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Preparation, Structural Characterization, Toxicity Test, and DNA Release Study of Low Molecular Weight Chitosan-*g*-L-Phenylalanine/DNA Complex

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ABSTRACT

The preparation of low molecular weight chitosan-*g*-L-phenylalanine (LMWCts-*g*-Phe) was successfully carried out by the coupling reaction using carbodiimide as a coupling agent. The complex of the LMWCts-*g*-Phe and DNA (LMWCts-*g*-Phe/DNA), prepared by a complex coacervation method, exhibited various shapes, i.e., sphere, square, rectangle, and rod with an average size of ~ 50-150 nm as observed by SEM and a zeta potential in the range from -5.9 to -8.9 mV. The *in vitro* cytotoxicity test showed that the LMWCts-*g*-Phe and LMWCts-*g*-Phe/DNA were less toxic to fibroblast cells with the %viability of 76-111. The *in vitro* release of DNA from the complex was very fast in high pH medium (tris buffer, pH 8.0) and completed within 3 days, while the sustained release was achieved in neutral (PBS, pH 7.4) and low pH media (citric acid/sodium citrate buffer, pH 3.0).

Key words: chitosan, phenylalanine, nanoparticle, DNA, complex, cytotoxicity, release

INTRODUCTION

For many decades, chitin-chitosan has been employed in various bio-based applications including gene and DNA deliveries (Bozkir and Saka, 2004; Koping-Hoggard *et al*., 2004; Son *et al*., 2004; and Kean *et al*., 2005) owing to its unique properties such as biocompatibility, bioactivity, biodegradability, and non-toxicity.

DNA, a negatively charged macromolecule, has been reported to form a complex with cationic polymers (Kircheis and Wagner, 2000 and Segura *et al*., 2003) including chitosan (Bozkir and Saka, 2004; Koping-Hoggard *et al*., 2004; and Son *et al*., 2004) to give polymeric vectors, which are useful in a nonviral delivery system. The complexes of chitosan/DNA (Bozkir and Saka, 2004), low molecular weight chitosan (LMWCts)/DNA and/or chitosan oligomer/DNA (Koping-Hoggard *et al*., 2004) as well as their derivatives/DNA (Son *et al*., 2004) were proposed to overcome the toxicity and low transfection efficiency of other cationic polymers, such as poly-L-lysine (Choi *et al*., 1998) and polyethylenimine (Godbey *et al*., 2001).

However, the use of LMWCts grafted with L-phenylalanine (LMWCts-*g*-Phe) as a condensing agent and/or carrier for DNA has not

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yet been reported. The present work, thus, focuses on the preparation and characterization of LMWCts-*g*-Phe/DNA complex, and also the cytotoxicity test and DNA release study to propose the LMWCts-*g*-Phe as an alternative carrier for DNA delivery.

MATERIALS AND METHODS

Materials

Chitosan (degree of deacetylation (DD) = 0.8) was purchased from TCI, Japan. L(-)-phenylalanine (Phe) was obtained from Wako Pure Chemical Industries, Ltd., Japan. 1-Hydroxy-1H-benzotriazole, monohydrate (HOBt), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (EDC), and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) were supplied by Dojindo, Japan. Trisodium citrate and sodium sulfate were purchased from Chameleon Reagent, Japan. DNA-Na-HP (Lot No. PS-1057) was supplied by Yuki Gosei Kogyo Co., Ltd., Japan. Dulbecco's modified eagle medium (DMEM) was purchased from Invitrogen/Gibco, UK. Fetal bovine serum (FBS) was obtained from Trace Scientific Ltd., Australia.

Preparation of low molecular weight chitosan-*g*-L-phenylalanine (LMWCts-*g*-Phe)

LMWCts (DD = 0.76, 0.1 g) was dissolved in distilled water (5 mL) at room temperature. Then, Phe (0.5 mole equivalent to pyranose ring), HOBt (1.5 moles equivalent to Phe), and EDC (1.5 moles equivalent to Phe) were added, respectively. The mixture was stirred at 4°C for 30 min, then at room temperature overnight, and reprecipitated in acetone. The precipitate was washed thoroughly with acetone several times and dried under reduced pressure overnight to give a yellowish powder.

