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O/W Emulsification for the Self-Aggregation and Nanoparticle Formation of Linoleic Acid—Modified Chitosan in the Aqueous System

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Chitosan was modified by coupling with linoleic acid through the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-mediated reaction to increase its amphipathicity for improved emulsification. The micelle formation of linoleic acid-modified chitosan in the 0.1 M acetic acid solution was enhanced by O/W emulsification with methylene chloride, an oil phase. The fluorescence spectra indicate that without emulsification the self-aggregation of LA-chitosan occurred at the concentration of 1.0 g/L or above, and with emulsification, self-aggregation was greatly enhanced followed by a stable micelle formation at 2.0 g/L. The addition of 1 M sodium chloride promoted the self-aggregation of LA-chitosan molecules both with and without emulsification. The micelles of LA-chitosan formed nanosize particles ranging from 200 to 600 nm. The LA-chitosan nanoparticles encapsulated the lipid soluble model compound, retinal acetate, with 50% efficiency.

KEYWORDS: Chitosan; linoleic acid; chemical modification; micelles; nanoparticle; emulsification; encapsulation

INTRODUCTION

Polyelectrolytes bearing a bulky hydrophobic group show a strong tendency of intra- or intermolecular aggregation in polar solvent (*1*, *2*). The polymeric micelles formed from the molecular assemblage have the ability to take water insoluble drugs or low molecular weight organic compounds and disperse them in the aqueous solution. The nanosize particulate micelles offer new potential applications in various fields, including pharmaceutics and cosmetics (*3*−*5*). Longer hydrophilic chains and bigger hydrophobic groups help stabilize the micelle structure and protect drug compounds from the environment (*6*). Because the hydrophobic core and hydrophilic shell need to be biodegradable and nontoxic, many investigations of hydrophobic polymers are focused on the natural biomaterials (*7*, *8*).

Chitosan, α-(1-4)-2-amino-2-deoxy-β-D-glucan, is a deacetylated form of chitin, an abundant natural polysaccharide present in crustacean shells. Its unique characteristics such as positive charge, biodegradability, biocompatibility, nontoxicity, and rigid linear molecular structure make this macromolecule ideal as a drug carrier and delivery material (*9*−*11*). There are reports on chitosan hydrophobic modifications and nanoparticle formation by self-aggregation in the aqueous solution (*12*, *13*). The gellike state of the chemically modified chitosan has a higher viscosity and is not stable for the distribution of drugs in the diluted solutions. The problem is, however, that during the self-aggregation process the longer linear molecules tend to form an inordinate gellike structure through cross-linking, for example, the self-aggregation of palmitic acid-modified glycol chitosan (*14*), deoxycholic acid-modified chitosan (*15*, *16*), and poly(*N*-isopropylacrylamide) chitosan (*17*).

In an effort to control the molecular self-aggregation and form a regular molecular assemblage or nanoparticles, Uchegbu et al. (*18*) prepared the nanovesicles 300−600 nm in size by mixing palmitic acid-modified glycol chitosan with cholesterol in the solution, where the presence of cholesterol molecules may interfere with encasing compounds to be encapsulated. Kim et al. (*19*), on the other hand, produced chitosan-based nanoparticles through self-aggregation, which was accomplished by initial controlled depolymerization of chitosan and subsequent hydrophobic modification with deoxychloic acid. In this case, depolymerization may result in weakening the micelle shell structure. The control methods of the molecular assemblage and the factors influencing the self-aggregation of the hydrophobic chitosan in solution without the depolymerization step need to be further studied.

