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Research paper

Cellular uptake mechanisms and intracellular distributions of polysorbate 80-modified poly (D,L-lactide-*co*-glycolide) nanospheres for gene delivery

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ABSTRACT

We previously developed chitosan (CS)-modified poly (D,L-lactide-*co*-glycolide) (PLGA) nanospheres (NS) by an emulsion solvent diffusion method as a gene delivery system. In this study, PLGA NS were modified using polysorbate 80 (P80) to improve their cellular uptake. We investigated the cellular uptake, intracellular distribution, and transfection efficiency of P80-modified PLGA NS (P80-PLGA NS) for a plasmid DNA delivery system in A549 cells. The cellular uptake and transfection efficiency of P80-PLGA NS were greater than CS-modified PLGA NS (CS-PLGA NS). The uptake of unmodified NS and CS-PLGA NS was mediated, predominantly, by clathrin-mediated endocytosis. In contrast, a specific endocytic pathway could not be determined for the cellular uptake of P80-PLGA NS. The intracellular distributions of PLGA NS depended on their surface properties. P80-PLGA NS were not cytotoxic for A549 cells. Thus, P80-PLGA NS could be used as an effective gene delivery system; the surface properties of PLGA NS are key parameters for optimal intracellular uptake and distribution.

1. Introduction

The development of carriers that effectively deliver genes into cells has attracted a great deal of attention in recent years. New modalities for gene delivery should circumvent some of the problems that can occur with viral vectors, such as endogenous viral recombination, oncogenic effects, and unexpected, undesirable immune responses. Further, non-viral delivery systems should be simple to use, should be easily manufactured for large-scale production, and should not induce specific immune responses [1].

Polymeric nanospheres (NS) have been used to deliver drugs because of their high stability and ability to target specific tissues or organs either by adsorption or by binding ligands attached to the particle surfaces [2]. In particular, biodegradable NS are available for delivering drugs and are degraded after passing a required specific site. Among these, poly (lactide) (PLA) and poly (D,L-lactide-*co*-glycolide) (PLGA) have been approved by the FDA for certain human clinical uses [3]. The degradation time of PLGA can be altered from days to years by varying the molecular weight and the lactic acid-to-glycolic acid ratio of the copolymer. PLGA NS have been suggested as good gene delivery carriers because of their safety and property for achieving sustained release [4].

We have also successfully developed PLGA NS by surface modification with chitosan (CS) to improve transfection efficiency of NS for gene delivery both *in vitro* and *in vivo* [5]. We found that these CS-modified PLGA NS (CS-PLGA NS) were useful for improving gene delivery via a pulmonary route. A particularly important feature of these PLGA NS is that due to their submicron sizes and positive zeta potentials, they can interact electrostatically with cell membranes, after which they are taken up into cells, and thereby, they transport the associated nucleic acids. These physicochemical properties of CS-PLGA NS that provide for intracellular penetration of drug molecules have great importance for gene delivery. In addition to particle size, surface properties and compositions also have a significant influence on particle stability and the intracellular fates of both the drug and carrier [6].

In this study, we modified the surfaces of PLGA NS using polysorbate 80 (P80) in order to improve the cellular uptake and transfection efficiency of NS, because it has been reported that P80-modified polybutylcyanoacrylate (PBCA) nanoparticles were taken up by brain endothelial cells much more rapidly and in significantly higher amounts (20-fold higher) than uncoated nanoparticles [7,8]. The aim of this study was to prepare surface-modified PLGA NS with P80, using an emulsion solvent diffusion (ESD) method and to evaluate these particles loaded with pDNA as a gene delivery system. The transfection efficiency, mechanism of cellular uptake, and the intracellular fates of surface-modified PLGA NS were evaluated using cultured A549 lung adenocarcinoma cells.

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2. Materials and methods

2.1. Materials

PLGA (lactide:glycolide = 75:25; MW = 5000) was purchased from Wako (Osaka, Japan). Polyvinylalcohol (PVA; MW = 25,000; hydrolyzation degree = 88.0%; polymerization degree = 500) was purchased from Kuraray (Osaka, Japan). Chitosan (MW = 20,000; deacetylation degree = 84.2%) was obtained from Katakurachikkarin (Tokyo, Japan). Polysorbate 80 was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). The fluorescent dye coumarin six laser grade, [3-(2-benzothiazolyl)-7-(diethylamine) coumarin] (6-coumarin), was purchased from MP Biomedicals (Solon, OH). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP, Sigma, St. Louis, MO) was used as a cationic complexing agent for the preparation of plasmid DNA (pDNA)-loaded PLGA NS. Luciferase-encoding pDNA (pCMV-luciferase) was a gift from 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 medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Basel, Switzerland). Filipin, cytochalasin D, and pronase (Pronase Type XIV from *Streptomyces griseus*) were purchased from Sigma. All other chemicals were of the highest grade available commercially.

