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# Improved cellular uptake of chitosan-modified PLGA nanospheres by A549 cells

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ABSTRACT

The authors have previously developed poly(DL-lactic-*co*-glycolic acid) (PLGA) nanospheres (NSs) as a nanoparticulate drug carrier for pulmonary administration. The present study demonstrates that chitosan (CS)-modified PLGA NSs (CS-PLGA NSs) are preferentially taken up by human lung adenocarcinoma cells (A549). PLGA NSs prepared using a water–oil–water emulsion solvent evaporation method were surface-modified by adsorption of CS. The physicochemical parameters of PLGA NS, including average size and surface charge, were measured to identify which parameter influenced cellular uptake of PLGA NS. Uptake was confirmed using fluorescence spectrophotometry and was visualized in A549 cells with confocal laser scanning microscopy (CLSM). The cytotoxicities of non- and CS-PLGA NS systems were compared *in vitro* by MTS assay. Cellular uptake of PLGA NS increased with decreasing diameter to the submicron level and with CS-mediated surface modification. Cellular uptake of PLGA NS was energy dependent, as shown by a reduction in uptake at lower incubation temperatures and in hypertonic growth medium used as an inhibitor of clathrin-coated pit endocytosis. CS-PLGA NSs were taken up by A549 cells in an energy-dependent manner, suggesting a clathrin-mediated endocytic process. CS-PLGA NS demonstrated low cytotoxicity, similar to non-PLGA NS.



## 1. Introduction

Biodegradable polymeric nanospheres (NSs) have been developed as a unique drug carrier due to their characteristic colloid-like behavior and sustained drug-release property (Lobenberg et al., 1997). Poly(DL-lactide-*co*-glycolide) (PLGA) has been widely accepted as a biocompatible and biodegradable material for drug delivery systems, such as subcutaneously injectable microspheres for luteinizing hormone-releasing hormone, the most successful delivery system for this drug (Ogawa et al., 1988). NSs of this biodegradable polymer can provide sustained, controlled, and targeted drug delivery to improve the therapeutic effects and reduce the side effects of formulated drugs. Furthermore, the polymeric matrix prevents degradation of the drugs (Feng, 2004). A number of research groups have encapsulated various types of therapeutic agents, from low-molecular-weight drugs to macromolecular drugs, e.g., proteins and plasmid DNA (Panyam and Labhasetwar, 2003; Jeon et al., 2007; Nafee et al., 2007; Vasir and Labhasetwar, 2007). Moreover, polymeric NSs have been used to deliver medicines because of their high stability, ease of cell uptake via endocytosis, and ability to target specific tissues or organs by adsorption or ligand-mediated binding to the surfaces of the particles (Lobenberg et al., 1997; Panyam and Labhasetwar, 2003; Rejman et al., 2004; Vasir and Labhasetwar, 2007).

The authors have previously developed mucoadhesive PLGA NSs through surface modification with chitosan (CS) and used them for pulmonary peptide delivery with an improved pharmacological effect (Kawashima et al., 1998, 2000). CS-modified PLGA NS (CS-PLGA NS) is useful for improving peptide delivery via the pulmonary route due to prolonged mucoadhesion for sustained drug release at the absorption site and due to the absorption-enhancing action of CS (Yamamoto et al., 2005). Moreover, CS improves drug absorption (Schipper et al., 1997; Huang et al., 2002).

The purpose of the present study was to investigate the uptake of CS-PLGA NSs in monolayers of A549 human lung adenocarcinoma cells, an in vitro model of the pulmonary epithelium having the characteristic features of alveolar Type II cells (Huang et al., 2002). Physicochemical properties of PLGA NS, such as particle size and zeta potential, were investigated to determine cellular uptake and to both quantify and elucidate the mechanism of interaction between CS-PLGA NSs and epithelial 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) was purchased from Kuraray (Osaka, Japan). Chitosan (MW = 20,000; degree of deacetylation 84.2%) was obtained from Katakurachikkarin (Tokyo, Japan). The fluorescent dye coumarin 6 laser grade, [3-(2-benzothiazolyl)-7-(diethylamine) coumarin] (6-coumarin), was purchased from MP Biomedicals (Solon, OH, USA). A549 human lung adenocarcinoma cells (A549) were purchased from RIKEN Gene Bank (Ibaraki, Japan). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Basel, Switzerland). Filipin and cytochalasin D were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of the highest grade available commercially.

