Characterization and Modification of Low Molecular Water-Soluble Chitosan for Pharmaceutical Application

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The low molecular water-soluble chitosan nanoparticles (LMWSC-NPs) were prepared, which was modified with hydrophilic and hydrophobic moieties to evaluate the potential for pharmaceutics application. The synthesis of LMWSC-NPs was identified by FT-IR and ^{1}H-NMR spectra. Also, we measured the photon correlation spectroscopy (PCS), transmission electron microscope (TEM) and atomic force microscope (AFM) to investigate the characteristics and morphology of the LMWSC-NPs. At the PCS measurement, the more increase the number of substitutive group, the more decrease the positive charge of LMWSC-NP surface. From the results of TEM and AFM, spherical morphologies were observed, and their sizes were 30-150 nm. Resultantly, LMWSC-NPs prepared in this experiment will be expected as a suitable device for the drug targeting system.

Key Words : Low molecular water-soluble chitosan, Nanoparticles, Drug carriers, AFM, TEM

Introduction

Generally, nanoparticles are useful as a device for carrying drugs or bioactive agents in the human body due to their small particle size. However, conventional nanoparticles still have several problems such as surfactant removal in process, undesirable side effects, rapid clearance by the reticuloendothelial system (RES), thermal instability, short blood circulation, and lower loading efficiency.[1,2] In a previous study,[3] we first synthesized nanoparticles without surfactant by the dialysis method and then solved the problem of toxicity by surfactant in the body. Until now, materials used to prepare the nanoparticles were PLGA and diblock copolymers composed of poly(aspartic acid) or poly(β-benzyl-L-aspartate) as hydrophobic component and poly(ethylene glycol) as hydrophilic component.[4-7] Unlike these synthetic polymers, we used low molecular water soluble chitosan (LMWSC) having a free amine group synthesized in our laboratory.[8]

High molecular weight chitosan (HMWC) results in poor solubility in water and organic solvents, and thus serious disadvantage to actual use. Thus, to increase the solubility of chitosan, it was modified with PEG. PEG-g-chitosan has been investigated as a potential carrier for the delivery of anionic drugs by Tatsuro Ouchi *et al*.[9] Ick chan Kwon *et al*. showed its potential as a drug carrier of self-aggregates formed by chitosan modified with hydrophobic group, bile acid.[10] However, the remaining chitosan solution dissolved in acetic acid, which presents a major drawback due to bioactive agents. In this study, we prepared LMWSC-NPs with a hydrophobic core using LMWSC developed in our laboratory. The LMWSC-NPs were formed as introduce hydrophilic group, polyethylene glycol (PEG), and hydrophobic group, cholesterol, at chitosan chains. PEG chains can prevent cell adhesion by entropically driven steric repulsion and increasing hydrophilicity of carrier surfaces. Also, the introduction of the cholesterol can enhance not only the association behavior of chitosan but also the stability and activity of the hydrophobic drug as formation of hydrophobic core of the LMWSC-cholesterol derivatives.

We expect that LMWSC-NPs with hydrophobic core will be found effective in serving as drug carriers for hydrophobic anticancer agents.

