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Linolenic Acid-Modified Chitosan for Formation of Self-Assembled Nanoparticles

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Chitosan was modified by coupling with linolenic acid through the 1-ethyl-3-(3-dimethylaminopropyyl)-carbodiimide-mediated reaction. The degree of substitution was measured by ^{1}H NMR, and it was 1.8%, i.e., 1.8 linolenic acids group per 100 anhydroglucose units. The critical aggregation concentration (CAC) of the self-aggregate of hydrophobically modified chitosan was determined by measuring the fluorescence intensity of the pyrene as a fluorescent probe. The CAC value in phosphate-buffered saline (PBS) solution (pH 7.4) was 5×10^{-2} mg/mL. The average particle size of self-aggregates of hydrophobically modified chitosan in PBS solution (pH 7.4) was 210.8 nm with a unimodal size distribution ranging from 100 to 500 nm. A transmission electron microscopy study showed that the formation of near spherical shape nanoparticles had enough structural integrity. The loading ability of hydrophobically modified chitosan (LA-chitosan) was investigated by using bovine serum albumin (BSA) as a model protein. Self-aggregated nanoparticles exhibited an increased loading capacity (19.85 ± 0.04 to 37.57 ± 0.25%) with an increasing concentration of BSA (0.1−0.5 mg/mL).

KEYWORDS: Chitosan; linolenic acid; nanoparticles; self-aggregates; BSA

INTRODUCTION

Hydrophobically modified water soluble polymers, also known as hydrophobized polymers, are water soluble polymers that are modified by hydrophobic groups such as alkyl, arolkyl, and deoxycholic acid. When they are dissolved in water, hydrophobized polymers can self-aggregate due to their intro- and/or intermolecular hydrophobic interactions. By this process, unique hydrogel nanoparticles with a hydrophobic core and hydrophilic shell can be prepared, which is a new approach for preparing the monodispersed hydrogel nanoparticles (*1*, *2*). This kind of hydrogel structure is suitable for trapping hydrophobic substances, such as fluorescent probes and various proteins, under mild conditions (*3*). Because the hydrophobic core and hydrophilic shell need to be biodegradable and nontoxic, several investigations have been performed to prepare hydrophobic polymers based on natural biomaterials (*4*−*9*).

Chitosan, β-(1,4)-link glucosamine unit, is produced by deacetylation of chitin, which is extracted from the shells of crabs, shrimp, and krill. It has several characteristics such as biocompatibility, biodegradability, positive charge, nontoxicity, and bioadhesivity, which makes this macromolecule act as an ideal drug delivery material (*10*−*12*). In earlier studies, the preparation of hydrogel nanoparticles based on hydrophobically modified chitosan has been reported (*4*, *8*, *9*, *13*, *14*). To fulfill a wide utility in drug delivery applications, nanoparticles with different sizes and structures were prepared by different methods. Kim et al. (*8*) prepared hydrogel nanoparticles using different molecular masses of chitosan whose sizes ranged from 130 to 300 nm and reported varied in vitro transfection efficiencies. Liu et al. (*15*) reported that N-alkylated chitosan and nanoparticles loaded with DNA ranged in size from 250 to 330 nm. They found that upon elongating the alkyl side chain, the transfection efficiency would be increased and would level off after the number of carbons in the side chain exceeded eight. In our previous work (*16*), linoleic acid chitosan nanoparticles were prepared by an oil-in-water emulsification method with methylene chloride, which can be used to encapsulate lipid soluble compounds. In this case, methylene chloride was used as an oil phase, which is not suitable for the preparation of nanoparticles that act as protein delivery carriers. However, nanoparticle preparation using LA-chitosan by self-aggregation in the aqueous solution and in the absence of methylene chloride needs to be studied further.

In this paper, chitosan was modified by linolenic acid. Linolenic acid is a natural product with a hydrophobic chain, and it is also an essential fatty acid required for fatty acid metabolism in the human body (*17*). Linolenic acid can provide the hydrophobic group that makes LA-chitosan strongly am-

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phipathic, and the resulting amphiphilic polymer might exhibit a better compatibility with the biological systems and interact favorably with proteins, enzymes, or lipid soluble substances. The objective of our study was to prepare and characterize the self-assemblied nanoparticles using hydrophobic chitosan and their feasibility as a delivery carrier for proteins.

