LOW MOLECULAR WEIGHT CHITOSAN-*G* -L-PHENYLALANINE: PREPARATION, CHARACTERIZATION, AND FEASIBLE APPLICATION

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Abstract

The grafting of L-phenylalanine onto low molecular weight chitosan (LMWCts-*g*-Phe) was successful as confirmed from the ester peak at 1747 cm^{-1} (FT IR technique) and the aromatic proton peak at 7.3-7.5 ppm (^{1}H NMR technique). By increasing the amount of phenylalanine in feed, the amount of phenylalanine grafting on LMWCts (% grafting) was enhanced, e.g., 48 and 341 % for phenylalanine in feed of 0.5 and 5 mole(s) equiv. to pyranose unit, respectively. LMWCts-*g*-Phe formed spherical particles with an average size of ~80 nm, when the % grafting was ~ 123 corresponding to phenylalanine in feed of 1 mole equiv. to pyranose unit. This compound showed the ability to form complex with DNA as clarified from the turbid solution. The complex appeared various shapes, i.e., sphere, rectangle, square, and rod with an average size of ~ 50-150 nm. The % viability of LMWCts-*g*-Phe/DNA complex was ~ 93-108 implying non-toxicity to fibroblasts. The release of DNA from LMWCts-*g*-Phe/DNA complex was very fast in carbonate (pH=9.5) and tris buffers (pH=8), but more controllable in PBS (pH=7.4) and citric acid buffers (pH=3). The release reached the plateau within 69 hours for carbonate and tris buffers, while more than 333 hours for PBS and acetic acid buffers. The results suggested that LMWCts-*g*-Phe was a promising carrier for DNA delivery.

Introduction

For many decades, chitosan has been reported as a bio-potential material for medical and pharmaceutical applications, due to its biodegradability, biocompatibility, bioactivity, and non-toxicity [1-3]. However, the development of this biomaterial is limited owing to its high crystallinity and low solubility in most common organic solvents including water at neutral pH, which resulting from high molecular weight and high inter- and intramolecular hydrogen bonds. The reduction of molecular weight and the chemical modification are alternative approaches to overcome those problems. Low molecular weight chitosan, oligochitosan, and/or water soluble chitosan, which can be achieved by enzymatic degradation, photo-irradiation, acid-base hydrolysis, etc., are easy to handle and further modify [4-5]. The introduction of bulky groups onto chitosan backbone provides the derivatives with not only the solubility enhancement, but also the controlled molecular structure and the versatile properties [6-7].

The grafting of amino acids onto chitosan have been recently studied for the specific purposes, such as modifying the surface of chitosan beads for a selective removal of immunoproteins [8], improving the chondrogenesis of a poly(D,L-lactic acid) membrane surface by coating with chitosan-amino acid for tissue engineering [9], and using amino acid as a spacer arm for conjugating dihydronicotinamide groups onto chitosan [10]. However, the preparation of chitosan-amino acid in a particulate form for bio-based applications has not yet been reported.

Phenylalanine, one of the twenty common amino acids used to construct proteins, consists of not only two reactive functional groups, i.e., amino and carboxyl groups, which can be chemically modified, but also benzyl side chains, which provide hydrophobicity to a molecule.

The present work, thus, focused on the preparation and characterization of low molecular weight chitosan grafted with L-phenylalanine (LMWCts-*g*-Phe). We also studied on the complex formation of LMWCts-*g*-Phe and DNA (LMWCts-*g*-Phe/DNA complex) including the *in vitro* cytotoxicity and DNA release in order to propose this compound as a DNA condensing agent for gene delivery system.

Materials and Methods

Materials

Chitosan (DD = 0.8) was purchased from TCI, Japan. L(-)-phenylalanine, hydrogen peroxide, ethanol, sodium hydroxide, acetone, citric acid, tris(hydroxymethyl)aminomethane, hydrochloric acid, sodium carbonate, sodium bicarbonate, sodium hydrogen phosphate, potassium chloride, potassium phosphate monobasic, sodium chloride, and trypsin were products of 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 from Dojindo, Japan. Trisodium citrate and sodium sulfate were purchased from Chameleon Reagent, Japan. Deuterium oxide and acetic acid-d4 were supplied from Cambridge Isotope Laboratories, Inc., USA. DNA-Na-HP (Lot No. PS-1057) was a product of Yuki Gosei Kogyo Co., Ltd., Japan. Dulbecco's modified eagle medium (DMEM) was supplied from Invitrogen/Gibco, UK. Fetal bovine serum (FBS) was purchased from Trace Scientific Ltd., Australia. All chemicals were used as received.

