

Table 1: Compositions of amphiphilic copolymers

2.3 Characterizations

Fourier transform infrared (FT-IR, Nicolet model Magna IR 550, Madison, WI) spectroscopy was used to confirm the synthesis of amphiphilic copolymer. The average particle size and the size distribution of the nanospheres were determined using a Zetasizer (Malvern-zetasizer 3000hs, Malvern, UK) at 25 °C. The measurement was performed after diluting the nanosphere suspension with deionized water. The surface charge of the nanospheres was determined from zeta potential measurements (Malvern-zetasizer 3000hs, Malvern, UK). The nanospheres were dispersed in deionized water. The dispersion was sonicated in a bath ultrasonicator for 1 min before analysis.

2.4 Encapsulation of Protein

The chitosan/ursolic acid copolymers were dissolved in dionized water. The mass of BSA loaded in the inner core of a micelle was determined by measuring the UV absorbance using a UV-visible spectrophotometer after treating it with BCA agents. The entrapped BSA content in the nanosphere cores was calculated from the weight of initial drug-loaded nanospheres and the mass of incorporated drug using the following equation.

Drug loading efficiency (DLE)

$$= \frac{\text{Amount of BSA in nanospheres}}{\text{Amount of BSA loaded nanospheres}} \times 100 \qquad (1)$$

$$= \frac{\text{BSA}}{\text{BSA} + \text{Polymer}} \times 100$$

The drug encapsulation efficiency (DEE) was defined as the ratio of the mass of the encapsulated drug to the mass of the drug used for nanosphere preparation using the following equation.

Drug encapsulation efficiency (DEE)

$$= \frac{\text{Amount of encapsulated BSA}}{\text{Amount of BSA used for nanosphere preparation}} \times 100 \qquad (2)$$

3 RESULTS AND DISCUSSION

3.1 Amphiphilic Nanoparticles

Figure 1 shows the molecular structure of the chitosan and ursolic acid. Ursolic acid could be coupled with, and so form amide linkages with, the amino group of chitosan using EDC and NHS. The amphiphilic block was composed of hydrophobic and hydrophilic parts, and could be self-assembled to form micelles in a selective solvent that was a precipitant for one of the copolymer components and a good solvent for the other component. The core-shell-type polymeric nanosphere systems consisted of a hydrophobic inner core and a hydrophilic outer shell.

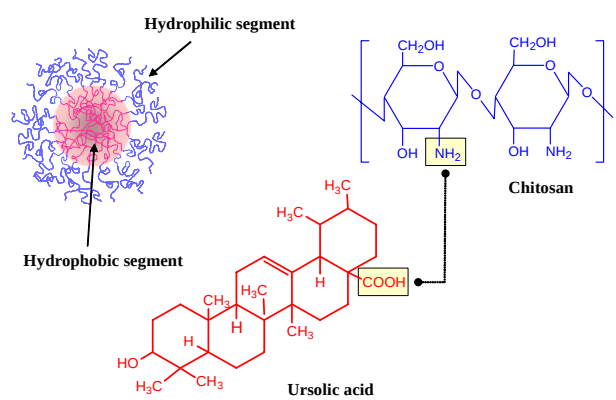

Figure 1: Molecular structure of the chitosan and ursolic acid.

The synthesis of amphiphilic copolymer composed of chitosan and ursolic acid was confirmed using FT-IR spectroscopy, as shown in Figure 2. The FT-IR spectrum of chitosan indicated that peaks appeared at 1637 cm^{-1} and 1512 cm^{-1} could be assigned to a carbonyl stretching vibration (amide I) and N-H bending vibration (amide II) of a primary amino group, respectively. In addition, Figure 2-(c), ursolic acid, shows characteristic peak at 1703 cm^{-1}, which can be attributed to the characteristic peaks of carboxylic acid group. Thus, in the case of the chitosan-*g*-ursolic acid copolymer (Figure 2-(b)), the formation of