MATERIALS AND METHODS

Materials. Chitosan (low molecular weight) was purchased from Aldrich. Linolenic acids, pyrene, BSA, and 1-ethyl-3-(3-dimethylaminopropyyl)carbodiimide (EDC) were purchased from Sigma Chemicals (St. Louis, MO).

Preparation of LA-Chitosan. Linolenic acid was coupled to chitosan by the formation of amide linkages through the EDC-mediated reaction following the method of Chen et al. (*16*). Chitosan (1 g) was dissolved in 1% (w/v) aqueous acetic acid solution (100 mL) and diluted with 85 mL of methanol. Linolenic acid was added to the chitosan solution at 0.54 mol/mol glucosamine residue of chitosan followed by a dropwise addition of 15 mL of EDC methanol solution (0.07 g/L) while stirring at room temperature. The 1:1 mole ratio of EDC to linolenic acid was used in this study. After 24 h, the reaction mixture was poured into 200 mL of methanol/ammonia solution (7/3, v/v) with stirring. The precipitated material was filtered, washed with distilled water, methanol, and ether, and then dried under vacuum for 24 h at room temperature.

Fourier Transform Infrared (FT-IR) and ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy. The IR spectrum of LA-chitosan was recorded on a FT-IR spectrometer-430 (Jasco Co., Tokyo, Japan) at room temperature following the method of Shigemasa et al. (*18*). About 2 mg of LA-chitosan was mixed with 100 mg of KBr, and prepared pellets were used for studies.

^{1}H NMR spectra were recorded on a Bruker ARX 300 spectrometer at 25 °C. The sample was dissolved in 1% CD_3OOD of D_2O (v/v) solution to give the concentration of 30 mg/mL. The measurement conditions were as follows: a spectral window of 500 Hz, 32k data points, a pulse angle of 30°, an acquisition time of 2.03 s, and 32 scans with a delay of 1 s between scan (*19*).

Preparation of LA-Chitosan Nanoparticles. The modified chitosan was suspended in phosphate-buffered saline (PBS) (pH 7.4) at 37 °C for 24 h and sonicated using a probe type sonifier (Utralsonic Homogenizer UH-600) at 20 W for 3 min. The sonication was repeated two times to get an optically clear solution using pulse function (pulse on, 10.0 s; pulse off, 2.0 s). The clear solution of nanoparticles was filtered through a filter (Whatman) to remove dust. The solutions of different concentrations were obtained by diluting the 1% (w/w) stock solution with PBS buffer.

Fluorescence Measurement. Pyrene, used as a hydrophobic probe, was purified by repeated recrystallization from ethanol and vacuum-dried at 20 °C. Purified pyrene was dissolved in ethanol at the concentration of 0.4 mg/mL. About 20 μL of this solution was added into a 20 mL test tube, and the ethanol was driven off under a stream of nitrogen gas. Two milliliters of LA-chitosan nanoparticles solution was added to the test tube, bringing the final concentration of pyrene to 2 μM. The mixture was incubated for 3 h in a water bath at 65 °C and shaken in a BS-10 skaking water bath (Jeio Tech, Seoul, Korea) overnight at 20 °C. Pyrene emission spectra were obtained using a Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu Co., Kyoto, Japan). The probe was excited at 343 nm, and the emission spectrum was collected in the range of 360−500 nm at an integration time of 1.0 s. The excitation and emission slit opening were 15 and 1.5 nm, respectively (*20*).

Particle Size Distribution. The average particle size and size distribution were determined by quasielastic laser light scattering with a Malvern Zetasizer (Malvern Instruments Limited, United Kingdom) (*21*). Nanoparticle distilled water solutions of 3 mL (1 mg/mL) were put into polystyrene latex cells and measured at a detector angle of 90°, a wavelength of 633 nm, a refractive index of 1.33, a real refractive index of 1.59, and a temperature of 25 °C.

