Improvements in Transfection Efficiency with Chitosan Modified Poly(DL-lactide-*co*-glycolide) Nanospheres Prepared by the Emulsion Solvent Diffusion Method, for Gene Delivery

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This study sought to evaluate the *in vitro* transfection efficiency of plasmid DNA (pDNA)-loaded chitosan-modified poly(DL-lactide-*co*-glycolide) nanospheres (CS-PLGA NS) in a gene-delivery system. Using the emulsion solvent diffusion (ESD) method, pDNA-loaded PLGA NS was prepared and the surface of the PLGA NS was modified by binding to CS. Gene transfection ability of CS-PLGA NS was examined in A549 cells. The luciferase gene was used as a reporter gene. The pattern of luciferase activity by pDNA-loaded CS-PLGA NS was initially weak, but gradually grew stronger before decreasing activity. These phenomena should be in accordance with the sustained-release profile of pDNA from PLGA NS in the cytosol and the pDNA protection against DNase. Positively charged CS-PLGA NS was found, by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay, not to exhibit cytotoxicity on A549 cells. These results suggest that CS-PLGA NS are potential contributors to efficient pDNA delivery due to their increased interactions with cells and lack of cytotoxic effects.

Key words poly(DL-lactide-*co*-glycolide); chitosan; plasmid DNA; nanosphere; emulsion solvent diffusion method

An ideal gene-delivery carrier safely transports genetic material into the host cell, without generating toxic effects or an immune response.[1)] Initially, gene-delivery research was conducted exclusively using viral vectors, most commonly retroviruses and adenoviruses,[2)] but an increasing number of studies are being reported that examine nonviral vectors modified with fusogenic peptides,[3)] cationic lipids,[4)] or cationic polymers.[5)] It has been reported, however, that some cationic compounds are cytotoxic.[6)]

Polymeric nanospheres (NS) have been used to deliver drugs, because of their high stability, the ease with which they are taken up into cells by endocytosis, and the accuracy with which they target specific tissues and organs for adsorption or binding with ligands on the surface of the particles.[7)] A particular feature of polymeric NS is that they are biodegradable and degrade after passing the required specific site. Two polymeric NS—poly(lactide) (PLA) and poly(DL-lactide-*co*-glycolide) (PLGA)—have been approved by the U.S. Food and Drug Administration (FDA) for limited clinical use. The degradation time of PLGA can be altered from days to years by varying the molecular weight, the lactic acid-to-glycolic acid ratio in copolymer, or the structure of the NS. PLGA NS are viewed as reliable gene-delivery carriers, because of their safety and capacity for sustained release.[8)] We have developed an emulsion solvent diffusion (ESD) method in water for preparing PLGA NS[9)]; with ESD, NS can be prepared by a simple process under mild conditions, without the use of sonication or being subjected to high-shear agitation with a homogenizer, either of which causes DNA degradation. The ESD method was used to develop the PLGA NS platform, which encapsulates a wide variety of nucleic acids as well as low-molecular weight drugs.[10)]

We have also successfully developed PLGA NS by surface modification with chitosan (CS) as a cellular drug delivery system.[11)] Both PLGA and CS are biocompatible, biodegradable, and nontoxic polymers; furthermore, CS aids in the transport of DNA across the cell membrane.[12)] For all these reasons, CS and its derivatives are preferred choices for demonstrably safe and useful cationic materials for gene delivery. This study sought to evaluate the *in vitro* transfection activity of plasmid DNA (pDNA)-loaded CS-modified PLGA NS manufactured by ESD, as a gene-delivery system.