Preparation of Low Molecular Weight Chitosan (LMWCts)

Low molecular weight chitosan (LMWCts) was prepared as reported elsewhere [11-12]. Briefly, chitosan (1 g) was dispersed in distilled water (30 mL) at ambient. The solution of H_2O_2 (30%, 5 mL) was gradually added, while the mixture was heated to 60°C and stirred for 6 h. The mixture was neutralized with 1 M NaOH solution following by filtration. The supernatant was concentrated by rotary evaporator, and then reprecipitated in ethanol. The precipitate was washed thoroughly with ethanol and dried under vacuum to give a yellowish fine powder.

Preparation of LMWCts -g-L-Phenylalanine (LMWCts-g-Phe)

LMWCts (0.1 g) was dissolved in distilled water (5 mL) at ambient. L-phenylalanine (0.5, 1.0, 2.0, 3.0, and 5.0 mole(s) equiv. to pyranose unit), HOBt (1.5 moles equiv. to Phe), and EDC (1.5 moles equiv. to Phe) were added in LMWCts solution, respectively. The mixture was, then, stirred at 4°C for 30 min following by room temperature overnight. The solution was centrifuged and reprecipitated in acetone. The precipitate was collected and dried under vacuum to provide a yellowish powder.

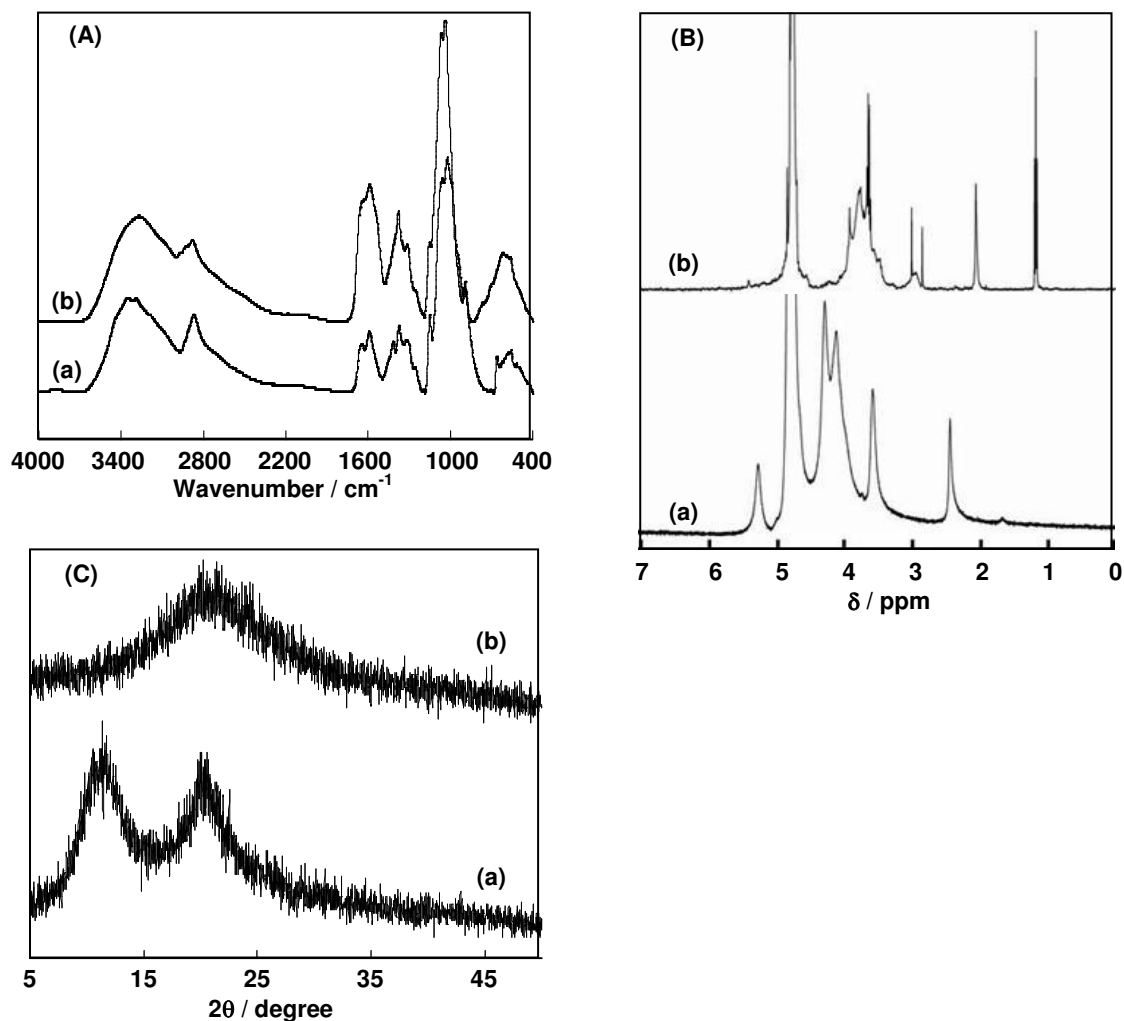### *Preparation of LMWCts-g-Phe/DNA Complex*