In this paper, chitosan was modified by coupling linoleic acid, as a hydrophobic group, to the chitosan molecule by chemical reaction. Linoleic acid (LA) was chosen to provide a hydro-

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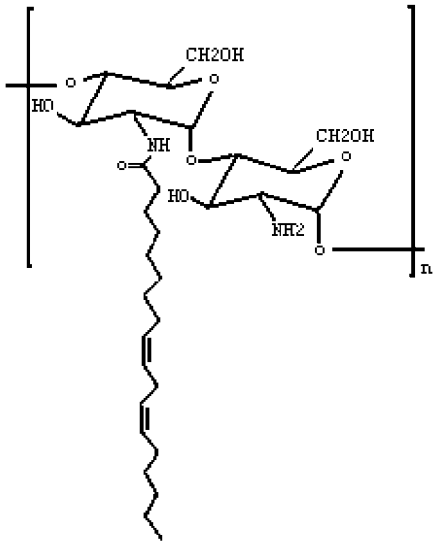

Figure 1. Chemical structure of linoleic acid-modified chitosan.

phobic group that makes LA-chitosan strongly amphipathic and thus suitable for emulsification and is an essential fatty acid required for the fatty acid metabolism in human body (*20*). The objectives of our study were to investigate the chemical characteristics and the self-aggregation properties of the linoleic acid-modified chitosan (LA-chitosan, **Figure 1**), the effect of O/W emulsification and sodium chloride on the process of nanoparticle formation of the amphipathic chitosan in aqueous solutions, and the encapsulation properties of emulsified LA-chitosan.

MATERIALS AND METHODS

Materials. Chitosan of 100 mesh, degree of deacetylation 90%, molecular weight 28 KDa, was made from crab shell and obtained from Biotech Co. (Mokpo, Korea). LA, pyrene, retinal acetate, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma Chemicals (St. Louis, MO).

Preparation of LA-Chitosan. LA was coupled to chitosan by the formation of amide linkages through the EDC-mediated reaction following the method of Lee et al. (*16*). Chitosan (1 g) was dissolved in 1% aqueous acetic acid solution (100 mL) and diluted with 85 mL of methanol. LA was added to the chitosan solution at 0.34 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 20 °C. The 1:1 mole ratio of EDC to LA was used in this study. After 24 h, the reaction mixture was poured into 200 mL of methanol/ammonia solution (7/3, v/v) while stirring. The precipitated material was filtered, washed with distilled water, methanol, and ether, successively, and dried under vacuum for 48 h at 20 °C. The yield of LA-chitosan was 88% (w/w, LA-chitosan/chitosan).

Fourier Transform Infrared Spectroscopy (FT/IR) and ^{1}H NMR Spectroscopy. The IR spectrum of LA-chitosan was recorded on an FT/IR-430 Fourier transform infrared spectrometer (Jasco Co., Tokyo, Japan) at 20 °C following the method of Shigemasa et al. (*21*). For the IR spectroscopic analysis, 2 mg of LA-chitosan was mixed with 100 mg of KBr and made into pellets.

^{1}H NMR spectra of samples were recorded on a Bruker ARX 300 spectrometer at 25 °C. The sample was dissolved in 1% CD_3OOD of D_2O solution (v/v) 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 each scan (*22*).

Preparation of LA-Chitosan Nanoparticles and Dispersion of Model Compounds. Ten milligrams of LA-chitosan was dissolved in 2 mL of 0.1 M acetic acid solution. Varying amounts of methylene chloride (served as an oil phase with concentration range of 0−7%, v/v) were added to the LA-chitosan acetic acid solution while stirring to vary the O/W ratio and homogenized (5 min, 13 000 g) with an ULTRA-TURRAX T-25 dispersing machine (Kushu Kika Kogyo Co., Japan). Methylene chloride was chosen because of its ability to diffuse into the aqueous phase at a rapid rate facilitating particle formation upon evaporation (*23*). The solution was held under vacuum for 30 min at 20 °C to remove methylene chloride and then 1 mL of 0.25% sodium tripolyphosphate (STPP) solution was added as a cross-linking reagent. To determine encapsulation properties of the LA-chitosan, the lipid soluble model compounds were incorporated into the solution by first dissolving in methylene chloride (10 g/L) at the O/W ratio of 1% (v/v) and subsequently emulsifying as previously described. The model compounds were retinal acetate, tocopherol acetate (liquid form), and stearic acid methyl ester (solid form), respectively.