2.2. Preparation of surface-modified PLGA nanospheres by the emulsion solvent diffusion method

PLGA NS loaded with 6-coumarin as a fluorescent label were prepared by the previously reported ESD method in an aqueous solution [9]. PLGA (100 mg) and 6-coumarin (1 mg) were dissolved in 3 mL of an acetone/ethanol mixture (acetone:ethanol = 2:1). For the preparation of pDNA-loaded PLGA NS, pDNA complexation with a cationic compound was used [10]. To prepare the DOTAP/pDNA complex, 50 μL of pDNA solution (2 μg/μL, in Milli-Q water) was added to the same volume of DOTAP solution (20 μg/μL, in Milli-Q water) and then mixed by pipetting. PLGA (100 mg) and 100 μL of DOTAP/pDNA complex were dissolved in 3 mL of an acetone/ethanol mixture (acetone:ethanol = 2:1). The resulting organic solution was poured into 25 mL of an aqueous PVA solution (2% w/v, in distilled water) and stirred at 400 rpm using a propeller type agitator with three blades at room temperature. The entire dispersed system was then centrifuged (43,400*g* for 10 min), and the sediments were resuspended in distilled water. This process was repeated, and the resulting dispersion was freeze-dried. For the preparation of CS- or P80-modified PLGA NS, either a CS (0.25% w/v, in 0.5 M acetate buffer, pH 4.4)–PVA (1% w/v) or a P80 (1% w/v, in distilled water)–PVA (1% w/v) solution was used as the dispersing phase for the ESD process.

2.3. Analysis of NS physicochemical properties

Particle size and zeta potential were determined using a Zetasizer 3000 HSA (Malvern Instruments Ltd., Malvern, UK). 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 as follows. NS (10 mg) were dissolved in acetonitrile (1 mL) to which acetate buffer (pH 4.4, 0.5 mL) containing sodium dodecyl sulfate (SDS) (0.1% w/v) was added. This precipitated the polymer in the resulting aqueous mixture and dissolved the pDNA. The pDNA content was determined by quantifying the amount of fluorescent PicoGreen (Molecular Probes, Eugene, OR, USA), with excitation at 480 nm and emission at 520 nm. The loading efficiency of nucleic acids was calculated using the following equation:

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

2.4. Cell lines and cell culture

A549 cells were grown in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and streptomycin at 37 °C in an incubator with 95% humidity and 5% CO_2. After confluent growth, A549 cells were trypsinized and seeded on plates for each experiment. Experiments used cells at passage numbers 85–105. A549 cells were grown on Lab-Tek® II chamber slides (Nalge Nunc International, Naperville, IL, USA) with 5.0×10^4 cells/well for microscopic studies, on 12-well plates with 2.0×10^5 cells/well for uptake studies, on 6-well plates with 5.0×10^5 cells/well for cell binding and intracellular distribution studies, or on 96-well plates with 2.0×10^4 cells/well for cytotoxicity assays. Experiments were conducted once the cells had formed confluent monolayers, as determined by light microscopy.

2.5. Cytotoxicity assay

NS cytotoxicity was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay [11]. Cytotoxicity was assessed using a CellTiter 96® Aqueous One solution assay (Promega, Madison, WI, USA). The solution reagent contained MTS and an electron coupling reagent (phenazine ethosulfate). After confluent growth of A549 cells on a 96-well plate, different concentrations of samples were added to the wells. The cultures were incubated for 4 h after which 20 μL of the Aqueous One solution reagent was added to the culture wells. After 1-h incubation, absorbance was measured using a Model MTP-100 microplate reader (Corona Electric, Tokyo, Japan) at a test wavelength of 490 nm and a reference wavelength of 660 nm. The quantity of formazan product, as determined by absorbance at 490 nm, was directly proportional to the number of viable cells. Cell viability (%) relative to the control wells containing cell culture medium without test samples was calculated by the following equation: $[A]_{test}/[A]_{control} \times 100$, where $[A]_{test}$ is the absorbance of the test sample, and $[A]_{control}$ is the absorbance of the control sample.

2.6. Microscopic studies

After confluent growth, the growth medium was replaced with a suspension of NS, containing 6-coumarin as a fluorescent dye, in DMEM then incubated for 4 h at 37 °C. After incubation, the cell monolayers were washed three times with cold PBS and then fixed with 4% paraformaldehyde. For F-actin staining, fixed cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and subsequently incubated with Alexa Fluor® 488-conjugated phalloidin (Molecular Probes, Eugene, OR) in PBS for 60 min at room temperature. After washing with PBS, cover slips were mounted on slides using the SlowFade anti-fade kit (Molecular Probes, Eugene, OR, USA). Fixed cells were observed with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Goettingen, Germany) equipped with a Zeiss Plan-Neofluar × 100/1.3 oil immersion objective lens using an argon ion laser (458–514 nm) and a helium/neon laser (543 nm).