The solutions of LMWCts-*g*-Phe in water and DNA in 50 mM Na_2SO_4 were separately prepared. After preheating at 45-50°C, the solution of LMWCts-*g*-Phe was gradually dropped into DNA solution during vortex at the highest speed. The mixture solution was, then, placed at room temperature for 30 min and washed with water several times. Some particles were dispersed in water for SEM observation, while the other was rinsed with ethanol and dried under vacuum at room temperature overnight. Dried complexes were applied for the *in vitro* cytotoxicity test and *in vitro* DNA release study.

Results and Discussion

Preparation and Characterization of LMWCts

LMWCts was prepared by H_2O_2 oxidative degradation [11-12]. Although the obtained product showed the reduced weight average molecular weigh (M_w, ~ 4405 Dalton), the chemical structure should be investigated, before applying as a starting material for the next step, to confirm whether *N*-acetyl-D-glucosamine and D-glucosamine units were still retained in the structure. Here, FT IR and ^{1}H NMR techniques were applied to find that the hydrolyzed product shows the peaks at 3266 (OH), 2860 (C-H stretching), 1617 (C-O, amide I), 1573 (NH, amide II), 1029 (C-O-C), and 879 cm^{-1} (pyranose ring) (Figure 1Ab) as well as the proton peaks at 2.08 (CH_3 acetamide), 3.03 (H-2), 3.79 (H-5,6), 3.95 (H-3,4,6), and 4.59 ppm (H-1) (Figure 1Bb), which are similar to those of native chitosan (Figure 1Aa and 1Ba). This implied that the primary structure of the product still be chitosan. Here, it was concluded that LMWCts was successfully prepared. The degree of deacetylation (DD) of LMWCts was ~ 0.76 as determined by ^{1}H NMR.

The packing structure was observed by XRD. Generally, chitosan shows two peaks at 2θ 10 and 20 degree (Figure 1Ca). But LMWCts gives only a broader peak at 2θ 20 degree implying the decrease in crystallinity.

Figure 1 : (A) FT IR spectra, (B) ^{1}H NMR patterns, and (C) XRD patterns of (a) chitosan and (b) LMWCts.