Fluorescence Spectroscopy. 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 pipetted into a 20 mL test tube, and the ethanol was driven off by under a stream of nitrogen gas. Two milliliters of LA-chitosan solution (with or without added 1 M sodium chloride) was added to the test tube, bringing the final concentration of pyrene to 2 μM. The ionic strength of 1 M NaCI was chosen based on a previous study (*24*). 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 (*24*).

Transmittance Spectrophotometer. The transmittance of the solution was recorded on a DU-650 spectrophotometer (BECKMAN Co., U.S.A.) using a quartz cell with an optical path length of 1 cm at 600 nm.

Size Distribution. The particle size was measured using Zatasier S (Malven Instrument, Malvern, U.K.) at detector angle of 90°, 670 nm, and 25.2 °C.

Encapsulation Efficiency Determination. Retinal acetate was determined by high-performance liquid chromatography (HPLC) method under the following conditions: Waters 501 HPLC pump, Waters 486 UV detector (280 nm), ODS-2 column 150 mm × 4.6 mm (Waters Corporation, Miford, U.S.A.). The flow rate of the mobile phase (acetonitrile:methanol = 75:25, v/v) was 1 mL/min.

Sample Preparation. The nanoparticle solution was centrifuged at 8000−9000*g* for 30 min at 4 °C. The precipitate was freeze-dried. Three to five milligrams of the dried material was soaked in 1 mL of methylene chloride in a 5 mL screw-capped tube and shaken in a BS-10 skaking water bath for 30 min at 20 °C, and 2.5 mL of mobile phase solution was added. The mixture was centrifuged as above. The supernatant was vacuum-treated for 30 min to remove methylene chloride, and the resulting solution was used for the HPLC analysis. The calibration curve was obtained with the mobile solution containing retinal acetate at the concentration of 1−60 ng/mL. The encapsulation efficiency of retinal acetate was calculated by the equation of $Y = w_2/w_1 \times 100\%$. Y is the encapsulation efficiency; w_1 is the total amount of retinal acetate; and w_2 is the amount of retinal acetate resulted from HPLC analysis.

RESULTS AND DISCUSSION

Synthesis and Characteristics of LA-Chitosan. EDC is a "zero-length" cross-linker, which forms an amide linkage between the carboxyl group of LA and the amino group of chitosan without leaving a spacer molecule (*15*). The yield of LA-chitosan was 88% (w/w). The product was a white powder and soluble in dilute aqueous acetic acid. The increase of the amide II band at 1655 cm^{-1} in the IR spectra of the product confirms the formation of an amide linkage between amino groups of chitosan and carboxyl groups of LA. **Figure 2** shows the ^{1}H NMR spectra of the chitosan and the LA-chitosan. The

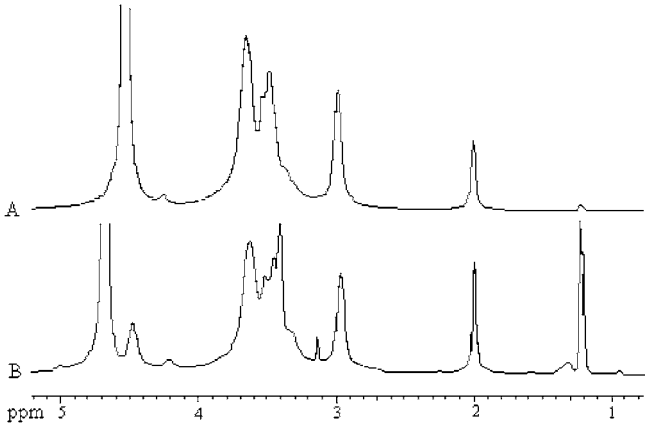

Figure 2. ^{1}H NMR spectra of (A) chitosan and (B) LA-chitosan in D_2O and CD_3COOD.

proton assignment of chitosan (**Figure 2A**): $\delta_{2.0} = CH_3$ (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 2B**): $\delta_{1.2} = CH_3$ (methyl group of linoleyl); $\delta_{2.0} = CH_3$ (acetyl group of chitosan); $\delta_{2.95} =$ CH (carbon 2 of chitosan); $\delta_{3.15} = CH_2$ (linoleyl protons); $\delta_{4.2-4.5} =$ CH (carbon 1 of chitosan). The ^{1}H NMR spectrum confirms the presence of major functional groups linked to chitosan on LA-chitosan as previously reported (*14*, *25*).