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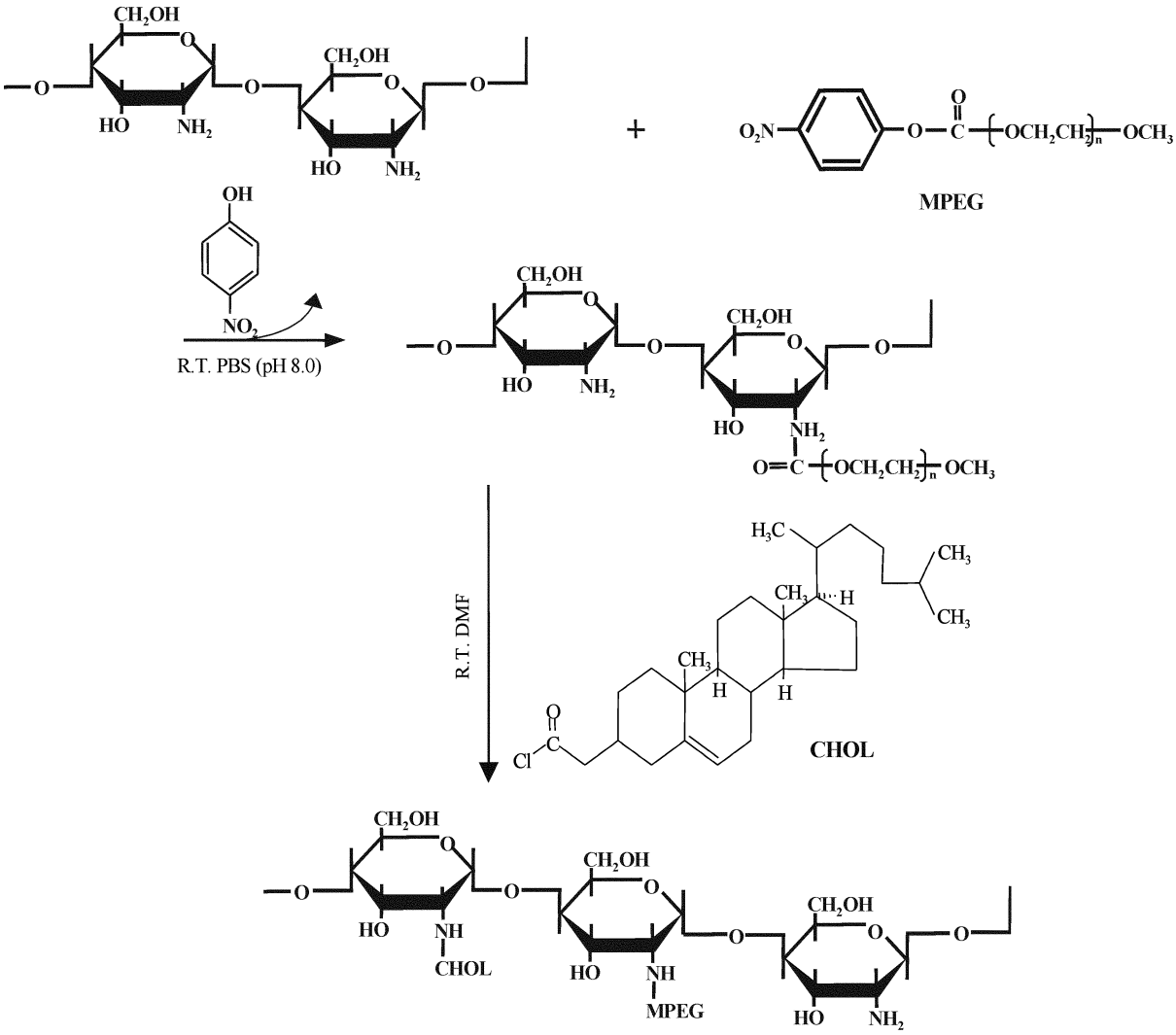
Experimental Section

Materials. LMWSC with a molecular weight of 19 kDa and degree of deacetylation (DDA) of 93% was supplied from KITTOLIFE Co. KOREA. Methoxy poly(ethylene glycol) p-nitrophenyl carbonate (MPEG-pNP)(Mw 5000), hydrophilic moiety, was purchased from Sigma Co. and cholesteryl chloroformate, hydrophobic group, was purchased from Aldrich Co. Dialysis tubing (MWCO 12000) was commercially obtained from Spectrum. Dimethylformamide (DMF) of reagent grade was used without further purification and double distilled water was used in all of the experiments.