2.7. Cellular uptake mechanisms of surface-modified PLGA NS

To study the effects of various inhibitors on PLGA NS uptake [6], cells were first preincubated with an inhibitor and then with a suspension of PLGA NS (100 μg/mL), which also contained the respective inhibitor at the same concentration used during preincubation. The inhibitors used were as follows: (1) 450 mM sucrose (inhibitor of clathrin-mediated endocytosis) for 1 h, (2) 1 μg/mL filipin (inhibitor of caveolae-mediated endocytosis) for 30 min, and (3) 30 μM cytochalasin D (inhibitor of macropinocytosis) for 30 min. After cells were preincubated, the inhibitor solution was replaced with a suspension of NS containing 6-coumarin as a florescent dye in serum-free DMEM and then incubated for 2 h at 37 °C. Uptake was terminated by washing the cells three times with ice-cold PBS and solubilizing the cells with 0.2 mL of reporter cell lysis reagent (Promega). The fluorescent dye was extracted from NS by mixing each sample with 3 mL of methanol/chloroform (1:1). The samples were centrifuged (1400*g* for 10 min) and 6-coumarin concentrations were determined with a fluorescence spectrophotometer (F-3010, Hitachi, Tokyo, Japan; emission wavelength 490 nm; excitation wavelength 520 nm). The protein contents of cell lysates were measured using the Pierce BCA protein assay (Rockford, IL, USA). Uptake was expressed as the amount of NS (μg) per unit weight (mg) of total cellular protein. Results were expressed as percentages against control, which was the cellular uptake by A549 cells without inhibitor treatment.

2.8. Cell binding and intracellular distribution of PLGA nanospheres

A suspension of the PLGA NS containing 6-coumarin in serum-free DMEM was added to a A549 cell monolayer at a final concentration of 2.5 mg/mL. After incubation for 30 min at 37 °C, the PLGA NS suspension on the cell monolayer was replaced with culture medium and incubated for different times (0, 60, and 120 min). At the end of each incubation period, the culture medium was removed, and the cell monolayer was briefly washed three times with cold PBS. Cell surface proteins were digested for 15 min at 4 °C with 0.1% (w/v) pronase (Protease XIV, Sigma, St. Louis, MO, USA) in a protection buffer (0.25 M sucrose, 20 mM pH 7.2 HEPES, 2 mM potassium phosphate, 0.24 mM EGTA, 10 mM $MgCl_2$). As previously reported [12], this treatment also resulted in detaching the cells from the culture dish. The cells were homogenized, mixed with 1 mL of DMEM medium supplemented with 10% FBS to stop pronase action, and centrifuged (500*g* for 5 min at 4 °C). The supernatant, corresponding to the pronase-sensitive fraction (membrane-bound NS), was collected. The pellet was washed with 1 mL of cold PBS. Any remaining cell-associated material was considered as the pronase-resistant fraction (internalized NS). Subcellular fractions were extracted from the pronase-sensitive fraction with a ProteoExtract™ subcellular proteome extraction kit (S-PEK, Calbiochem, EMD Biosciences Inc., Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol [13]. For each fraction, the amount of 6-coumarin fluorescence activity was measured with a fluorescence spectrophotometer as described above.

2.9. Luciferase activity

After confluent growth of A549 cells on a 12-well plate, the growth medium was replaced with a suspension of the NS containing pDNA in serum-free DMEM (3 μg pDNA/2 mL per well), and then incubated for 48 h at 37 °C. Luciferase assay was performed according to the manufacturer's instructions (Promega). The cells were rinsed three times with ice-cold PBS, solubilized with 0.2 mL of reporter cell lysis reagent (Promega), and centrifuged at 17,970*g* for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment, 100 μL of luciferase substrate was added to 20 μL of supernatant in the tubes. The luciferase activity was determined with a luminometer (MiniLumat LB 9506; PerkinElmer, Bad Wildbad, Germany). Cell protein assays used a BCA protein assay (Pierce) to convert the data to luciferase activity (RLU) per milligram of protein. Results were expressed as percentages of the luciferase activity relative to unmodified PLGA NS (non-PLGA NS).

3. Results

3.1. Physicochemical properties of surface-modified PLGA NS

The physicochemical properties of the surface-modified PLGA NS are summarized in Table 1. PLGA NS were prepared by an emulsion solvent diffusion method in water and modified with two kinds of polymers: CS and P80. PLGA NS particle sizes ranged from 250 to 300 nm, depending on the type of surface modifier used. Particle sizes of CS-PLGA NS were increased due to adsorbed CS molecular layers that formed on the PLGA NS surfaces. Freeze-dried NS were readily dispersed in aqueous medium with shaking by hand, which gave nearly the same particle diameter as that before drying.