Transmission Electron Microscopy (TEM). Specimens were prepared by dropping the sample solution onto a copper grid. The grid was held horizontally for 20 s to allow the molecular aggregates to settle and then at 45° to allow excess fluid to drain for 10 s. The grid was returned to the horizontal position, and one drop of 2% phosphotungstic acid was added to give a negative stain (*22*). The grid was then allowed to stand for 30 s to 1 min before the excess staining solution was removed by draining as above. The specimens were air-dried and examined using a Philips EM 400 transmission electron microscope (Netherlands) at an accelerating voltage of 80 Kv.

BSA Loading Capacity and Efficiency. The BSA solution was added to 2 mL of nanoparticle solution in such a way that the concentration range of BSA ranged between 100 and 1000 μg/mL. Thus prepared solutions were incubated at 25 °C for 24 h. Then, these solutions were centrifuged at 30000*g* and 4 °C for 30 min to separate the unloaded BSA (supernatant) and BSA-loaded nanoparticles. The unloaded BSA present in the supernatant was determined by an UV spectroscopic method (280 nm) using supernatant solution of nonloaded nanoparticle solution as a blank. The BSA loading capacity (LC) of nanoparticles and BSA loading efficiency (LE) were calculated by using equations 1 and 2, respectively (*23*):

$$\mathrm{LC} = (A - B)/C \times 100 \quad (1)$$

$$\mathrm{LE} = (A - B)/A \times 100 \quad (2)$$

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

Statistical Analysis. The assays were performed at least in triplicate on separate occasions. The data collected in this study are expressed as the mean values ± standard deviations.

RESULTS AND DISCUSSION

Synthesis and Characteristics of LA-Chitosan. EDC is a "zero-length" cross-linker, which brings about the branches of an amide linkage between the carboxyl group of linoleic acid and the amino group of chitosan without leaving a spacer molecule (*14*, *16*). The increase of the amide I band at 1655 cm^{-1} in the IR spectra of the product confirmed the formation of an amide linkage between amino groups of chitosan and carboxyl groups of linoleic acid (*14*, *16*). **Figure 1** shows the ^{1}H NMR spectra of the chitosan and the LA-chitosan. The proton assignment of chitosan (**Figure 1A**) is as follows: $\delta_{2.0}$ = CH_3 (acetyl group of chitosan); $\delta_{2.95}$ = CH (carbon 2 of chitosan); $\delta_{3.3-3.7}$ = CH (carbon 3−6 of chitosan); $\delta_{4.2-4.5}$ = CH (carbon 1 of chitosan). The proton assignment of LA-chitosan (**Figure 1B**) is as follows: $\delta_{1.3}$ = CH_3 (methyl group of linolenyl); $\delta_{2.0}$ = CH_3 (acetyl group of chitosan); $\delta_{2.95}$ = CH (carbon 2 of chitosan); $\delta_{3.15}$ = CH_2 (linolenyl protons); $\delta_{4.2-4.5}$ = CH (carbon 1 of chitosan). A new peak at 1.30 ppm, which originates from the ^{1}H NMR spectra of LA-chitosan, is due to mainly characteristic methyl protons of the reacted chitosan with linolenic acid (*16*, *24*). This peak shows the presence of major functional groups linked to chitosan, and the degree of substitution (DS) was calculated by comparing the ratio of linolenyl methyl protons (δ = 1.3 ppm) to sugar protons (δ = 3.5−4.5 ppm) (*25*), which can be defined as the number of linolenic acid groups per 100 anhydroglucose units of chitosan. The obtained DS was 1.8.