### 2.2. Water–oil–water emulsion solvent evaporation method

CS-PLGA NSs containing 6-coumarin were prepared using a water–oil–water (w/o/w) emulsion solvent evaporation method (Wang et al., 1999). One hundred microliters Tris–EDTA buffer (pH 8.0) was emulsified in 500 μl chloroform containing 100 mg PLGA and 0.5 mg 6-coumarin by sonication (duty cycle 75%, output 2) for 10 s using a Branson Sonifier 250 (Branson Ultrasonic, Danbury, CT, USA). The resulting primary emulsion was added to 2 ml of 10% (w/v) PVA and sonicated for 5, 15, and 60 s to form a double emulsion. The resultant emulsion was added dropwise to 18 ml of 10% (w/v) PVA and agitated for 3 h at room temperature under evaporation to completely remove chloroform. PLGA NSs were collected by centrifugation at 43,400 × *g* for 10 min at 4 °C, washed three times with distilled water, and freeze-dried for 3 days. For preparation of CS-PLGA NSs, a mixed solution consisting of CS (0.25%, w/v, in 0.5 M acetate buffer, pH4.4) and PVA (10%, w/v, in distilled water) was used as the dispersing phase for the emulsion solvent diffusion process.

### 2.3. Physicochemical properties of PLGA NSs

Particle size and zeta potential measurements were conducted using a Zetasizer 3000 HSA (Malvern Instruments, Malvern, UK). Particle size was measured by photon correlation spectroscopy (PCS). Zeta potential determinations were based on the electrophoretic mobility of the NSs in serum-free DMEM.

### 2.4. A549 cell culture

A549 cells were grown in DMEM supplemented with 10% FBS and 50 μg/ml penicillin and streptomycin at 37 °C and incubated at 90% humidity and 5% $CO_2$. The cells were allowed to grow until confluence and were trypsinized and seeded in plates for each experiment. Experiments were performed with cells following 85–105 passages.

### 2.5. Cellular PLGA NS uptake

A549 cells were seeded in 12-well plates with a density of $2.0 \times 10^6$ cells/well. Two days after seeding, the growth medium was replaced with an NS suspension containing 6-coumarin in serum-free DMEM and incubated for 1–12 h at 37 °C. For uptake experiments performed at 4 °C, cells were preincubated with serum-free DMEM before incubation with the respective NS dispersion at the same temperature. Uptake was terminated by washing the cells three times with ice-cold phosphate buffered saline (PBS), and solubilizing the cells with 0.2 ml of reporter cell lysis reagent (Promega, Madison, WI, USA). The 6-coumarin fluorescence dye was extracted from the NSs by mixing each sample with 3 ml of methanol/chloroform (1:1). The samples were centrifuged at 1400 × *g* for 10 min and the 6-coumarin concentration was determined by fluorescence spectrophotometry (F-3010, excitation wavelength 490 nm, emission wavelength 520 nm; Hitachi, Tokyo, Japan). The protein content in the cell lysate was measured with a Pierce BCA protein assay (Rockford, IL, USA). Uptake was expressed as the amount (μg) of NSs associated with a unit weight (mg) of cellular protein. The uptake was represented as NS (μg) normalized per milligram total cell protein. To study the effects of various inhibitors on the uptake of PLGA NS (Panyam et al., 2002), cells were preincubated at 37 °C in a humidified 5% $CO_2$ incubator first with the medium containing each inhibitor: (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. And then the medium for preincubation was replaced with a suspension of PLGA NSs (100 μg/ml), which also contained the respective inhibitor at the same concentration as that used for preincubation.

### 2.6. Observation of cellular uptake by confocal laser scanning microscopy

A549 cells were grown on Lab-Tek® Chamber Slides (Nalge Nunc International, Naperville, IL, USA) at a density of $5.0 \times 10^5$ cells/well. On the second day after seeding, the growth medium was replaced with an NS suspension containing 6-coumarin in DMEM, incubated for 4 h at 37 °C, and the cell monolayers were fixed with 0.5 ml of 4% paraformaldehyde. Cellular actin was counterstained with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (Sigma–Aldrich) at room temperature. The fixed cells were observed using an LSM 510 confocal laser scanning microscope (Carl Zeiss, Goettingen, Germany) equipped with Zeiss Plan-Neofluar 40/0.75× and Zeiss Plan-Neofluar 100/1.3× oil as objective lenses at 40× and 100× magnifications, respectively.