Non-PLGA NS, which were prepared using only PVA, a non-ionic polymer, had a negative zeta potential (−31.0 mV) due to the dissociation of the PLGA carboxyl group in distilled water. CS-PLGA NS had highly positive zeta potentials (+18.6 mV) in distilled water due to protonation of the amino group. The physicochemical properties of non-PLGA NS and P80-modified PLGA NS (P80-PLGA NS) were very similar.

3.2. Cytotoxic effects of surface-modified PLGA NS

PLGA is a material that is well tolerated by cells because it is a biodegradable and biocompatible polymer. A mitogenic assay (MTS) showed that at NS concentrations of <5 mg/mL, within the dose range used for gene transfection studies, there were no adverse effects on cell viability (Fig. 1). None of the NS formulations were toxic for cells after incubation for 4 h in the tested dose ranges. However, the CS and P80 solutions were highly toxic for cells at high concentrations.

3.3. Effects of surface modifiers on cellular uptake of PLGA NS

The cellular uptake of surface-modified PLGA NS was observed visually using confocal laser scanning microscopy (CLSM), as

Table 1
Physicochemical properties of surface-modified PLGA NS.

Modifier	Particle size (nm)		Zeta potential (mV)	Polydispersity	pDNA loading efficiency (%)
	Before freeze dry	After freeze dry			
Unmodified (non)	251.2	254.5	−31.0	0.098	9.5
Chitosan (CS)	298.5	309.1	18.6	0.190	20.4
Polysorbate 80 (P80)	247.4	248.1	−30.9	0.084	9.3

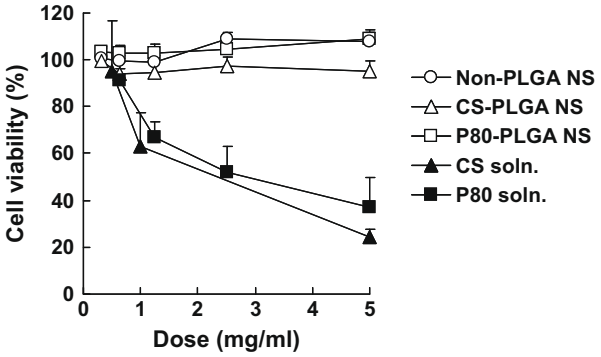

Fig. 1. Cytotoxicities of the different PLGA NS formulations and surface modifier solutions for A549 cells. Viability of treated cells was determined by MTS assay. Symbols represent means ± SD ($n = 6$).

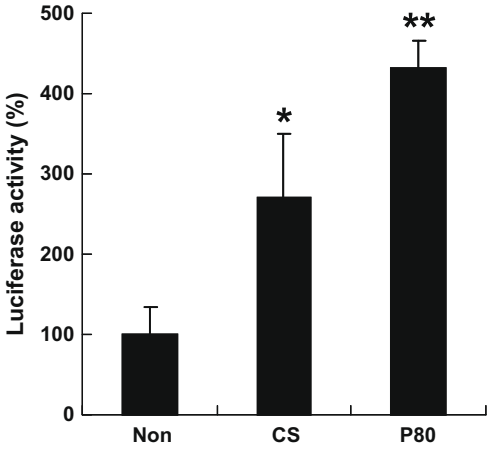

Fig. 3. Effects of surface properties of pDNA-loaded PLGA NS on luciferase activity. Doses are the amounts of pDNA equivalents in 3 μg/well. Results are means ± SD of three experiments; statistically significant differences from non-PLGA NS are denoted by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$).

shown in Fig. 2. PLGA NS were loaded with fluorescent dye 6-coumarin (green fluorescence). F-actin in the A549 cells was stained with Alexa Fluor® 488-conjugated phalloidin (red fluorescence). CLSM for A549 cells exposed to 6-coumarin-labeled PLGA NS showed fluorescence activity within the cells during incubation with the PLGA NS suspension. The yellow color in the images indicates the co-localization of 6-coumarin loaded PLGA NS (green fluorescence) and F-actin (red fluorescence) within the A549 cells. Thus, NS internalization within cells could be confirmed by observing yellow dots in the cells.

The images shown are *z*-sections through the centers of the cells, which confirmed that the PLGA NS taken up were localized inside the cells. After 4-h incubation under serum-free conditions, the cellular uptake of surface-modified PLGA NS increased when compared to non-PLGA NS. The fluorescence from the surface-modified PLGA NS could only be observed in the cytoplasm or around the nucleus when analyzing the CLSM images after the uptake experiments. It was observed that among the PLGA NS that interacted with A549 cells, most of the PLGA NS were found inside the cells; however, a few particles were found on the cell surface. A549 cells showed higher uptake amounts of surface-modified PLGA NS than non-PLGA NS. In particular, P80-PLGA NS showed the highest fluorescence activity among the evaluated PLGA NS.

3.4. Luciferase activity of PLGA NS

Transfection efficiencies for the particle preparations were determined by quantifying the expressions of luciferase-encoded plasmids. As shown in Fig. 3, luciferase activity increased after any surface modification and was the highest for P80-modified NS. The transfection efficiency of surface-modified PLGA NS appeared to be correlated with the amount of NS taken up by cells (Fig. 2).