Preparation and Characterization of LMWCts-g-Phe

The grafting reaction of phenylalanine onto LMWCts was carried out in water at room temperature using EDC as a coupling agent and HOBt as a catalyst. The obtained product gives new peaks at 2936 (C-H stretching), 1747 (ester bond), and 700 and 525 cm^{-1} (aromatic ring) (Figure 2Ac) as well as the proton peaks at δ 3.05-3.35 (CH$_2$), 4.0 (CH), and 7.3-7.5 ppm (aromatic ring) (Figure 2Bc) implying the successful grafting reaction. It should be noted that the grafting reaction occurred randomly at both hydroxyl and amino groups as clarified from the new ester peak and the higher intensity of amide I and II bands, respectively. XRD result also supports the success of reaction as illustrated from the peak combination of LMWCts (Figure 2Ca) and phenylalanine (Figure 2Cb) in the pattern of product (Figure 2Cc).
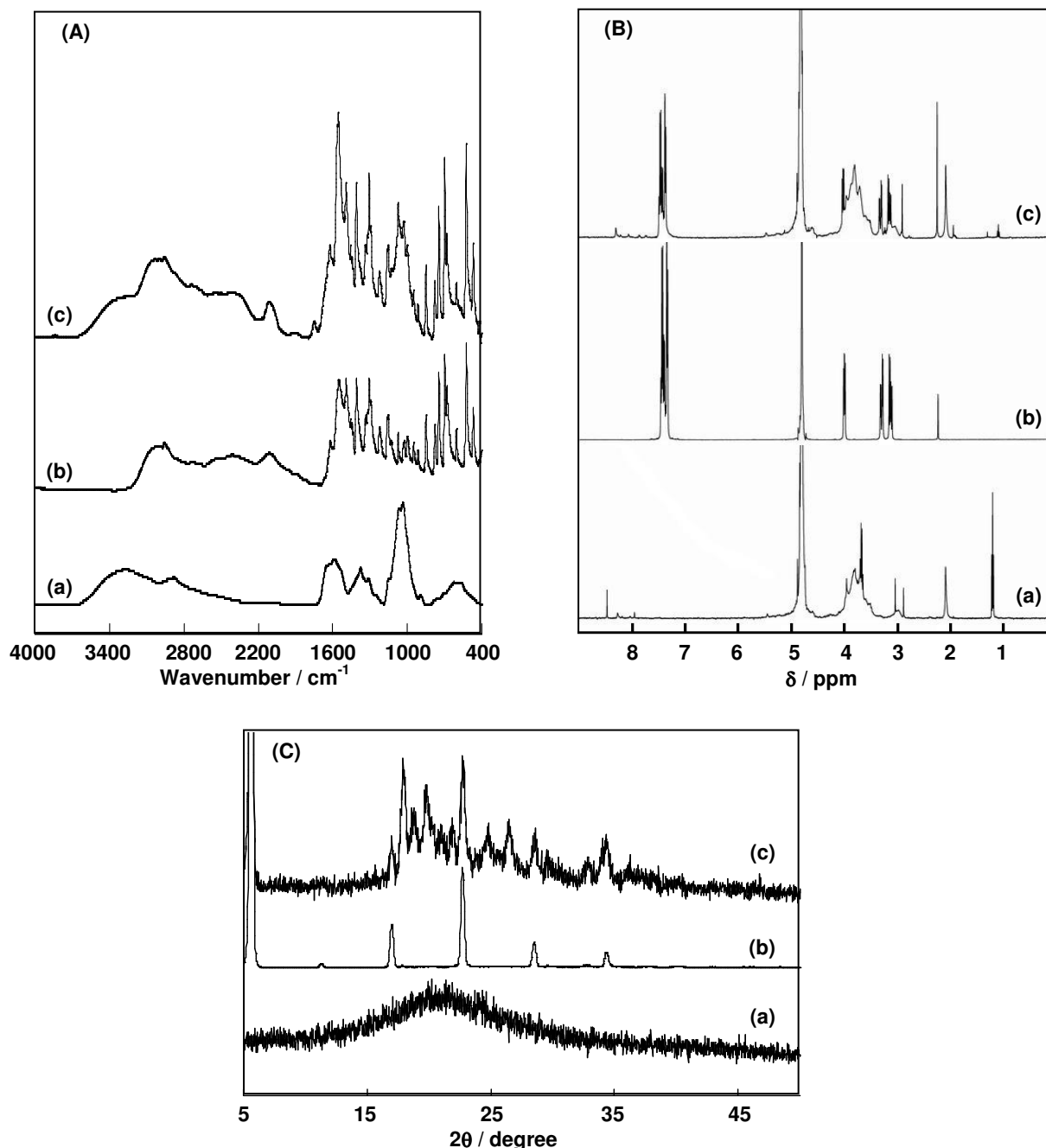

Figure 2 : (A) FT IR spectra, (B) ^{1}H NMR patterns, and (C) XRD patterns of (a) LMWCts, (b) phenylalanine, and (c) LMWCts-g-Phe.