Experimental

Materials PLGA (lactide : glycolide 75 : 25, molecular weight (MW) 5000; Wako, Osaka, Japan) was used as matrix material for the NS, polyvinyl alcohol (PVA; Kuraray, Osaka, Japan) was used as the dispersing agent, and chitosan (MW 20000, deacetylation degree 84.2%; Kataku-rachikkarin, Tokyo, Japan) was used as the modifying polymer applied to the surface of the PLGA NS. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP; Sigma, St. Louis, MO, U.S.A.) was used as a cationic complexing agent for the preparation of pDNA-loaded PLGA NS. Luciferase-encoding pDNA (plasmid cytomegalo virus (pCMV)-luciferase) was kindly provided by Professor M. Hashida (Kyoto University). The plasmid was propagated in *Escherichia coli* and purified by an EndoFree Plasmid Giga Kit (Qiagen, Hilden, Germany). Human lung adenocarcinoma cells (A549) were purchased from the RIKEN Gene Bank (Ibaraki, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Basel, Switzerland). All other chemicals were obtained commercially at the highest analytical grade available.

Preparation of pDNA-Loaded PLGA NS by the Emulsion Solvent Diffusion Method To prepare the DOTAP/pDNA complex, 50 μl of Tris–ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0) containing 100 μg of pDNA was added to the same volume of Milli-Q water containing 1000 μg of DOTAP and then stirred (nitrogen to phosphorus (N/P) ratio=4.72). To prepare the CS/pDNA complex, 50 μl of Tris–EDTA buffer containing 100 μg of pDNA was added to the same volume of 0.1 M sodium acetate buffer (pH 4.4) containing 1000 μg of CS and then stirred (N/P ratio=13.96). One hundred microliters of pDNA complex solution was added into 2 ml acetone dissolving 100 mg of PLGA; the resultant

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organic solution was poured into 50 ml of an aqueous polyvinyl alcohol (PVA) solution (2%, w/v) and then stirred at 400 rpm using a propeller-type agitator with three blades. The entire dispersed system was then centrifuged (43400×***g*** for 10 min) and resuspended in 10 ml Milli-Q water; the resultant dispersion was freeze-dried. To prepare CS-modified PLGA NS, a CS-PVA mixed solution (0.25% w/v and 1% w/v) was used in the dispersing phase for the ESD process.

Preparation of pDNA Complex In this study, three pDNA complexes (DOTAP/pDNA, lCS/pDNA and CS-PLGA NS/pDNA) were evaluated *in vitro*. One hundred microliters of DOTAP or CS solution (10 mg/ml) was added rapidly to an equivalent volume of Tris–EDTA buffer containing pDNA (1 mg/ml) and mixed with a vortex mixer for 30 s. In the case of the CS-PLGA NS/pDNA complex, 100 μl of 0.1 M sodium acetate buffer (pH 4.4) suspending in 1 mg of PLGA NS (10 mg/ml) was added rapidly to an equivalent volume of Tris–EDTA buffer containing pDNA (1 mg/ml) and mixed with a vortex mixer for 30 s.

Analysis of Physicochemical Properties of NS Particle size and zeta potential were obtained using a Zetasizer 3000 HSA (Malvern Instruments Ltd., Malvern, U.K.). Particle size was measured by photon correlation spectroscopy. Zeta potential determinations were based on the electrophoretic mobility of the NS in aqueous medium.

The amount of pDNA trapped in the NS was determined by dissolving the NS (10 mg) in acetonitrile (1 ml), to which 0.1 M sodium acetate buffer (pH 4.4, 0.5 ml) containing sodium dodecyl sulfate (SDS) (0.1% w/v) was added to precipitate the polymer and dissolve the pDNA in the resultant aqueous mixture. The pDNA content was determined by fluorescent PicoGreen quantitation (Molecular Probes, Eugene, OR, U.S.A.), with excitation at 480 nm and emission at 520 nm. The loading efficiency of nucleic acid was calculated *via* the following equation:

$$\text{loading efficiency (\%)} = \frac{\text{weight of nucleic acids in NSs}}{\text{weight of nucleic acids formulated in the system}} \times 100$$

A549 Cell Culture A549 cells were grown in DMEM supplemented with 10 % FBS and 50 μg/ml penicillin and streptomycin at 37 °C in a humidified incubator with 5% CO_2. After confluence, A549 cells were trypsinized and seeded in plates for each experiment. Experiments were performed with cells of passage numbers 85—105.