3.5. PLGA NS binding and uptake by A549 cells

To determine the amounts of PLGA NS that were taken up by cells, the fluorescence intensities of 6-coumarin, indicating the amounts of PLGA NS interacting with cells, were measured after the A549 cell surfaces were washed with cold PBS. Nearly all of the adsorbed NS on the cells' surfaces could be removed with PBS washing. However, even after washing once with PBS, a portion of the PLGA NS that had been strongly adsorbed on the surface and extracellular matrix of the cells remained in the cell pellet residue. Repeated washing with PBS for three times resulted in the effective removal of adsorbed PLGA NS, as the last wash values were close to the background. After three PBS washes, the remaining PLGA NS that strongly attached to the cell surface or the extracellular matrix were removed by pronase treatment, which degrades cell membrane proteins. Pronase appears to act only on the cell surface, leaving the intracellular contents intact. Subsequently, the 6-coumarin fluorescent marker was extracted using a methanol–chloroform mixture (1:1), and the 6-coumarin fluorescence intensity was measured.

As shown in Fig. 4, the amount of NS internalized within cells was much greater than the amount of NS adsorbed on the cell

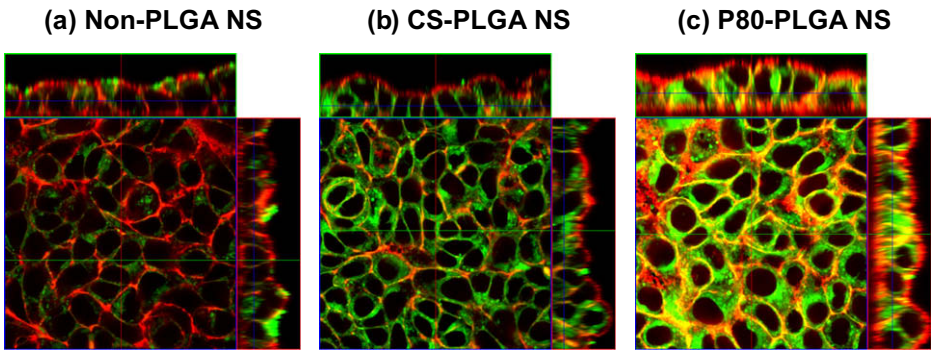

Fig. 2. Confocal laser microscope images of A549 cells incubated with surface-modified PLGA NS. After 4-h incubation with a suspension of 6-coumarin (green)-containing PLGA NS at 37 °C, A549 cell actin was stained with Alexa Fluor® 488-conjugated phalloidin (red) and examined using a confocal microscope. (a) Non-PLGA NS, (b) CS-PLGA NS, and (c) P80-PLGA NS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

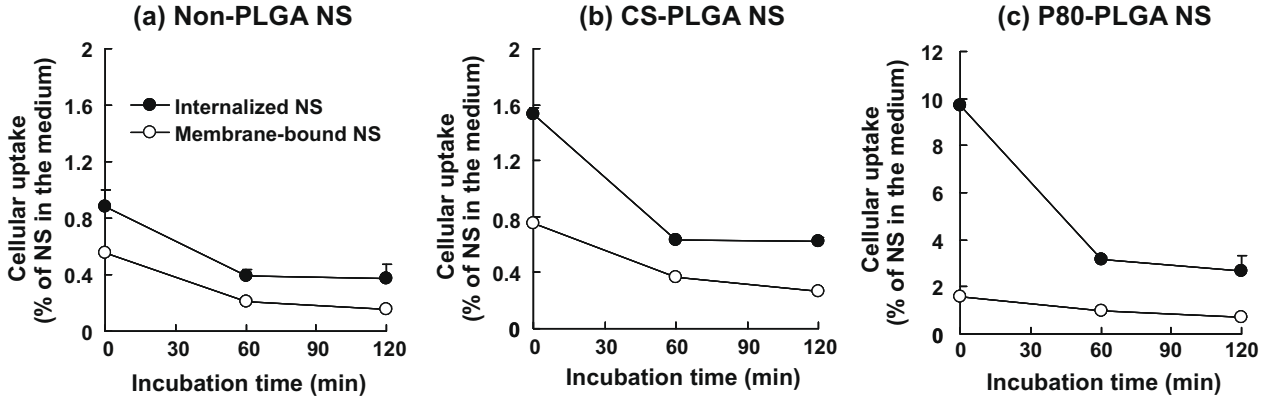

Fig. 4. Time course of cell binding and uptake of surface-modified PLGA NS by A549 cells. Different surface properties for (a) non-PLGA NS, (b) CS-PLGA NS, and (c) P80-PLGA NS. A549 cells were incubated with NS suspensions for 30 min, washed, and incubated with fresh medium (0 min time point). Medium was removed, and cells were washed and analyzed for NS cellular uptake levels at different time points (60 and 120 min). Results are percentages of total PLGA NS added in the system. Symbols represent means ± SD (n = 3).