Self-Aggregation and Micelle Characteristics. Figure 3a shows the fluorescence spectra of pyrene in LA-chitosan solution in 0.1 M acetic acid. Each spectrum corresponds to LA-chitosan concentration of 0.001−4 g/L. The peak height (I) at 372 nm is indicative of the sensitivity of pyrene to hydrophobic environment while the peak III at 384 nm is relatively unaffected (*24*). The peak III/I ratio can therefore be used to determine the reactivity or aggregation properties of amphipathic molecules to the change in environment hydrophobicity in the aqueous system (**Figure 4A**). When the LA-chitosan concentration was lower than 1 g/L, the peak III/I ratio remained constant near 0.56, meaning that there was a lack of hydrophobic environment with no occurrence of assemblage (**Figure 4A**,**B**) (*26*). When the concentration of LA-chitosan was increased to 1.0 g/L or above, the peak III/I ratio increased, indicating the occurrence of self-aggregation of LA-chitosan resulting from intermolecular hydrophobic interactions between the GlcNAc groups (*27*). A similar observation was made in the unmodified chitosan in 0.1 M acetic acid (*24*).

The fluorescence spectra of pyrene at various concentrations of the LA-chitosan emulsion are shown in **Figure 3b**,**c**. At the lower concentrations of LA-chitosan solution (0.0001−0.01 g/L), the spectra (**Figure 3c**) were similar to those of LA-chitosan solution without emulsification (**Figure 3a**). At the higher concentrations (0.1−5 g/L) (**Figure 3b**), they were drastically different from **Figure 3a**. The peak III/I ratio began to increase from 0.001 g/L LA-chitosan (**Figure 4C**) and leveled off when the LA-chitosan concentration reached 2.0 g/L. The increase in the peak III/I ratio for emulsified LA-chitosan at lower concentrations than the unemulsified one indicates the development of a hydrophobic environment resulting from the occurrence of more extensive self-aggregation under emulsification. The constant peak III/I ratio at the LA-chitosan concentration over 2.0 g/L indicates a stable microenvironment for the probe molecule, and the formation of LA-chitosan molecule micelles (*28*).