The obtained product was characterized by FT IR, ^{1}H NMR, and EA. FT IR (cm^{-1}): 3288, 3088 (OH, NH), 2936, 2884 (C-H stretching), 1730 (COO, ester), 1640 (C-O, amide I), 1557 (NH, amide II), 1033 (C-O-C), 948 (pyranose ring), and 700, 525 (aromatic). ^{1}H NMR (D_2O, 25±1°C, d, ppm): 2.08 (3H, C$\underline{H}_3$ of acetamide), 2.90 (1H, H-2), 3.13-3.35 (2H, C$\underline{H}_2$ of PLA) 3.70 (2H, H-5,6), 3.79 (3H, H-3,4,6), 4.00-4.03 (1H, C$\underline{H}$ of PLA) 4.58 (1H, H-1), and 7.34-7.47 ($C_6\underline{H}_5$). EA: Anal. Calcd for $(C_6H_{11}O_4N)_{0.57}$ $(C_8H_{13}O_5N)_{0.21}(C_{24}H_{29}O_6N_3)_{0.22}$ (%): C, 53.02; H, 6.56; and N, 8.59. Found (%): C, 44.77; H, 6.25; and N, 7.28.

Complex formation of LMWCts-*g*-Phe and DNA (LMWCts-*g*-Phe/DNA)

The complex of LMWCts-*g*-Phe and DNA (LMWCts-*g*-Phe/DNA) was prepared by a complex coacervation method. The solutions of LMWCts-*g*-Phe in water (1 mg/mL) and DNA in 50 mM aqueous sodium sulfate were preheated at 50°C. Then, the solution of LMWCts-*g*-Phe/water (250 μL) was gradually dropped into the DNA/50 mM Na_2SO_4 solution (250 μL) during vortex at the highest speed for 30 sec. The mixture was placed at room temperature for 30 min to allow a complete complex formation. The surface charge of the complex was analyzed by a zeta potentiometer at 20°C. The complex was collected, washed with water several times, rinsed with ethanol, and dried under reduced pressure overnight. The shape and size of the complex were evaluated by SEM and TEM techniques.

Cytotoxicity test of LMWCts-*g*-Phe/DNA

The *in vitro* cytotoxicity of the complex was evaluated using fibroblast cells (L929). Firstly, the fibroblasts were seeded into DMEM culture medium containing 10% FBS in a 24-well plate with the density of 5×10^4 cells per well and then incubated at 37°C with a humidified 5% CO_2 for 2 hours. After the cells had adhered to the well, the solution of sample (LMWCts-*g*-Phe, DNA, and LMWCts-*g*-Phe/DNA) in DMEM culture medium

(1 mg/mL) was fed into each well to get the final sample concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL and final volume of 2 mL. Then, the cells were incubated at 37°C in a humidified 5% CO_2 for additional 22 hours. The morphology of cells was observed by an optical microscope and the number of viable cells was determined by WST-1 method (Kean *et al.*, 2005). DMEM medium without sample was also prepared using the same protocol and used as a control (100 % viability). The %viability was calculated from the following equation; %viability = [A/B]×100 when A and B are the number of cells in a sample well and in a control well, respectively.

DNA release study

The release of DNA was studied in various buffers, i.e., citric acid/trisodium citrate buffer (pH 3.0), phosphate buffer saline (PBS, pH 7.4), and tris buffer (pH 8.0). Dried complex (3.9 mg) was incubated in the buffer solution (1.2 mL) at 37±0.5°C. At predetermined time intervals, the incubated solution was centrifuged at 3,000 rpm for 15 min, and 400 µL of supernatant was collected for evaluating the amount of released DNA by spectrophotometer at 258 nm. The equal volume of fresh buffer was, then, replaced into the solution and the same procedure was repeated for the next predetermined time interval.