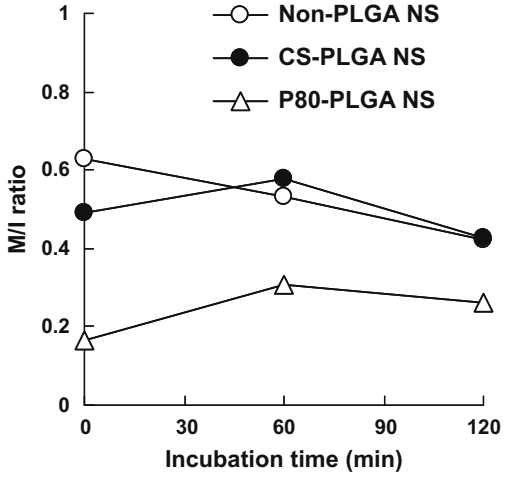

Fig. 5. Time course of changes in membrane-bound NS (M)/internalized NS (I) ratios. Symbols represent means ± SD (n = 3).

surfaces, irrespective of the surface modifier. Cellular uptake of PLGA NS, which included both membrane-bound NS and internalized NS, increased with surface modification. Moreover, after the NS suspension was removed from the cells' culture medium, a trend for decreased association of NS with cells was observed.

The ratio of the membrane-bound NS fraction (M) to the internalized NS fraction (I) was calculated for each NS preparation. Comparisons of these M/I ratios are shown in Fig 5. For each NS, the M/I ratio was <1, indicating that the proportion of internalized NS was greater than membrane-bound NS, which was independent of incubation time.

3.6. Intracellular distribution of surface-modified PLGA NS

The intracellular distributions of PLGA NS varied with the type of surface modifier, as shown in Fig. 6. Non-PLGA NS tended to accumulate in the cytoskeleton as time advanced. In contrast, nearly all of the internalized CS-PLGA NS were associated with the organelle fraction.

3.7. Mechanisms of surface-modified PLGA NS uptake

As shown in Table 2, the uptake mechanism for PLGA NS was an endocytic process, as there was reduced uptake at a lower incubation temperature (4 °C) irrespective of the NS type. The effects of

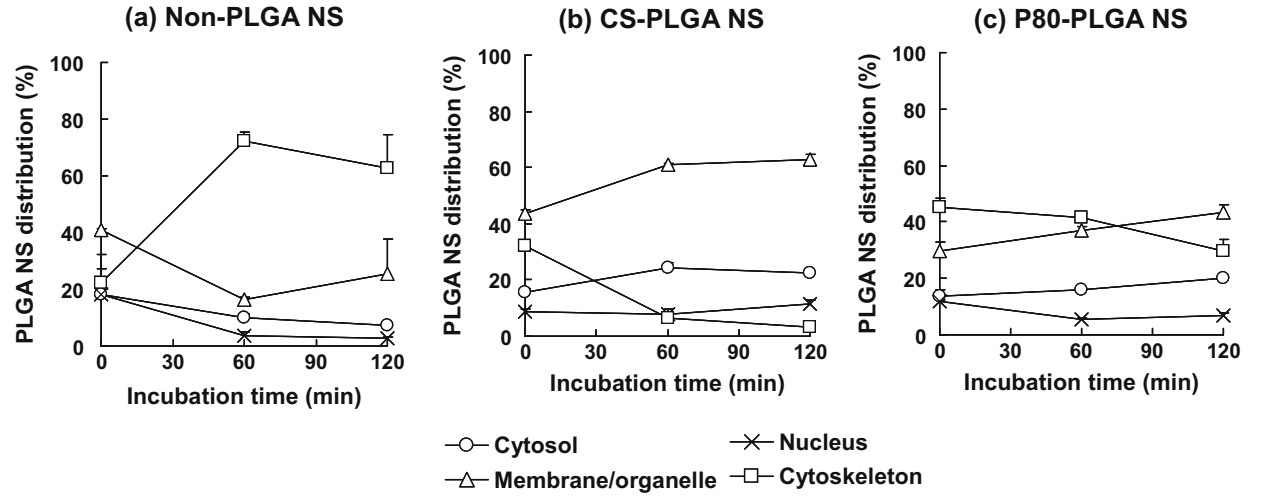

Fig. 6. Time course of intracellular distributions of surface-modified PLGA NS. Different surface properties for (a) non-PLGA NS, (b) CS-PLGA NS, and (c) P80-PLGA NS. A549 cells were incubated with NS suspensions for 30 min, washed, and incubated with fresh medium (0 min time point). Medium was removed, and cells were washed and analyzed for NS intracellular distributions at different time points (60 and 120 min). Symbols represent means ± SD (n = 3).