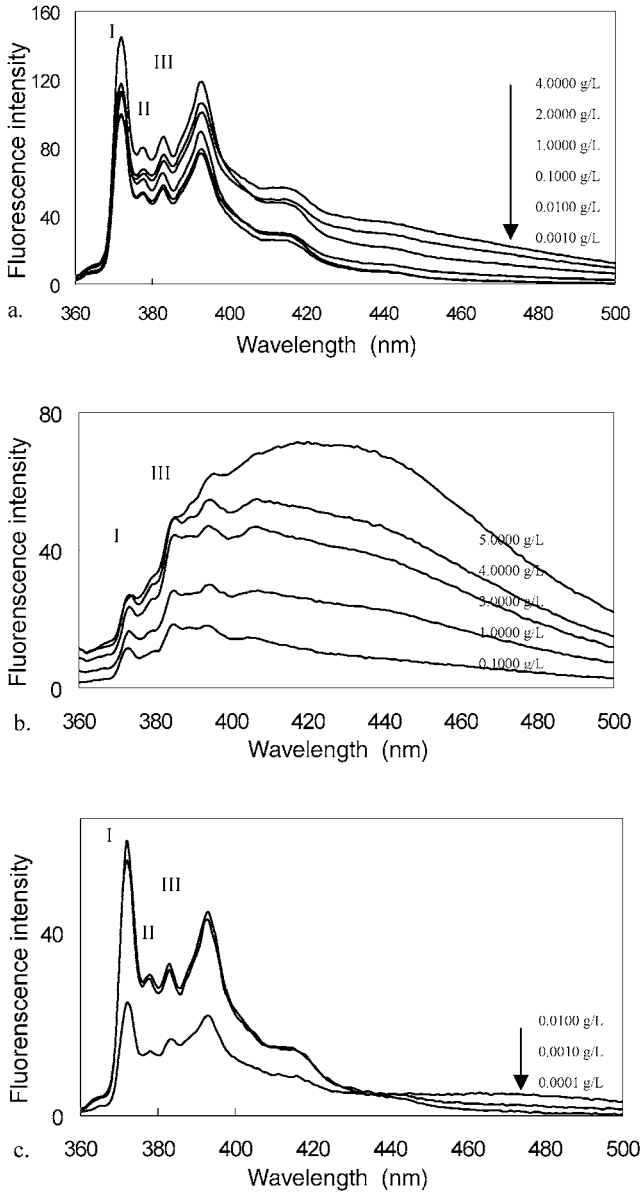

Figure 3. Fluorescence spectra of pyrene in (a) LA-chitosan original solution and (b, c) LA-chitosan solution with the O/W emulsification treatment with methylene chloride (1%, v/v). The concentrations of LA-chitosan were from 0.0005 g/L to 5.0 g/L in 0.1 M acetic acid with 2.0 μM pyrene.

The increasing ionic strength from 0 to 1.0 was reported to facilitate self-aggregation of chitosan in solution with the significant effect being shown only at 1 M NaCI (*24*). The impact of sodium chloride (1 M) on the aggregations of LA-chitosan at the concentration above 1 g/L is shown in **Figure 4B**. The peak III/I ratio slightly increased in the presence of sodium chloride when LA-chitosan concentration of over 1.0 g/L, maintaining the pattern of changes similar to that without sodium chloride. Upon emulsification with methylene chloride, sodium chloride enhanced the LA-chitosan molecule self-aggregation in the solution but could not bring the LA-chitosan molecule into micelles formation until the concentration of LA-chitosan reached 1 g/L (**Figure 4D**). The O/W emulsification with methylene chloride was found to be effective in the formation of LA-chitosan micelles in the solution.

Another phenomenon of LA-chitosan solution (5 g/L) that comes from the O/W emulsification with methylene chloride

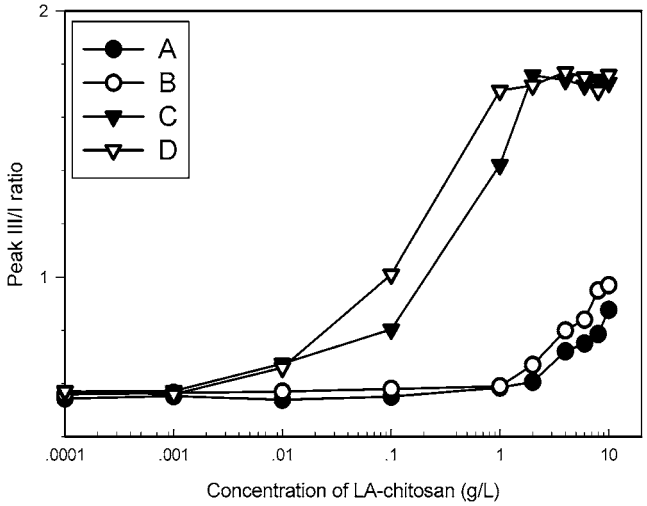

Figure 4. Peak III/I ratio of pyrene fluorescence as a functional of LA-chitosan concentration in 0.1 M acetic acid. (A) Original solution; (B) containing 1 M sodium chloride; (C) O/W emulsification treatment with methylene chloride (1%, v/v); (D) O/W emulsification treatment with methylene chloride (1%, v/v) containing 1 M sodium chloride. (The data were the average values of three group experiments.)