Cytotoxicity Assay The cytotoxicity of NS was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay. A549 cells were seeded in 96-well plates at a density of 2.0×10^4 cells/well. The cytotoxicity of NS was assessed using the CellTiter 96 AQueous One Solution Assay (Promega, Madison, WI, U.S.A.). The solution reagent contained a tetrazolium compound MTS and an electron coupling reagent (phenazine ethosulfate). At 24 h after seeding the cells, samples were added to the wells. The cultures were further incubated for 4 h or 48 h, and then 20 μl of the AQueous One Solution reagent was added directly to the culture wells. After 1 h of incubation, the absorbance at 490 nm was measured with a standard microplate reader (MTP-100; Corona Electric, Tokyo, Japan) at a test wavelength of 490 nm, with a reference wavelength of 660 nm. The quantity of formazan product, as measured by absorbance at 490 nm, was directly proportional to the number of living cells in the culture.

In Vitro* Transfection Study** Cells were always seeded at the same density of 2×10^5 cells per 35-mm dish. On the second day after seeding, the growth medium was replaced with a suspension of the NS containing pDNA in serum-free DMEM (1 μg pDNA per 2 ml, per 35-mm dish), and the system was incubated for 48 h at 37 °C. Suspension of the NS was replaced with culture medium, and the system was incubated for 2 to 9 d. Culture medium was renewed once 2 d. At various times after transfection, cells were rinsed three times with ice-cold phosphate buffered saline (PBS), solubilized with 0.2 ml of reporter cell lysis reagent (Promega, Madison, WI, U.S.A.), and centrifuged at 17970×g*** for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment, 100 μl of luciferase assay buffer (Promega) was added to 20 μl of supernatant in the tubes. The luciferase activity of these samples was measured with a luminometer (MiniLumat LB 9506; PerkinElmer, Bad Wildbad, Germany). Cellular protein assays were performed with a BCA Protein Assay (Pierce, Rockford, IL, U.S.A.), to allow for the conversion of the data into luciferase activity (RLU) per milligram of protein.

Results and Discussion

Physicochemical Properties of Various Types of pDNA Preparations There are two types of nanoparticle systems for delivering nucleic acid into the host cell: DNA or RNA loading systems[13] and complex systems.[14] A complex system utilizes ionic interaction between the cationic polymer and anionic nucleic acid. A nucleic acid loading system, meanwhile, is a reservoir-type NS system; its advantage is the protection of nucleic acid during its release into the host cell. In this study, we designed various types of pDNA preparations *in vitro*, to test their transfection efficiency (Fig. 1); these included naked pDNA, DOTAP/pDNA complex, CS/pDNA complex, DOTAP/pDNA complex-loaded PLGA NS (non-DOTAP/pDNA-loaded PLGA NS), DOTAP/pDNA complex-loaded PLGA NS modified with CS (CS-DOTAP/pDNA-loaded PLGA NS), CS/pDNA complex-loaded PLGA NS (non-CS/pDNA-loaded PLGA NS), CS/pDNA complex-loaded PLGA NS modified with CS (CS-CS/pDNA-loaded PLGA NS), and pDNA/CS-modifying *blank*-PLGA NS complex (CS-PLGA NS/pDNA complex).