Synthesis of LMWSC-NPs. This reaction was carried out in two steps, shown in Scheme 1. First, LMWSC (0.44 mmol) was dissolved in 2 mL of phosphate buffered saline (PBS, pH 8.0). MPEG-pNP (10 mmol) solution dissolved into 1 mL of PBS (pH 8.0) slowly dropped in LMWSC solution and reactant was stirred at room temperature for 2 h. After reacting, the solution was dialyzed using a molecular cut-off 12 000 g/mol dialysis tube against 1.0 L × 3 of cold distilled water for 3 h and then distilled water exchanged at intervals of 3-4 h during a 24 h. The solution was freeze-dried. For the second reaction, the product was dissolved in DMF. A Cholesteryl chloroformate (1.5 mmol) solution



Scheme 1. Synthetic route of LMWSC-NPs.

dissolved in 1 mL of DMF was dropped into the resulting solution. After 2 h, reactant was precipitated with large excess diethyl ether and centrifuged. This process was repeated three times. In this reaction, nonreacted cholesteryl chloroformate was completely removed from product because cholesteryl chloroformate is well soluble in diethyl ether. The final product was dried under reduced vacuum in 37 °C.

Measurement of FT-IR and ^{1}H-NMR spectroscopy. FT-IR(Shimadzu, FT-IR 8700) and ^{1}H-NMR spectrometer (Bruker, DRX-500MHz) was used to identify the synthesis of LMWSC-NPs modified with hydrophobic group and hydrophilic group. For ^{1}H-NMR measurement, LMWSC-NP was dissolved in $CDCl_3$ at a concentration of 10 mgmL^{-1} and the spectra were performed at 353 K. To investigate the structural characteristics of LMWSC-NP, ^{1}H-NMR spectra were measured in $CDCl_3$ and D_2O. The concentration of nanoparticles was 0.5 wt.% in $CDCl_3$ and D_2O.[11]

Morphologies observation of LMWSC-NPs. LMWSC-NP morphology was examined with a JEOL JEM-2000 FX-II transmission electron microscope (TEM) and PARK's Science Autoprobe CP Atomic Force Microscope (AFM). A drop of nanoparticles suspended in 0.01% of phosphotungstic acid was placed on a carbon film coated on a copper grid for TEM. For analysis of the AFM image, nanoparticles of 0.1 mg/mL in distilled water were placed on a silicon wafer surface and observed at room temperature with cantilever frequencies oscillating between 12 and 24 kHz.

Photon correlation spectroscopy (PCS) measurements. PCS was measured with a Zetasizer 3000 (Malvern instruments, England) with He-Ne laser beam at a wavelength of 633 nm at 25 °C (scattering angle of 90°) for the determination of surface charge.

Results and Discussion

Chitosan having a structure similar to cellulose is a suitable biomaterial for designing nanoparticles to carry bioactive agents in human body. However, a major disadvantage to actual use is its poor solubility in water and

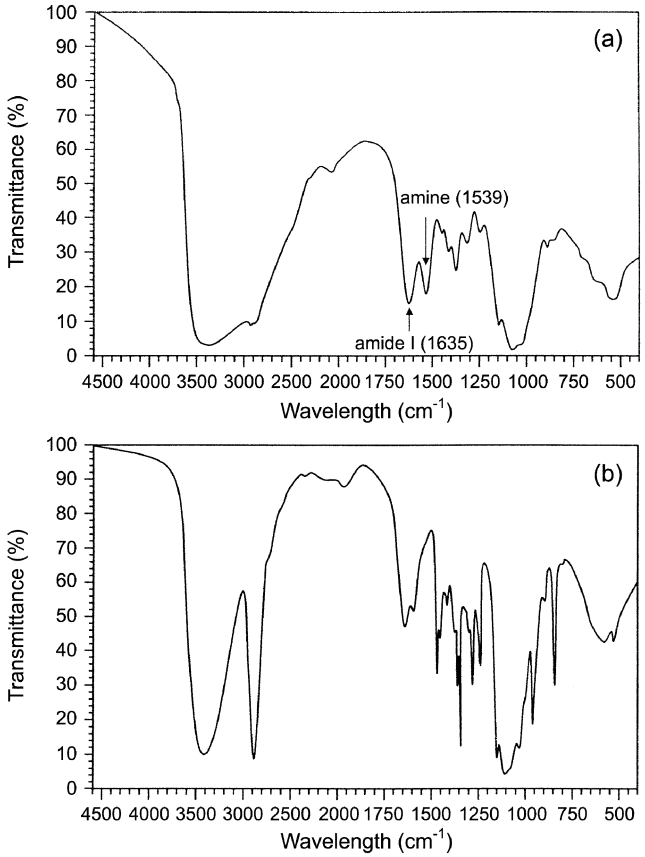


Figure 1. FT-IR spectra of LMWSC (a) and LMWSC-MPEG (b).