RESULTS AND DISCUSSION

Characterizations of low molecular weight chitosan-*g*-L-phenylalanine (LMWCts-*g*-Phe)

The grafting of Phe onto LMWCts was successful at both hydroxyl and amino groups as confirmed from new peaks at 2936 (C-H stretching), 1730 (ester bond), and 700 and 525 (aromatic ring) by FT IR technique (Figure 1c), as well as proton peaks at δ 3.05-3.35 (C<u>H</u>$_2$), 4.0 (C<u>H</u>), and 7.3-7.5 ppm (aromatic ring) by ^{1}H NMR technique (Figure 2c). The result from XRD technique also supported the success of grafting reaction as the peak combination of LMWCts (Figure 3a) and Phe (Figure 3b) appeared in the pattern of the product (Figure 3c). The grafting percentage of Phe onto LMWCts was 48 with the degree of substitution (DS, the number of grafting chains per 100 glucopyranose rings) of 21.6 and the degree of polymerization (DP, the number of

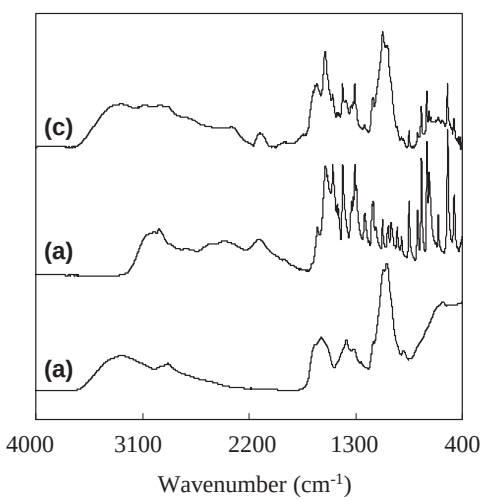

Figure 1 FT IR spectra of (a) LMWCts, (b) L-phenylalanine, and (c) LMWCts-*g*-Phe.

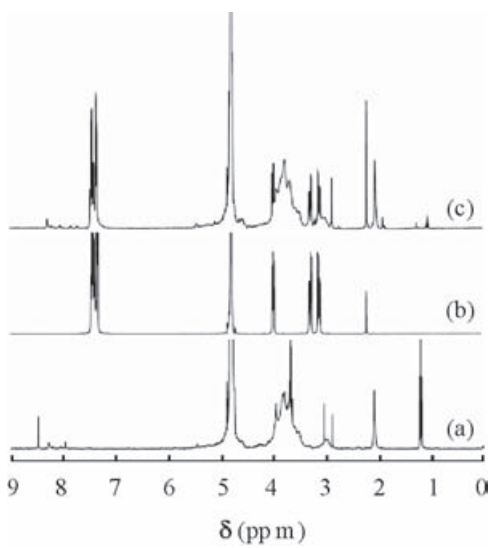

Figure 2 ^{1}H NMR patterns of (a) LMWCts, (b) L-phenylalanine, and (c) LMWCts-*g*-Phe.

Phe units per a grafting chain) of 2.2 as quantitatively determined by ^{1}H NMR.

The morphology of LMWCts-*g*-Phe was observed by SEM. The LMWCts-*g*-Phe exhibited spherical shape with an average size of ~ 80 nm (Figure 4b), which was relatively smaller than that of LMWCts (~ 100 nm) (Figure 4a).

Complex formation of LMWCts-*g*-Phe and DNA (LMWCts-*g*-Phe/DNA)

By dropping the solution of LMWCts-*g*-Phe/water into the solution of DNA/50 mM Na_2SO_4 with an equi-volume at 50°C during vortex at the highest speed, the mixture became turbid implying the complex formation. It was speculated that the complex was formed via the electrostatic interaction between the cationic amino group of LMWCts-*g*-Phe and the anionic phosphate group of DNA molecules.

The individual complex showed various shapes, i.e., sphere, square, rectangle, and rod with an average size of ~ 50-150 nm as observed by SEM (Figure 4c) and ~ 40-70 nm by TEM (Figure 5). Moreover, SEM micrograph revealed that some particles formed aggregates with an approximate size of ~ 300-500 nm.

The zeta potential of the obtained complexes measured in a 25 mM aqueous Na_2SO_4 solution at 20°C was in the range from -5.9 to -8.9 mV implying that the complex surface was negatively charged and mainly covered with DNA.