The % grafting of phenylalanine onto LMWCts was determined by ^{1}H NMR technique using the following equation.

$$\% \text{ grafting} = [(I_{\text{aromatic protons}}/5) / (I_{\text{H3-6(pyranose ring)}}/5)] \times 100$$

It was found that % grafting increases when the amount of phenylalanine in feed increases (Figure 3), e.g., 48.04 and 340.92 % when 0.5 and 5 mole(s) equiv. to pyranose unit of phenylalanine were fed to the reaction, respectively.
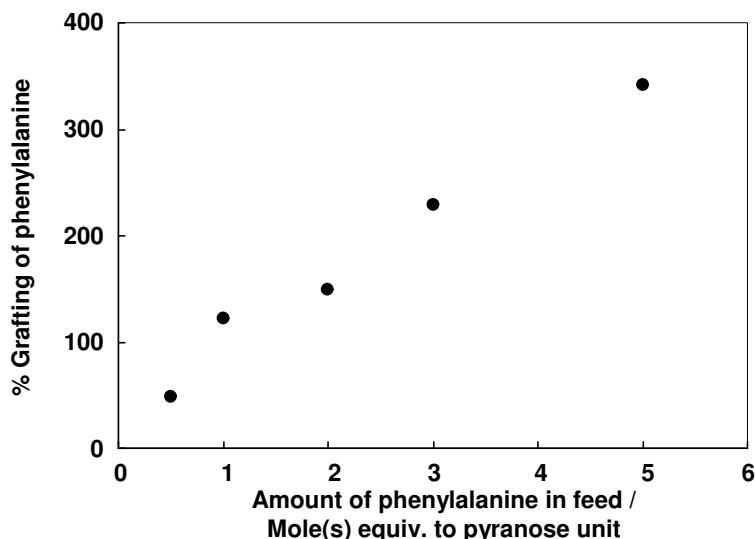

Figure 3 :   Grafting percentage of phenylalanine as a function of phenylalanine in feed.

The morphology of LMWCts-*g*-Phe was observed by SEM. Figure 4a shows the aggregation of LMWCts, whose individual particles are round shape with an average size of ~100 nm. LMWCts-*g*-Phe appears different shapes depending on the amount of phenylalanine in feed, or in other words, the % grafting of phenylalanine. For example, when % grafting is low, e.g., 123 (1 mole equiv. to pyranose unit of phenylalanine in feed), LMWCts-*g*-Phe gives spherical shape with an average size of ~80 nm (Figure 4b), but when % grafting is high, e.g., 343 (5 moles equiv. to pyranose unit of phenylalanine in feed), the appearance changes and seems particles combining each other (Figure 4c), which might be resulted from pi-pi stacking interaction.