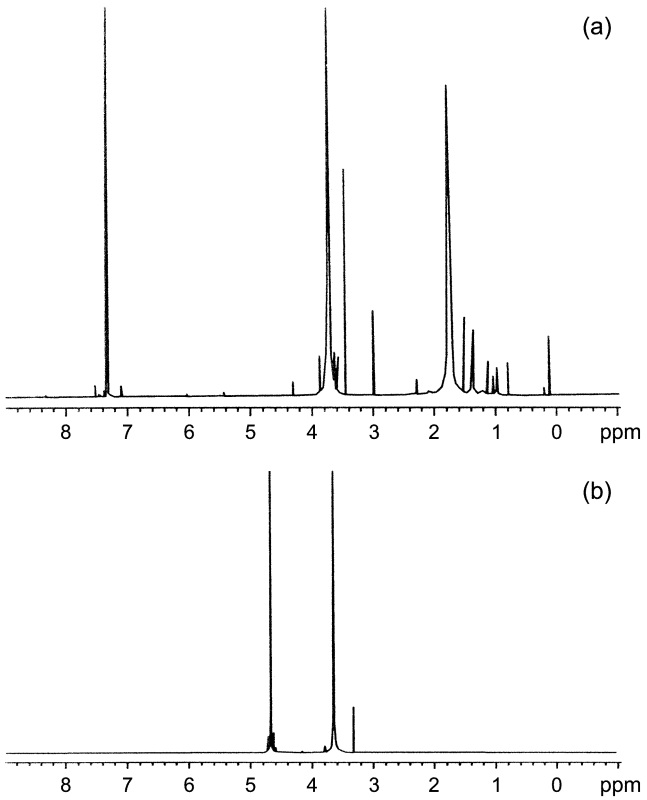


Figure 2. ^{1}H-NMR spectra of LMWSC-NP in $CDCl_3$ (a) and D_2O (b)

other organic solvents. Until now, most research has used HMWC solution in aqueous including acid for application as a biomaterial, and its use as a solvent is considered another limitation to actual use due to the fact that bioactive agents such as peptide and protein drugs, genetic material, and anticancer drugs may be affected by acetic acid.

In this study, we used LMWSC prepared by a novel method. LMWSC has a free-amine group at the C-2 position of the glucose amine unit. Therefore, we chose to modify the free-amine group, using MPEG-pNP as a hydrophilic group and cholesteryl chloroformate as a hydrophobic group.

Spectroscopic identification of LMWSC-NPs. Figure 1 (a) shows the FT-IR spectrum of LMWSC. Amide I and amine peaks showed at 1635 and 1539 cm^{-1}, respectively. However, we found that the amide I band at 1650 cm^{-1} increased and the amine peak at 1539 cm^{-1} decreased due to the formation of amide bonds between LMWSC and PEG (Figure 1(b)). Also, the salient peak of PEG by aliphatic -CH appeared at 2880 cm^{-1}. From ^{1}H-NMR data (Figure 2(a)), the characteristic peak by 14-H at cholesteryl chloroformate was observed at 5.4 ppm. The peak by acetamide group of chitosan and the peak by methoxy group of MPEG-pNP appeared at 2.0 and 3.3 ppm, respectively. The evidence for polymeric micelle formation of LMWSC-NP was obtained with ^{1}H-NMR spectra in $CDCl_3$ and D_2O. As shown in Figure 2(a) of $CDCl_3$ where micelle formation is not expected. All the peaks correspondence to LMWSC, MPEG as hydrophilic part and cholesterol as the hydrophobic part were detected in $CDCl_3$. But, in D_2O (Figure 2(b)), no signals corresponding to cholesterol were shown. These results indicated restricted motions of these protons within the micellar core and rigid structure of cholesterol core of the LMWSC-NP.