It is important to keep in mind that the surface charge should be considered when the complex is introduced into the body. For example,

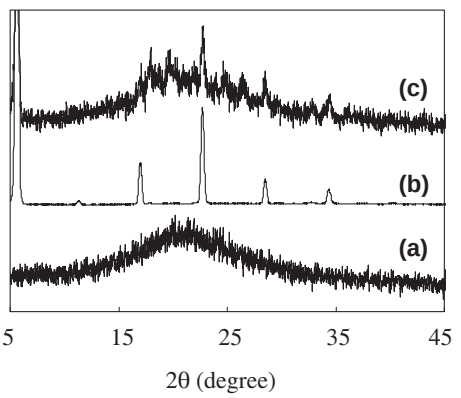

Figure 3 XRD patterns of (a) LMWCts, (b) L-phenylalanine, and (c) LMWCts-*g*-Phe.

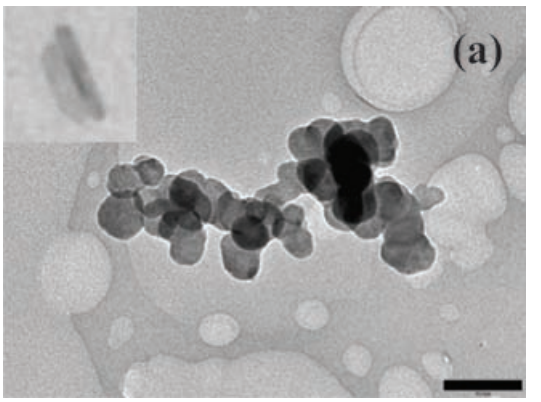

Figure 5 TEM micrographs of LMWCts-*g*-Phe/DNA; bar = 100 nm.

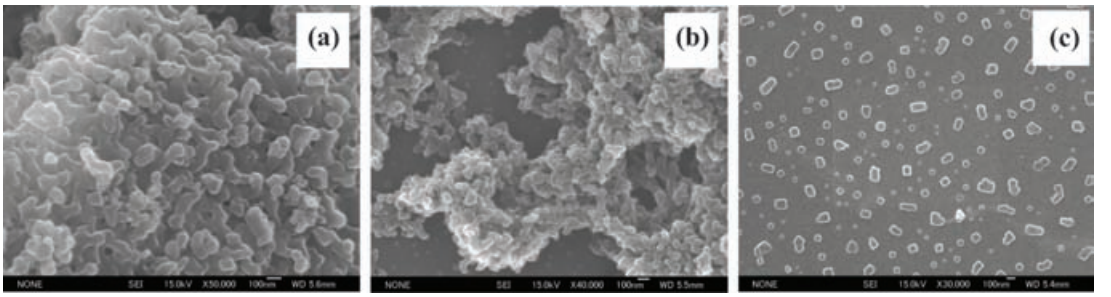

Figure 4 SEM micrographs of (a) LMWCts (50,000×), (b) LMWCts-*g*-Phe (40,000×), and (c) LMWCts-*g*-Phe/DNA (30,000×).

a highly positive charge (high zeta potential) should be avoided because it might be toxic to cells including nonspecific interactions between complex and cells, and also exchange reactions of DNA with negatively charged macromolecules existing in the body during delivery (Kircheis and Wagner, 2000 and Schatzlein, 2001).

The loading efficiency of DNA onto the complex was 90% based on the total amount of DNA added during the complex preparation step.

Cytotoxicity test of LMWCts-*g*-Phe/DNA

The toxicity of LMWCts-*g*-Phe and LMWCts-*g*-Phe/DNA was tested *in vitro* with fibroblast cells to confirm whether the material is suitable for biomedical applications. The morphology and viability of cells were evaluated after incubating the cell in a sample/DMEM culture medium solution at 37°C in a humidified 5% CO_2 for 22 hours. Figure 6 showed the appearances of cells in various samples. All samples gave a normal cell adhesion.