Key factors in the formulation to determine NS were ex-

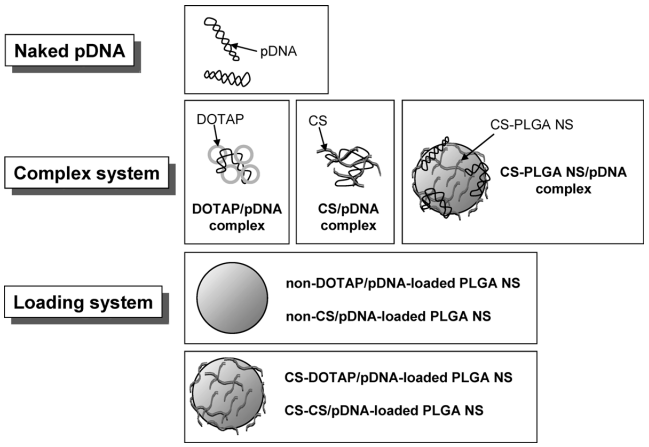

Fig. 1. Schematic Illustration of Various pDNA Delivery Systems

Table 1. Physicochemical Properties of the Different pDNA Preparations Studied

		Particle size (nm)	Polydispersity index	Zeta potential (mV)	pDNA loading efficiency (%)
Complex system	DOTAP/pDNA complex	280.2	0.170	42.6	—
	CS/pDNA complex	203.8	0.624	21.6	—
	CS-PLGA NS/pDNA complex	475.6	0.395	3.2	—
Loading system	non-DOTAP/pDNA-loaded PLGA NS	286.6	0.140	−25.8	21.5
	non-CS/pDNA-loaded PLGA NS	302.2	0.115	−28.4	6.3
	CS-DOTAP/pDNA-loaded PLGA NS	354.6	0.265	4.8	24.5
	CS-CS/pDNA-loaded PLGA NS	322.4	0.493	3.8	15.4

plored, to develop new biological applications. We assumed from our previous studies that critical factors influencing the transfection and cellular uptake are particle size and the physicochemical properties of the NS surface (Table 1).

Cationic lipid DOTAP used as a positive control is widely used as a non-viral vector, because it easily forms a complex with pDNA that has high positive zeta potential (+42.6 mV). Surface-modified PLGA NS were manufactured by ESD, which is a simple process conducted under mild conditions without the use of sonication, which otherwise causes nucleic-acid degradation. CS-PLGA NS was produced, using a PVA-CS blended aqueous solution for the ESD method. We were able to encapsulate pDNA in PLGA NS using ESD, which forms a complex with cationic compound (DOTAP or CS) and pDNA.[10] The particle size of pDNA-loaded PLGA NS prepared *via* the ESD method was about 200—300 nm. The zeta potential of unmodified PLGA NS (non-PLGA NS) was negative, with dissociation of the terminal carboxyl group of PLGA; the negative zeta potential of pDNA-loaded PLGA NS proved that DOTAP/pDNA or CS/pDNA complexes, which have positive zeta potentials, were encapsulated into the polymer matrix but not adsorbed on the surface of PLGA NS. CS-PLGA NS could be prepared by adding CS solution into the outer phase with PVA solution. The CS-PLGA NS had a positive charge due to the protonation of the CS amino group. The zeta potential of the NS shifting to positive values was a result of CS adsorption on the surface of the PLGA NS. The particle size of CS-PLGA NS increased, because molecular layers of CS had formed on the surface of PLGA NS; modification by CS also resulted in increased encapsulation efficiency.[10] The effect of modification by CS on loading efficiency might be caused by the prevention of pDNA leakage from the emulsion droplet during the diffusion process, with an ionic interaction between pDNA and CS. The positive zeta potential of the CS-PLGA NS/pDNA complex is lower than that of CS-PLGA NS without forming complex, because the nucleic acid had a strong negative charge derived from the dissociated phosphate group.