Morphologies observation. Figure 3(a), (b), (c) shows TEM photographs of LMWSC-NP with the ratio of 10 : 2 : 0.2, 10 : 2 : 0.4, 10 : 2 : 0.8, the ratio for the units of glucosamine : MPEG-pNP : cholesteryl chloroformate. The shape of the LMWSC-NPs is spherical and the diameters range from about 30 to 100 nm. When the MPEG ratio to glucosamine unit is constant, the more increase the ratio of cholesterol, the smaller the size of LMWSC-NP due to the increase of hydrophobicity. Figure 4(a), (b), (c) shows AFM observation of LMWSC-NPs with the ratio of 10 : 2 : 0.2, 10 : 2 : 0.4, 10 : 2 : 0.8. The shape of the nanoparticles was spherical and the sizes were ranged about 30-100 nm in diameter. From these results, it was identified that the morphology and size of LMWSC-NPs agree with the TEM results.

Zeta potential analysis. Table 1 shows the zeta potential of LMWSC-NPs according to the number of the substituted group at C-2 position of chitosan and the size of LMWSC-NPs by TEM, AFM measurements. As the results, the more increase in the number of substituted group, the more decrease in the positive charge of nanoparticle surface. This means that the positive charge having amine group of chitosan was neutralized by substitution with other groups.

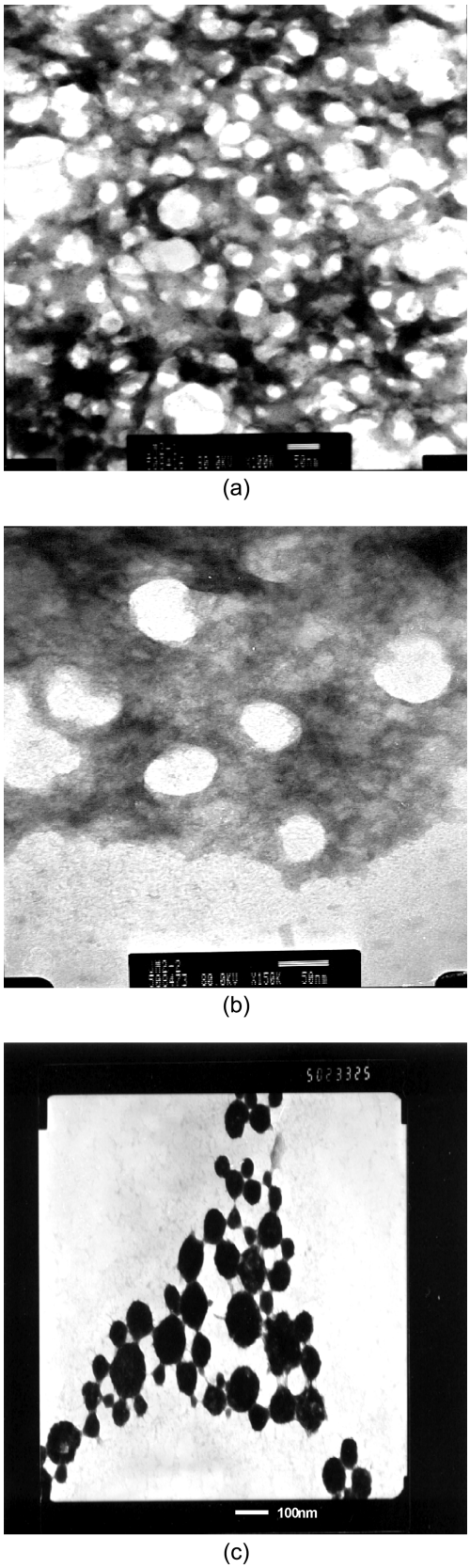


Figure 3. TEM of morphologies of LMWSC-NPs : (a) 10 : 2 : 0.2, (b) 10 : 2 : 0.4, (c) 10 : 2 : 0.8 (the units of glucosamine : MPEG-pNP : cholesteryl chloroformate).