The %viability of cells was evaluated by WST-1 method, which bases on the ability of mitochondrial dehydrogenases in viable cells to convert WST-1 into WST-formazan,[4] which can be quantitatively detected by spectrophotometer at λ_{max} of 450 nm. The %viabilities of cells were 86-95, 99-119, and 76-111 for LMWCts-*g*-Phe, DNA, and LMWCts-*g*-Phe/DNA, respectively. The results supported the fact that DNA is non-toxic and implied that LMWCts-*g*-Phe and its complex exhibited a very low toxicity to fibroblasts when the concentration of sample was 0.1-0.5 mg/mL. It should be noted that in the cases of LMWCts and DNA, the sample concentration hardly affected the %viability of cells. However, for LMWCts-*g*-Phe/DNA, the %viability decreased when the concentration was high, e.g., 0.4-0.5 mg/mL. It was suspected that the complex at high concentration might perform as a surfactant to remove the adhered cells and/or to impede the cell adhesion resulting in the decrease in viable cell number.

DNA release study

The DNA release studies provided us not only the release mechanism, but also the

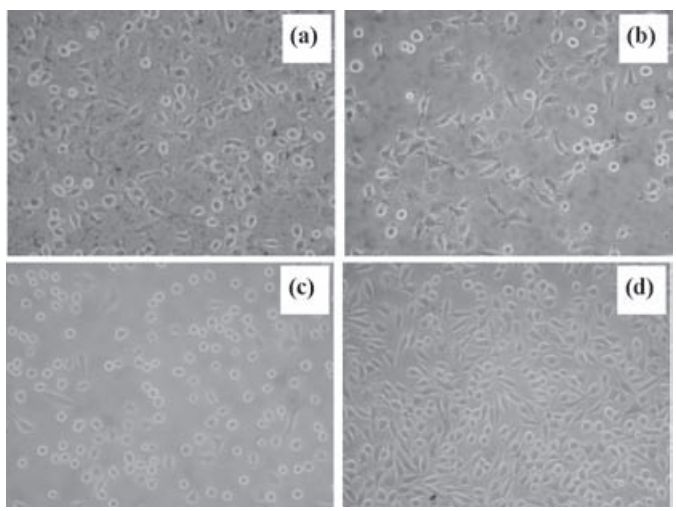

Figure 6 Optical micrographs (200×) of L929 fibroblast cells after incubation at 37°C in a humidified 5% CO_2 for 22 hours with DMEM solutions of (a) culture medium (control), (b) LMWCts-g-Phe, (c) DNA, and (d) LMWCts-g-Phe/DNA.

confirmation of the successful loading of DNA onto the complex. The release of DNA was investigated *in vitro* at 37±0.5°C for more than a month in various pH buffers. It was found that the complexes incubated in high pH medium, i.e., tris buffers, had swollen and completely disappeared within 4 days. In contrast, the complexes shrank and became darker in low pH medium, i.e., citric acid/trisodium citrate buffer. For PBS, even though the complexes swelled and disappeared little by little, some still existed through out the experimental period.

The amount of released DNA was measured by spectrophotometer at 258 nm. The release of DNA was very fast for the initial stage, i.e., the first 24 hours for tris buffer (Figure 8c) and 333 hours for citric acid/trisodium citrate (Figure 8a) and PBS buffers (Figure 8b). In other words, the initial release rate was high (burst effect) as confirmed by the slope of DNA releasing profiles (Figure 8). The mechanism of DNA release at this stage might be involved with the diffusion of DNA localized at the complex surface. The diffusion of DNA was enhanced in high pH medium due to the deprotonation of cationic LMWCts-*g*-Phe. As a result, the electrostatic interaction between the LMWCts-*g*-Phe and DNA was null and finally DNA was discharged very fast.