Cytotoxic Side Effects of CS-Modified PLGA NS Since PLGA is a biodegradable and biocompatible polymer, it is highly tolerated by cells. The MTS assay results suggested that NS at a concentration of 1 μg pDNA has no adverse effect on cell viability (Fig. 2A). Neither non- nor CS-PLGA NS exhibited cytotoxicity with respect to the A549 cells, whereas about 20% of cell death occurred when the A549 cells were treated with DOTAP/pDNA complex after sample incubation for 4 h. Even increasing incubation time of CS-DOTAP/pDNA-loaded PLGA NS until 48 h, cytotoxitiy could not be observed (Fig. 2B). This is a significant advantage compared to cationic polymers and lipids, most of which have a toxic effect on cells at the higher concentrations commonly used for gene transfection. Positively charged CS-PLGA NS showed no cytotoxicity in the A549 cells; this result suggests that CS existed and strongly adsorbed only on the particulate surface of PLGA NS, and unbounded CS was removed by centrifugation during the preparation process. CS-PLGA NS constitutes a suitable system for the intracytoplasmic delivery of genes, because both PLGA and CS are biodegradable and biocompatible.

Sustained Gene Expression with PLGA NS in A549 Cells The *in vitro* transfection abilities of the different pDNA preparations were evaluated in human lung adenocarcinoma (A549) cells, using the pCMV-luciferase plasmid (Fig. 3). The results of the luminescence assay—used to detect luciferase activity in transfected cells—indicated relatively high transfection efficiencies with high reproducibility. The plasmid DNA in solution (naked pDNA) alone showed a negligible level of transfection. The transfection efficiency of CS-PLGA NS, meanwhile, was higher and more prolonged than that of non-PLGA NS; this result correlates with those of cellular uptake studies,[15] which indicate that the higher cellular uptake ability of CS-PLGA NS may be due to electrostatic interaction. This was confirmed by a study that

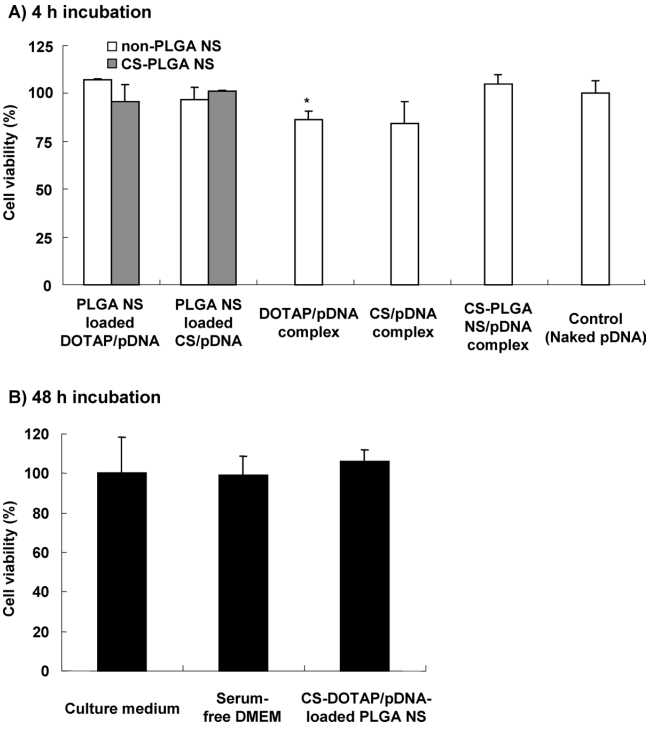

Fig. 2. Cytotoxic Side Effects of Different pDNA Preparations on A549 Cells

The viability of the treated cells was determined with an MTS assay. Doses are presented as the amount of pDNA equivalent in 1 μg/well. The results are provided as the mean±S.D. of six experiments. Cell viability was measured at 4 (A) and 48 h (B) after addition of pDNA preparations. Statistically significant differences from naked pDNA (control) are denoted with an asterisk (* $p<0.05$).