Formation of Self-Aggregated Nanoparticles. The formation of aggregates in aqueous solution was studied by using a florescence spectroscopy method where pyrene was used as a molecular probe. Pyrene is poorly soluble and a self-quenching agent in a polar environment but strongly emits radiation when self-aggregates or other hydrophobic microdomains are formed in an aqueous solution, as it prefers to be close to (or inside)

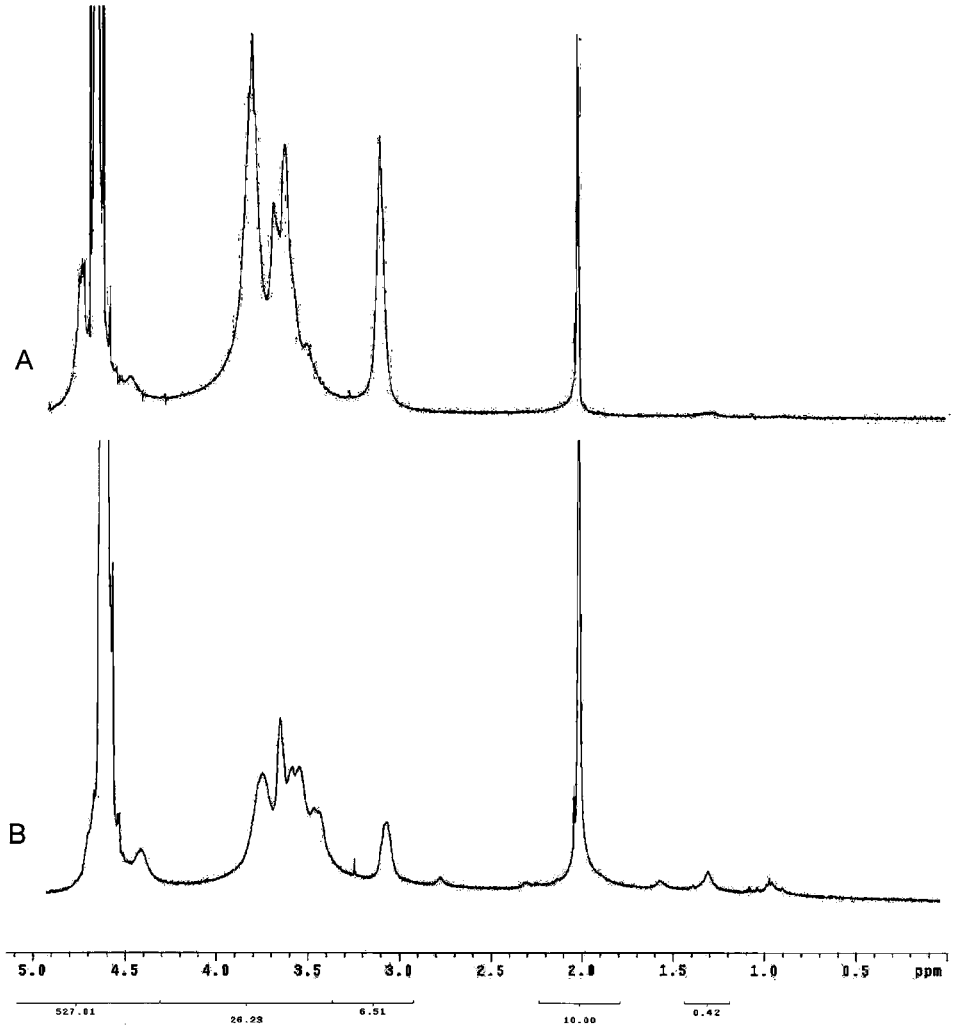

Figure 1. ^{1}H NMR spectra of (**A**) chitosan and (**B**) LA-chitosan in D_2O and CD_3COOD.

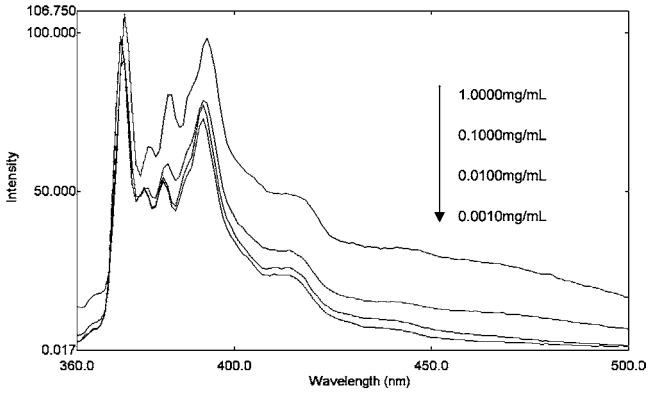

Figure 2. Fluorescence spectra of pyrene in LA-chitosan nanoparticle solution. The concentrations of LA-chitosan were from 0.001 to 1.0 mg/mL in 0.1 M PBS buffer with 2.0 μM pyrene.