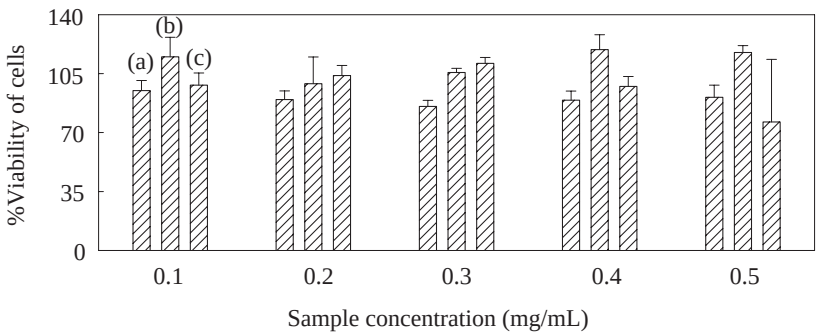

Figure 7 %Viability of cells at various concentrations of (a) LMWCts-*g*-Phe, (b) DNA, and (c) LMWCts-*g*-Phe/DNA. The data are expressed as mean values ± standard deviation (n = 3-4).

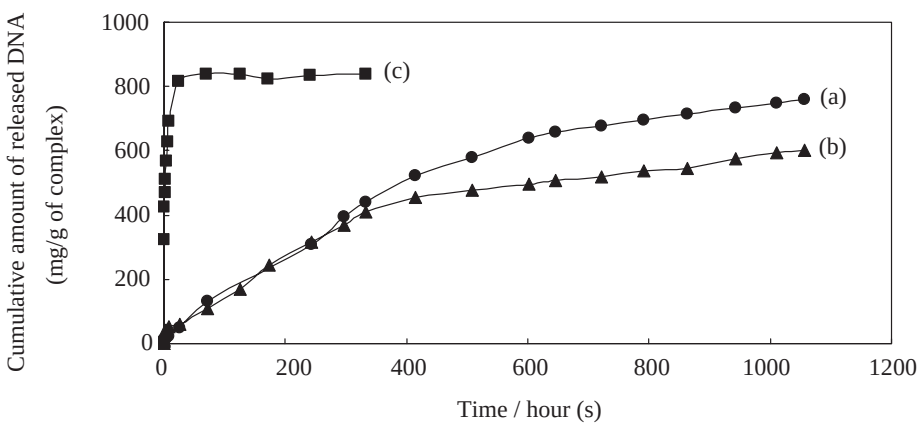

Figure 8 *In vitro* releasing profiles of DNA from LMWCts-*g*-Phe/DNA at 37±0.5°C in various buffers; (a) citric acid/trisodium citrate buffer (pH=3), (b) phosphate buffer saline (PBS, pH=7.4), and (c) tris buffer (pH=8).

For the second stage, the release rate was relatively slow, especially in the case of tris buffer (Figure 8c). This might be due to the small amount of DNA left inside and/or at the surface of the complex. The small release of DNA at this stage might occur via the degradation of cationic material. The release of DNA in tris buffer reached the plateau within 69 h, while the ones in PBS and citric acid/trisodium citrate buffers required longer period of time (> 44 days) to reach the plateau. In other words, the release of DNA from LMWCts-*g*-Phe/DNA complex finished within 3 days in tris buffer, but more than 44 days in PBS and citric acid/trisodium citrate buffers. This might be caused by the stronger electrostatic interaction between cationic material and DNA resulting in the longer release period.

However, it was expected that the amount of released DNA in citric acid/sodium citrate buffer should be lower than that in PBS, but the result was contrary, especially after 241 hours. This might result from the higher degradation and/or dissolution rate of the cationic material in the lower pH medium after 10 day-incubation.

CONCLUSION

The complex of LMWCts and DNA (LMWCts/DNA), having various shapes, i.e., sphere, square, rectangle, and rod with nano-size as observed by SEM (~ 50-150 nm) and TEM (~ 40-70 nm) and negatively charged surface (-5.9 to -8.9 mV), was successfully prepared. The cytotoxicity of LMWCts/DNA complex to fibroblast cells was very low as confirmed by the normal cell adhesion and the number of viable cells (76-111%). The release of DNA from the complex was more sustainable in PBS, pH 7.4 and citric acid/trisodium citrate buffer, pH 3.0 (more than a month) than in tris buffer, pH 8.0 (within 3 days). The results suggest that LMWCts-*g*-Phe is suitable as an alternative non-viral carrier for DNA delivery, owing to its nano-size, biodegradability, very low toxicity, and characteristic releasing profiles.

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