Table 2
Effects of incubation temperature and endocytosis inhibitors on uptake of PLGA NS into A549 cells.

	37 °C (Non-treated)	4 °C	Sucrose	Filipin	Cytochalasin D
Non-PLGA NS	100.0 ± 18.1	11.7 ± 1.4**	66.3 ± 6.9*	125.1 ± 6.6*	97.1 ± 2.8
CS-PLGA NS	100.0 ± 8.2	21.6 ± 6.2**	67.4 ± 3.4**	113.0 ± 2.3*	90.5 ± 15.9
P80-PLGA NS	100.0 ± 0.8	8.3 ± 1.7**	20.5 ± 1.6**	79.6 ± 3.6**	17.2 ± 1.4**

Two different temperatures (4 and 37 °C) were used to evaluate the uptake of surface-modified PLGA NS. Cellular uptake of PLGA NS by A549 cells in the presence of hypertonic growth medium (inhibitor of clathrin-mediated endocytosis), filipin (inhibitor of caveolae-mediated endocytosis), and cytochalasin D (inhibitor of macropinocytosis). Results are percentages of control (non-treated A549 cells). Results are means ± SD ($n = 3$), $^{**}p < 0.01$, $^{*}p < 0.05$, significantly different compared with control.

three types of inhibitors for endocytosis (clathrin-mediated, caveola-coated pit endocytosis, and macropinocytosis) on the uptake of surface-modified PLGA NS into the A549 cells were evaluated. We previously discussed the uptake mechanisms for non- and CS-PLGA NS [6]. Briefly, the uptakes of submicron-sized non- and CS-PLGA NS were mediated, predominantly, by clathrin-mediated endocytosis, at least in A549 cells. In this study, the cellular uptake of P80-PLGA NS was decreased regardless of the particular endocytosis inhibitor that was used. Therefore, the specific endocytic process for the cellular uptake of P80-PLGA NS could not be identified using inhibitor studies.

4. Discussion

We have been investigating surface-modified PLGA NS as a non-viral vector for possible gene delivery system [10]. In this study, PLGA NS were modified using P80, as it has been shown that polymeric nanoparticles modified by P80 were taken up rapidly by brain microvessel endothelial cells [7,8]. Therefore, we considered that P80 might be a candidate PLGA NS surface modifier for use in a cellular drug delivery system. The effect of P80 as a surface modifier on the delivery of macromolecules was evaluated by the cytosolic delivery of PLGA NS.

Surface-modified PLGA NS could be prepared using the ESD method (Table 1). For the case using a cationic polymer (chitosan) as a surface modifier, the zeta potential of the NS was shifted from negative to positive. This proved that PLGA NS surfaces could be modified by adding a CS solution to the outer phase with a PVA solution. The zeta potential of P80-PLGA NS was very similar to that of non-PLGA NS. Thus, we could not confirm the modification of PLGA NS with P80 from the zeta potential data. However, we could confirm that P80-modified the NS surfaces by a colorimetric method based on a quantitative test for poly(ethylene oxide) with ammonium cobaltothiocyanate (data not shown).

After modifying with CS, the encapsulation efficiency for pDNA in PLGA NS increased. We previously reported that the effect of modifying with CS on the loading efficiency might be due to the formation of an ionic complex between pDNA and CS at the interface, which prevented pDNA leakage from emulsion droplets during the diffusion process [10].

One concern with using CS and P80 to improve PLGA NS delivery is possible cytotoxicity (Fig. 1). Several studies have found no cytotoxic effects following treatments with PLGA NS. However, CS and P80 solutions have been shown to be toxic for cells, depending on their concentrations. Our studies indicated that at the concentrations used, surface-modified PLGA NS do not induce significant cytotoxicity. This was possibly due to the small amounts of free CS or P80 in the NS suspensions, as any excess surface modifier not adsorbed to the NS surface was removed during the centrifugation step of the PLGA NS preparation. In general, cytotoxicity when using a surfactant results at concentrations higher than the critical micelle concentration. P80 on the PLGA NS surfaces did not form micelles. Therefore, cytotoxicity might not be observed. While a free CS or P80 solution resulted in significant cytotoxicity at high doses, no toxic effects were observed at doses equivalent to those used for NS.

In general, several biological barriers must be overcome in order to achieve an efficient, non-viral gene delivery system [14]. These barriers include binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, and overcoming the nuclear envelope [15]. The appropriate design of a non-viral gene carrier requires thorough understanding of the carrier's properties and the uptake mechanisms by the targeted cells. The uptake mechanisms are, in general, closely linked to the intracellular trafficking and fate of the carriers [16]. Understanding the uptake mechanisms and intracellular distributions of the PLGA NS is very important in order to design the PLGA NS for gene delivery systems [17,18]. Therefore, we attempted to evaluate the intracellular fates of surface-modified PLGA NS.