amide groups was confirmed by the peak disappearances of 1703 cm^{-1}

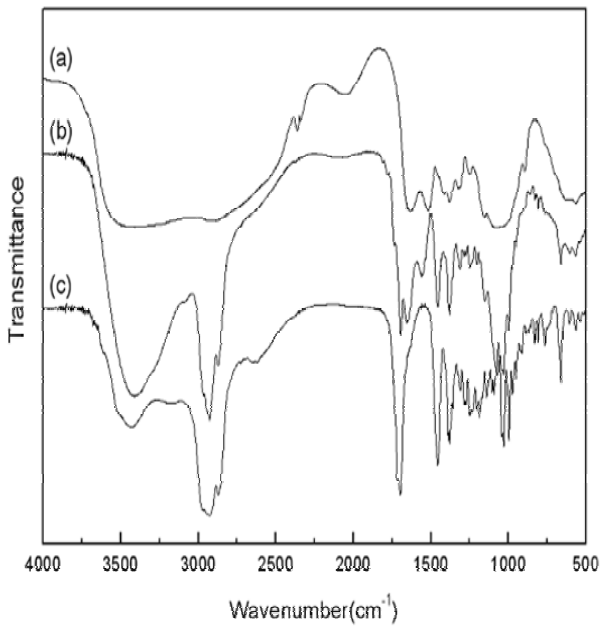

Figure 2: FT-IR spectra for (a) chitosan, (b) CsU-1 and (c) ursolic acid.

3.2 pH-dependant Particle Size

Figure 3 shows pH-sensitive characteristics of nanoparticles, which are investigated by particle size analyzer under various pH ranges between 3 and 9.

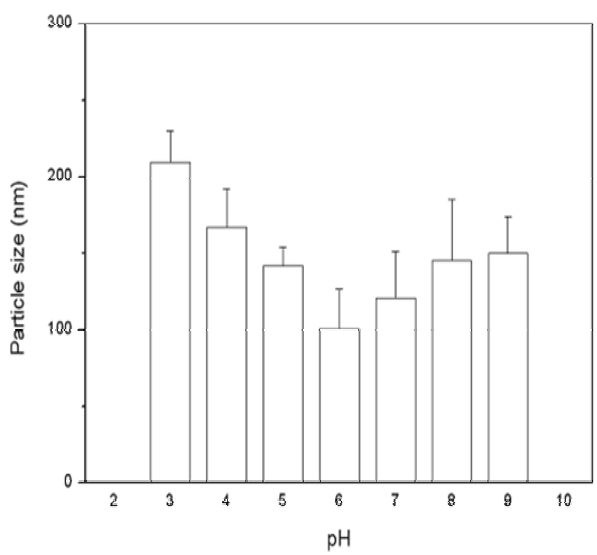

Figure 3: Particle size of nanoparticles under various pH ranges at 25°C.

The pH sensitivity is mainly affected by chitosan amino groups, which is a weak base with an intrinsic pK_a of about 6.5; namely, the chitosan hydrogels swelled at low pH due to the ionic repulsion of the protonated amine groups, and collapsed at high pH because of the influence of unprotonated amine groups. As the pH value of the buffer solution increases, ionized NH_3^+ groups become NH_2 groups, and the resulting neutralization of ionic groups causes the hydrogels to be precipitated. However, as shown in Figure 3, the particle size continuously increased above the pH 6 due to the ionization of hydroxyl group of chitosan and ursolic acid

3.3 pH-dependant BSA Encapsulation

Figure 4 shows pH-sensitive characteristics of nanoparticles, which are investigated by loading efficiency of BSA under various pH ranges between 3 and 9.

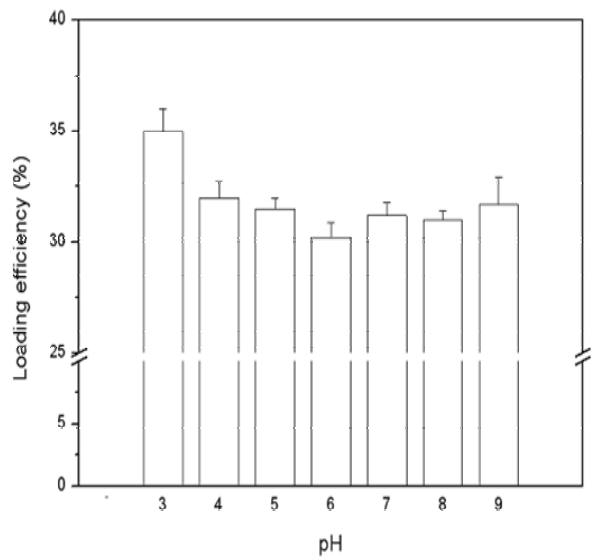

Figure 4: BSA loading efficiency of nanoparticles under various pH ranges at 25°C

Compared with Figure3 which is related with particle size, the amount of BSA loading increased with particle size of nanoparticles.