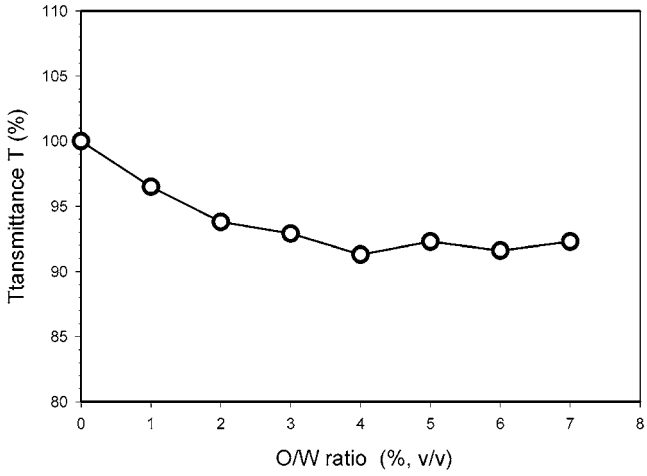

Figure 5. Effect of methylene chloride (v/v) on the transmittance of the O/W emulsion of LA-chitosan. Concentration of LA-chitosan 5 g/L in 0.1 M acetic acid, *T* at 600 nm. (The data were the average values of three group experiments.)

was the decrease in light transmittance (*T*, %, 600 nm) (**Figure 5**). With the increase of methylene chloride in the O/W emulsion, the *T* value of the LA-chitosan solution decreased. The reason for the *T* change was believed to be the transformation of LA-chitosan molecule from the inordinate state to the ordinary micelle state formed by the O/W emulsification with methylene chloride. The methylene chloride representing the oil phase in the emulsion attracted the linoleyl groups of LA-chitosan around the vacuole, and the chitosan moiety was at the outer part of the vacuole.

After the methylene chloride was removed by vaporization under vacuum, the ordered micelles were formed with the linoleyl groups. The ratios of methylene chloride/LA-chitosan solution (v/v) influenced the degree of formation. Beyond the ratio 4% (v/v) of methylene chloride to LA-chitosan solution, the light transmittance remained constant. The 4% amount was considered the critical ratio to form an O/W emulsion of LA-chitosan with methylene chloride.

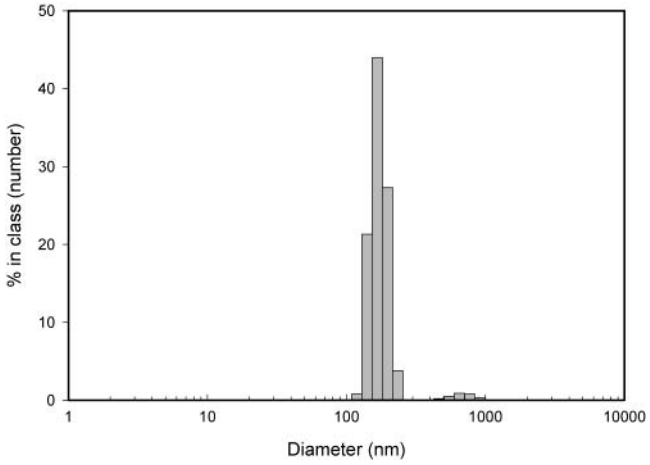

Figure 6. Distribution of LA-chitosan nanoparticles in number ($\theta = 90°$; $\lambda = 670$ nm; $T = 25.2$ °C).

Nanoparticles and the Distribution. Figure 6 shows the size distribution of the micelle particles formed by LA-chitosan (5 g/L) in the 0.1 M acetic acid solution after O/W emulsification with methylene chloride (1%, v/v) and cross-linking with STPP. There are two peaks in the histogram, and the majority number of the particles was around 200 nm in size. The small numbers of large particles (~600 nm) were believed to be formed by aggregation between the small particles.

Encapsulation of Model Compounds. Retinal acetate was examined for its encapsulation efficiency in the LA-chitosan nanoparticles, and the efficiency was found to be 50%.

In summary, the assemblage of LA-chitosan in the solution can be controlled by the O/W emulsification with methylene chloride. The critical value of methylene chloride to LA-chitosan solution for micelle formation was 4.0% (v/v). The micelles of LA-chitosan formed nanosize particles of 200−600 nm. Sodium chloride (1 M) promoted the self-aggregation of LA-chitosan molecule both with and without O/W emulsification with methylene chloride. The LA-chitosan nanoparticle system can be used to encapsulate lipid soluble compounds.

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