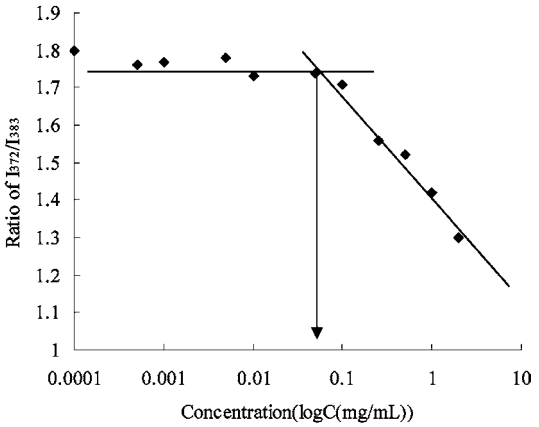

Figure 3. Plot of the intensity ratio of I_{372}/I_{383} from excitation vs logC of nanoparticles.

the microdomain (*26*). **Figure 2** shows the fluorescence spectra of pyrene at various concentrations of LA-chitosan in PBS (pH 7.4) after sonication. For instance, with the increase in the concentration of LA-chitosan, the total emission intensity was increased indicating that the probe is transferred from aqueous media to the less polar microdomains such as the interior of the self-aggregate. The critical aggregation concentration (CAC), which is the threshold concentration of self-aggregation formation by intra- and/or intermolecular association, can be determined by observing the change in the intensity ratio of the pyrene in the presence of polymeric amphiphiles (*27*). **Figure 3** exhibits the changes of the I_{372}/I_{383} value as a function of concentration of LA-chitosans. At low concentrations, the I_{372}/I_{383} value remains nearly unchanged. However,, further increasing its concentration, the intensity ratios begin to decrease, implying the onset of self-association from LA-chitosans. Furthermore, CAC was determined by the interception of two straight lines and its value was 5×10^{-2} mg/mL. In the earlier studies, the CAC of the polymeric amphiphiles, such as deoxycholic acid-modified chitosan (*4*) and deoxycholic acid-

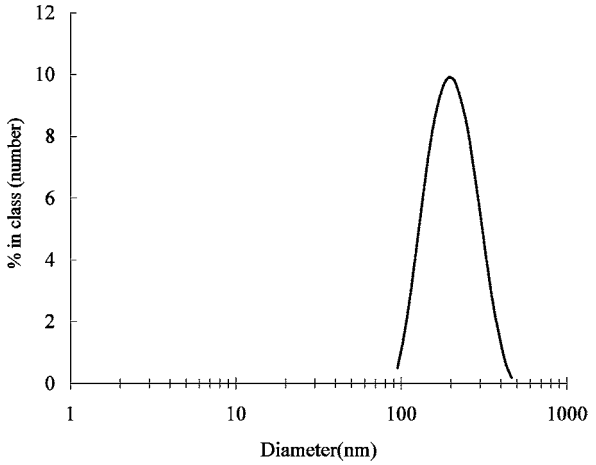

Figure 4. Distribution of LA-chitosan nanoparticles in number ($\theta = 90°$, $\lambda = 670$ nm, and $T = 25$ °C).

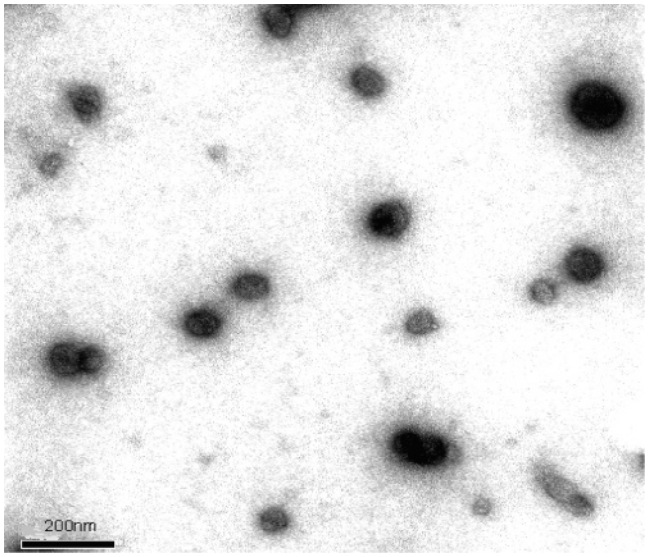

Figure 5. Transmission electron micrograph of the naoparticles of LA-chitosan.