3.4 Pulsatile pH-dependant Particle Size

Figure 5 shows the pulsatile particle size behavior of the nanoparticles at 25 °C with solution pH values alternating between 3 and 6.

The particle size was also measured in ten-minute steps. After ten minutes, a pH-dependent pulsatile behavior of particle size was observed due to the amino groups of the chitosan. In addition, the changeable process of particle size proved to be repeatable and rapidly responded to pH change.

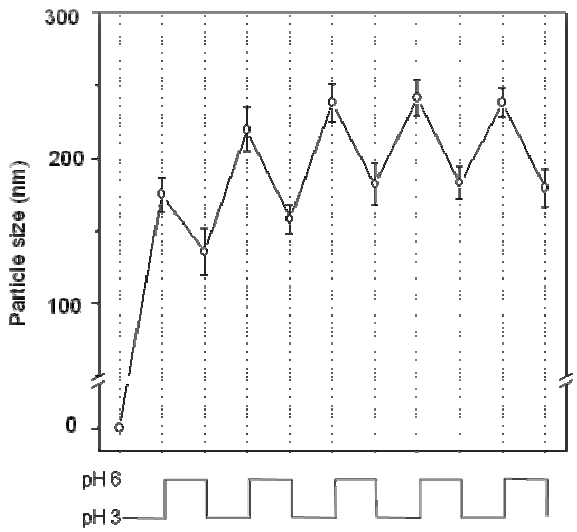

Figure 5: Pulsatile particle size behavior of the nanoparticles at 25 °C.

3.5 Hydrogel-typed Nanoparticles

To cross-linking the chitosan, the nanoparticles formed in deionized water were poured in the glutaaldehyde solution of 0.25%. The surface of particle was cross-linked and was similar with the structure of the hydrogel which was consisted of water soluble polymer with cross-linking points.

The particle size of the cross-linked nanoparticle was almost same with non-cross-linked nanoparticles at pH 7, whereas the particle size of non-cross-linked nanoparticles at pH 3 was almost changed, indicating the cross-linking restricted the swelling of chitosan at low pH.

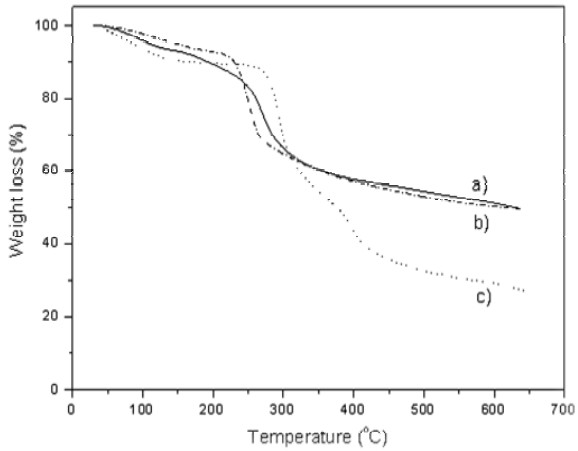

Figure 6: Thermalgravimetric analysis of (a) chitosan, (b) cross-linked CsU-1 and (c) CsU-1

Thermal stabilities of chitosan alone and nanoparticle were measured using thermogravimetric analysis (TGA) analysis. Figure 6 shows the weight loss curves recorded with a heating rate of 10 ^{o}C/min in nitrogen between 30 and 650 ^{o}C. The non-cross-linked nanoparticles show a faster thermal decomposition in comparison with that of cross-linked nanoparticles, because the introduction of the ursolic acid inside of matrix decreased thermal stability caused by the breakdown of crystalline region of chitosan. On the other hand, the thermal degradation profile of cross-linked nanoparticles is similar to that of chitosan.

4 CONCLUSIONS

A novel amphiphilic ursolic acid-grafted chitosan copolymer was prepared and could form the polymeric micelles. The properties of the micelles were changed according to pH conditions. The particle size of nanoparticle increased at low pH and high pH due to the ionized amine groups and hydroxyl group of chitosan, respectively. The amount of protein loading increased with particle size of nanoparticles. The cross-linked nanoparticle showed the lower pH-sensitive, however, the higher thermal stability than the non-cross-linked nanoparticles. Thus, it can be useful as carriers for active agents such as small molecules, proteins, peptides, and nucleic acids.

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