Preparation and Characterization of LMWCts-g-Phe/DNA Complex
The obtained LMWCts-*g*-Phe was expected as a carrier for delivery bioactive molecules, such as drugs, proteins, vaccines, DNAs, etc. to the target organ and release those molecules controllably. DNA was chosen as a model molecule in the present work, since it is a negatively charged macromolecule used in gene therapy and has been reported to form complex with cationic polymers via electrostatic interaction [13-15]. It should be noted that LMWCts-*g*-Phe used in this study was LMWCts-*g*-Phe1 (phenylalanine in feed = 1 mole equiv. to pyranose unit) owing to its small regular shaped particle (Figure 4b) and low substitution degree at amino groups.
The complex of LMWCts-*g*-Phe1 and DNA was successfully prepared by complex coacervation method. The complex formed soon after dropping the solution of LMWCts-*g*-Phe/water into DNA/50 mM Na$_2$SO$_4$ as observed from the turbidity of a mixture solution. SEM micrograph shows that the complex appears various shapes, e.g., sphere, square, rectangle, and rod with an average size of ~ 50-150 nm (Figure 4d).
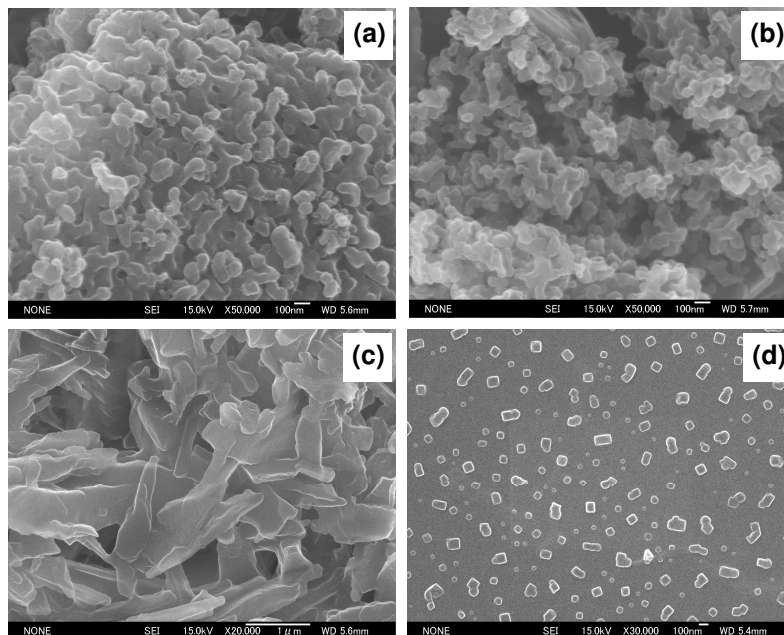
Figure 4 : SEM micrographs of (a) LMWCts (50,000×), (b) LMWCts-*g*-Phe1 (50,000×), (c) LMWCts-*g*-Phe5 (20,000×), and (d) LMWCts-*g*-Phe1/DNA (30,000×).

In Vitro Cytotoxicity Test of LMWCts-g-Phe and LMWCts-g-Phe/DNA Complex

The cytotoxicity of LMWCts-*g*-Phe and LMWCts-*g*-Phe/DNA complex was tested *in vitro* using fibroblasts (L929) as model cell. Fibroblast cells (density of ~ 5×10^4 cells per well) were pre-incubated in DMEM culture medium in a 24-well plate at 37°C in a humidified 5% CO_2/95% air atmosphere for 2 hours. The solution of samples (LMWCts-*g*-Phe or LMWCts-*g*-Phe/DNA) in DMEM culture medium was, then, added into those wells to get the final sample concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL. The incubation was continued at 37°C in a humidified 5% CO_2/95% air atmosphere for 22 hours. The % viability was evaluated by WST-1 method and calculated using the following equation.

% Viability = [cell number in a sample well/cell number in a control well] × 100
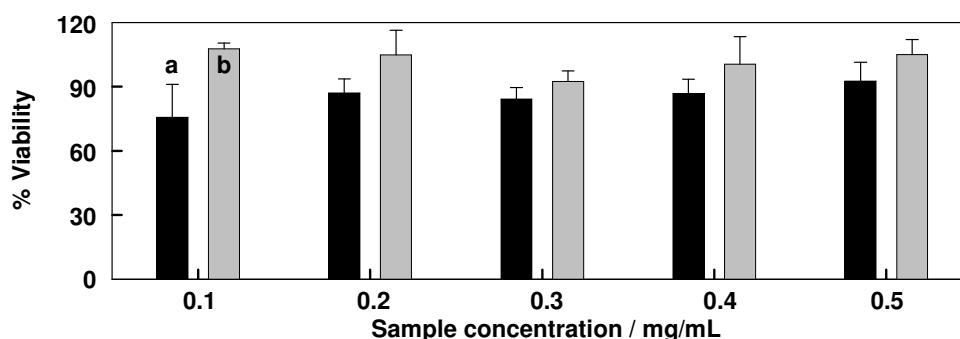
Figure 5 : % Viability of (a) LMWCts-*g*-Phe1 and (b) LMWCts-*g*-Phe1/DNA. The data are expressed as mean values ± standard deviation (n = 3-5).

The % viability of LMWCts-*g*-Phe is in the range of 76-93, while the one belonging to LMWCts-*g*-Phe/DNA complex is 93-108 implying the much reduced toxicity to fibroblasts (Figure 5). For all studied sample concentrations, the % viabilities of LMWCts-*g*-Phe/DNA complex are higher than those of LMWCts-*g*-Phe. This might be due to the fact that DNA is non-toxic and biocompatible molecule. The concentration of sample does not significantly affect to the % viability.