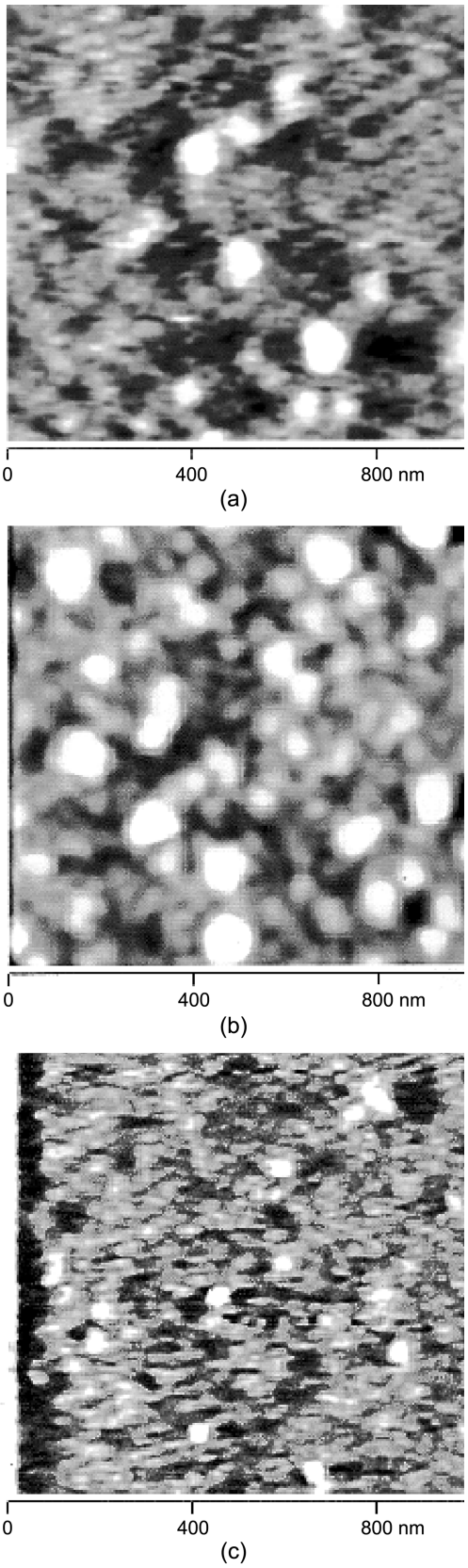


Figure 4. AFM of morphologies of LMWSC-NPs : (a) 10 : 2 : 0.2, (b) 10 : 2 : 0.4, (c) 10 : 2 : 0.8 (the units of glucosamine : MPEG-pNP : cholesteryl chloroformate)

Table 1. Characterization of LMWSC-NPs according to the ratio of hydrophobic group

LMWSC-NPs	The number of substituted group per 10 units of glucosamine (the units of glucosamine : MPEG-pNP : cholesteryl chloroformate)	Zeta potential (mV)	Particle size (nm)
LMWSC-NP1	2.2 (10 : 2 : 0.2)	11.5	70-150
LMWSC-NP2	2.4 (10 : 2 : 0.4)	3.2	50-100
LMWSC-NP3	2.8 (10 : 2 : 0.8)	1.1	30-50

Conclusions

This study shows the synthesis of LMWSC-NP with a hydrophobic core for delivery of hydrophobic drugs such as Taxol. LMWSC was modified with MPEG-pNP and cholesteryl chloroformate. These polymers are known to be biocompatible, biodegradable and nontoxic. The resulting synthesis was identified by FT-IR and ^{1}H-NMR spectrum analysis and the morphology was also revealed through AFM and TEM. It was shown that LMWSC-NP prepared successfully in this experiment can yield the necessarily modified chitosan nanoparticles suitable for drug delivery systems. Therefore, it is expected that LMWSC-NP modified with hydrophobic groups will increase the solubility of hydrophobic drugs.

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