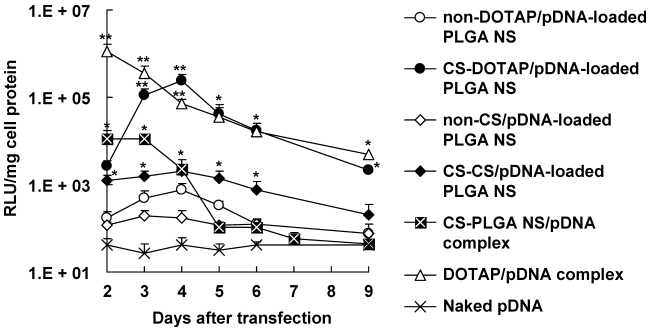

Fig. 3. Sustained Gene Expression with pDNA-Loaded PLGA NS in the A549 Cell Line

Doses are presented as the amount of pDNA equivalent in 1 μg/well. The results are provided as the mean±S.D. of three experiments. Statistically significant differences from naked pDNA (control) are denoted with asterisks (* $p<0.05$, ** $p<0.01$).

showed an increase in the cellular uptake of dye (6-coumarin)-loaded PLGA NS when the surface was modified by CS, compared to the uptake of non-PLGA NS.[15)]

The escape of the expression vector from the *endo*-lysosomal compartment is a key step in gene expression. Ishii *et al.*[16)] reported that CS/pDNA complexes uptaken into the cells were possibly released from endosome by rupturing endosomes after swelling of complexes. CS-PLGA NS has chitosan molecule on the surface of NS, therefore, it is possible that endosomal escape of CS-PLGA NS might be caused by the same mechanism as CS/pDNA complex.

The DOTAP/pDNA complex initially showed strong luciferase activity, but the transfection efficiency gradually decreased. This pattern of transfection efficiency, which is generally observed in conventional gene transfection system, is quite different from that of CS-PLGA NS, where the transfection efficiency of pDNA-loaded CS-PLGA NS was initially weak, but gradually became stronger before decreasing activity. The expression of luciferase by CS-DOTAP/pDNA-loaded PLGA NS was quantitatively evaluated using a moment analysis method[17)] and the parameters of mean retention time (*MRT*) were compared with DOTAP/pDNA complex. The *MRT* were calculated by integration to infinite time following below reference. The *MRT* of CS-DOPTA/pDNA-loaded PLGA NS (48.9 h) was longer than that of DOTAP/pDNA complex (17.6 h). A sustained-release profile of pDNA from CS-modifying PLGA NS in PBS was also confirmed after an initial burst released 40% of the pDNA as previously reported.[10)] These results suggested that gene transfection with CS-PLGA NS could be sustained. In contrast, the adsorbed pDNA on the surface of the NS (CS-PLGA NS/pDNA complex) did not last through luciferase activity. Most of pDNA dissociated from the surface of the NS in the medium before NS was taken up into the cells, resulting in low transfection efficiency without prolongation. The luciferase activity of CS/pDNA complex was more than 1000-fold lower compared to DOTAP/pDNA complex at 2 d after transfection (data not shown). The CS/pDNA complex-loaded PLGA NS also showed a lower luciferase activity level than DOTAP/pDNA-loaded PLGA NS. It is possible that pDNA might become more tightly twisted with CS than with DOTAP, because CS is a cationic polymer of glucosamine (MW＝20000). It has been suggested that the inability of pDNA to dissociate from CS results in the delayed release of pDNA from PLGA NS. The transfection efficiency of various patterns can be designed by combining the carriers to show quick release of pDNA with the carriers such as PLGA NS to show the delayed release of pDNA. This possibility suggests that CS-PLGA NS may have numerous applications to therapeutics, with respect to various diseases.

Conclusion

Using the ESD method—which allows pDNA to be encapsulated in PLGA NS—we prepared a gene-delivery system of pDNA-loaded PLGA NS and evaluated its activity *in vitro*. The pattern of luciferase activity exhibited by pDNA loaded in CS-PLGA NS was initially weak, but gradually grew stronger. These phenomena, newly found in the present study, should be explained by the sustained release of pDNA from PLGA NS in the cytosol and the protection of pDNA by DNase. CS-PLGA NS are highly recommended as desirable carriers for gene delivery, because of their increased interaction with cells and lack of cytotoxicity.

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