In Vitro DNA Release Study of LMWCts-g-Phe/DNA Complex

The study of DNA release was carried out at 37±0.5°C for 36 days in various buffers, i.e., carbonate buffer (pH=9.5), tris buffer (pH=8), phosphate buffer saline (PBS, pH=7.4), and citric acid buffer (pH=3). The released amount was measured by spectrophotometer at 258 nm.

It was found that the release rate is very fast for the initial stage, i.e., for the first 24 h in carbonate and tris buffers, and 333 h in PBS and citric acid buffers (Figure 6). The percentages of released DNA (calculation based on the maximum released DNA in each buffer) for the first day are 92, 84, 12, and 7 % when carbonate, tris, PBS, and citric acid buffers are applied as media, respectively. The initial release mechanism might be explained by the diffusion of DNA localized at the complex surface. This diffusion was enhanced in high pH media, i.e., carbonate and tris buffers, due to the deprotonation of LMWCts-*g*-Phe1; as a result the electrostatic or ionic interaction between LMWCts-*g*-Phe1 and DNA was null and DNA was eventually discharged very fast. In contrast, the electrostatic interaction between LMWCts-*g*-Phe1 and DNA was strong in low pH media (pH < pK_a), i.e., citric acid buffers, owing to the protonation of amino groups along LMWCts-*g*-Phe1 backbone. Here, it should be noted that pK_a of general native chitosan is ~ 6.0-6.5 [16], while the one belonging to LMWCts might be higher as confirmed from the solubility in wide range of pH, PBS was, thus, in the low pH media group. For the second stage, the release rate is slow in all media, especially in carbonate and tris buffers (Figure 6). The release mechanism for this stage might be involved with the degradation and/or the dissolution of LMWCts-*g*-Phe1 matrix.

By considering the release in high pH media, the amount of released DNA in tris buffer is higher than that in carbonate one (Figure 6). It was speculated that carbonate buffer at pH 9.5 was too severe for DNA to stay in its native form. For the release in low pH media, it was expected that the release of DNA in citric acid buffer should be lower than that in PBS, but the result is contrary, especially in the second stage of release. This might be involved with the difference in degradation and/or dissolution rate of LMWCts-*g*-Phe1 after 2 week-incubation.
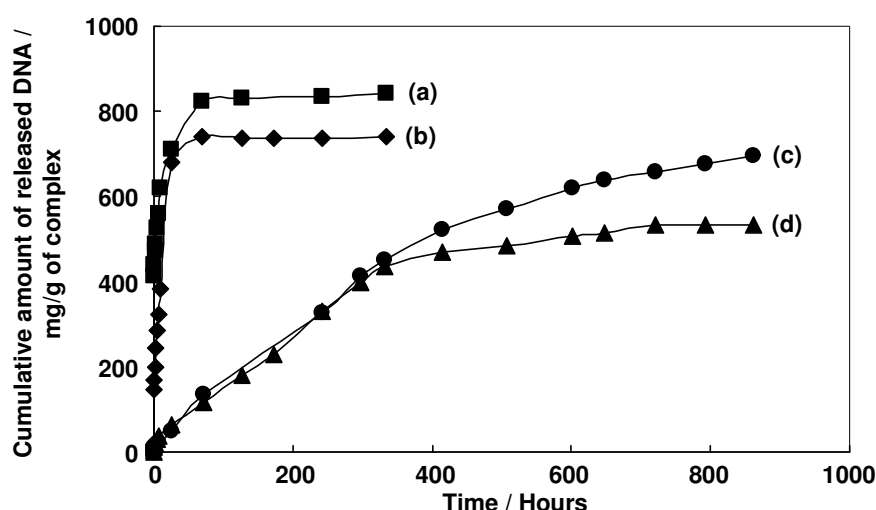
Figure 6 : DNA releasing profiles of LMWCts-*g*-Phe1/DNA complex in various buffers; (a) tris buffer (pH=8), (b) carbonate buffer (pH=9.5), (c) citric acid buffer (pH=3), and (d) phosphate buffer saline (PBS, pH=7.4).

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