modified dextran (*7*), was lower than the critical micelle concentration of various low molecular weight surfactants, i.e., 0.65 mg/mL for alkyl benzenesulfonate (C_{10-13} alkyl group) in water and 0.3 mg/mL for C_{15-18} α-olefinsulfonate in water. It indicates that self-aggregates are stable in aqueous solution.

Characteristics of Self-Aggregated Nanoparticles. The prepared nanoparticles were analyzed by a laser light scattering technique, and they demonstrated a unimodal particle size distribution (see **Figure 4**). The size of the particles was between 100 and 500 nm with a mean hydrodynamic diameter of 210.8 nm. The nanoparticles formed were of a spherical shape, which indicates that prepared nanoparticles have good structural integrity (see **Figure 5**).

The size of the nanoparticles was determined by TEM and the laser light scattering method. The size of the nanoparticles was different for both of the methods. For instance, the size measured by the TEM method (values not shown) was smaller than the laser light scattering method (**Figure 4**). This is mainly due to the process involved in the preparation of sample. In the case of the TEM method, TEM images (**Figure 5**) depicted the size at the dried state of the sample, whereas the laser light scattering method involves the measurement of size in the hydrated state. In other words, the size determined by TEM is an actual diameter (dry state) of the nanoparticles, whereas the size measured by the laser light scattering method is a hydrodynamic diameter (hydrated state) of the nanoparticles. Therefore, in the hydrated state, the nanoparticles will have a higher hydrodynamic volume due to solvent effect; hence, the size measured by the laser light scattering method was higher than the TEM method. A similar finding is reported by Prabha (*28*).

Table 1. Influence of BSA Concentration on Loading Efficiency and Loading Capacity[a]

BSA final concn (mg/mL)	loading efficiency (%)	loading capacity (%)
0.1	99.23 ± 0.49	19.85 ± 0.04
0.2	70.73 ± 0.18	28.29 ± 0.02
0.5	37.57 ± 0.54	37.57 ± 0.54
1	19.5 ± 0.06	38.74 ± 0.25

[a] Values are means ± standard deviation ($n = 3$).

BSA Loading Capacity and Efficiency of the Nanoparticles. The loading efficiency of BSA was affected by its concentration, and obtained results are presented in **Table 1**. As the concentration of BSA increases, the loading efficiency decreases. On the other hand, the protein loading capacity was enhanced dramatically from 19.85 ± 0.04 to 37.57 ± 0.25% by increasing the concentration from 0.1 to 0.5 mg/mL. However, the protein loading did not increase significantly at 1 mg/mL indicating that nanoparticles were almost saturated by BSA at 0.5 mg/mL. The formation of protein−carrier association may be due to a protein−polysaccharide electrostatic interaction at pH 7.4. BSA has a negative charge and interacts with amino groups of chitosan (*23*). On the other hand, the hydrophilic chitosan backbone and hydrophobic domains of associated LA of nanoparticles play an important role during the complexion with BSA, which has both hydrophobic and hydrophilic patches on its surface (*29*). The mechanism of association between the protein and the carrier needs to be studied further.

In summary, hydrophobically modified chitosan using linolenic acid as the hydrophobic group has been prepared. Nanoparticles were successfully prepared using the hydrophobically modified chitosan. Nanoparticles were small in size (mean diameter of ca. 210.8 nm) with a unimodal size distribution. The hydrogel nanoparticles formed with hydophobized chitosan can be used as water soluble proteins such as BSA carriers. Further investigations on these nanoparticles as protein carriers are in progress.

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