The cellular uptake amounts of P80-PLGA NS were much higher than that of non-PLGA NS (Fig. 2). CS modification also increased the cellular uptake amount, although it was lower than with P80 modification. There was a clear correlation between the cellular uptake of 6-coumarin-loaded PLGA NS and the transfection efficiency of pDNA-loaded PLGA NS (Fig. 3). Therefore, pDNA-loaded PLGA NS interacted with cell membranes and entered the cytosol while retaining pDNA inside the NS. Modifying the PLGA NS surfaces could improve their transfection efficiency.

The P80-PLGA NS showed the highest transfection efficiency among the NS prepared in this study. The transfection efficiency of CS-PLGA NS was higher than that of non-PLGA NS, because CS-PLGA NS had a positive zeta potential, and thus, could associate with negatively charged cell membranes via an electrostatic interaction. This raises an interesting question regarding these phenomena: why does P80 used as a surface modifier increase the cellular uptake and transfection efficiency of PLGA NS despite having similar physicochemical properties to non-PLGA NS?

To address this question, the mechanisms of cellular uptake of surface-modified PLGA NS were examined from the perspective of different endocytic pathways (Table 2). Each of the surface-modified PLGA NS was taken up into A549 cells via an endocytic process, as the amount of cellular uptake decreased at 4 °C [17]. We previously reported that the uptakes of non- and CS-PLGA NS were primarily via the clathrin-coated pit endocytic pathway [6]. It was difficult to determine the specific endocytic pathway involved for P80-PLGA NS. Therefore, a number of possibilities might explain the mechanisms of cellular uptake of P80-PLGA NS. One possibility is that P80 on the surfaces of PLGA NS may increase cell membrane fluidity, leading to endocytosis activation. Additional investigations will be required in order to elucidate the effects of P80 as a surface modifier before applying the present system to cell drug delivery.

The cellular uptake of PLGA NS was increased when the NS suspension was maintained in the extracellular medium [6]. However, when the NS suspension was replaced with fresh medium, the intracellular levels of NS decreased (Fig. 4). Although the NS levels decreased, the amount of NS in the cells reached a constant level from 60 to 120 min. After the culture medium was replaced, the NS elimination rate predominated, as the NS concentration inside

the cell was higher than the NS concentration outside the cell. The NS concentration in the culture medium increased gradually, and the uptake rate also increased. From 60 to 120 min, the elimination and uptake rates might have become equal.

The amount of membrane-bound NS was lower than that of internalized NS, irrespective of the surface modifier used (Fig. 5). The ratio of the membrane-bound NS fraction (M) to the internalized NS fraction (I), the M/I ratio, for P80-PLGA NS had the lowest value, which indicated that the P80-PLGA NS that were associated with cells were internalized into the cells more readily compared to the other NS.

CLSM studies demonstrated the uptake of PLGA NS into A549 cells (Fig. 2). NS were preferentially located in the cytoplasm or around the nucleus, irrespective of the surface modifier used. These results were also confirmed by studying the intracellular distributions of NS (Fig. 6). The intracellular distributions of the PLGA NS with different surface properties were also evaluated. Internalized non-PLGA NS, with a negative zeta potential due to dissociation of the PLGA carboxyl group, accumulated in the cytoskeleton. In the other cell fractions (cytosol, membrane/organelle, and nucleus), there were only small amounts of non-PLGA NS. The "membrane/organelle" fraction included endosomal components, and thus, many non-PLGA NS that were taken up into the cells by endocytosis might have been released from endosomes and escaped degradation by lysosomes. Internalized CS-PLGA NS, with a positive zeta potential due to protonation of the chitosan amino group, accumulated in the membrane/organelle fractions. The endosomal escape by CS-PLGA NS may be more efficient than by non-PLGA NS, as the transfection efficiency of CS-PLGA NS was higher than that of non-PLGA NS. Therefore, it is possible that CS-PLGA NS in the cells may be associated with organelles (e.g., mitochondria and Golgi fraction), except for the endosomal components. Although P80-PLGA NS and non-PLGA NS had similar particle sizes and zeta potentials, their intracellular distributions were different. Although the mechanism for endosomal escape by PLGA NS is not yet fully understood, P80 used in the formulation may play a role in endosomal membrane destabilization. Fig. 6 clearly demonstrates that the surface properties of the NS are key parameters for intracellular uptake and delivery. From the above results, the cellular uptake and intracellular distribution of NS depended on their surface properties. This suggests that PLGA NS can be used as a carrier for intracellular organelle targeting, which could be controlled in consideration of the intracellular target and be applied to therapeutics for various diseases by changing the surface properties.

5. Conclusion

Our studies showed that surface modifications of NS, especially P80 modification, significantly enhanced the cellular delivery of plasmid DNA and the NS itself. Surface-modified PLGA NS are highly recommended as a carrier for gene delivery due to their increased interactions with cells and lack of cytotoxic effects.

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