### 2.7. Cytotoxicity assay

NS cytotoxicity was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Braydich-Stolle et al., 2005). A549 cells were seeded in 96-well plates at a density of $2.0 \times 10^5$ cells/well. Cytotoxicity was assessed using the CellTiter 96® $AQ_{ueous}$ One solution assay (Promega). The solution reagent contained MTS and an electron-coupling reagent (phenazine ethosulfate; PES). Twenty-four hours after seeding, various concentrations of NSs were added to the wells. The cultures were further incubated for 4 h and then 20 μl of the $AQ_{ueous}$ One Solution reagent was directly added to the culture wells. After 1 h of incubation, the 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 the formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in the culture.

## 3. Results

### 3.1. Physicochemical properties of chitosan-modified PLGA NSs

The mean diameters and zeta potentials of the non- and CS-PLGA NSs prepared by the w/o/w emulsion solvent evaporation method are shown in Table 1 ($n = 3$). The particle size of PLGA

**Table 1**
Particle size and zeta potential of surface-modified PLGA NSs prepared using the w/o/w emulsion solvent evaporation method (mean ± SD, $n = 3$).

| | Sonication time (s) | Particle size (nm ± SD) | Zeta potential (mV ± SD) |
|---|---|---|---|
| Non-PLGA 1000 | 5 | 939 ± 23.9 | −30.8 ± 3.8 |
| Non-PLGA 400 | 15 | 410.0 ± 26.3 | −33.9 ± 2.2 |
| Non-PLGA 200 | 60 | 215.3 ± 0.2 | −28.5 ± 1.1 |
| CS-PLGA 1000 | 5 | 1109.1 ± 20.7 | −3.8 ± 0.6 |
| CS-PLGA 400 | 15 | 475.2 ± 16.0 | −4.6 ± 1.1 |
| CS-PLGA 200 | 60 | 248.9 ± 4.1 | −3.1 ± 0.7 |

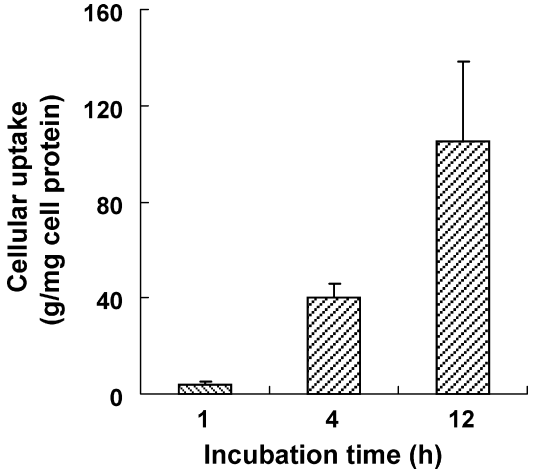


**Fig. 1.** Effects of incubation time on cellular uptake of PLGA NS (400-nm) at 37 °C by A549 cells. The concentration of PLGA NS (400-nm) was 2.5 mg/ml. Symbols represent mean ± SD ($n = 3$).

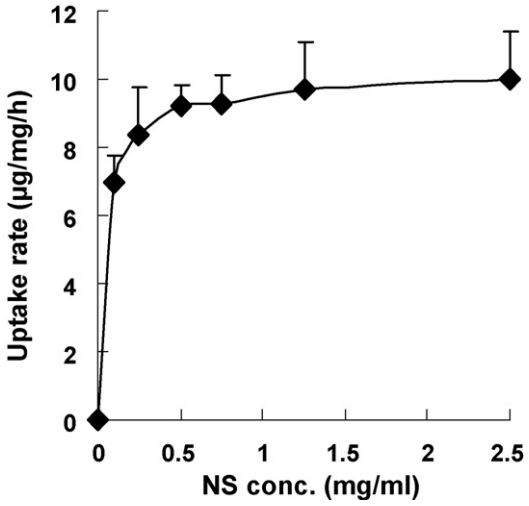


**Fig. 2.** Rate of uptake of PLGA NSs by A549 cells after 4-h incubation at 37 °C as a function of dose. Various PLGA NS (400-nm) concentrations ranging from 0–2.5 mg/ml were applied to A549 cells for 4 h. Symbols represent mean ± SD ($n = 3$). Symbols were fit to a curve using the Michaelis–Menten equation.

NSs, analyzed by photon correlation spectroscopy, was found to be controlled by sonication time, and varied from approximately 1000–200 nm. The particle sizes of both non- and CS-PLGA NSs were approximately 1000, 400, and 200 nm using sonication times of 5, 15, and 60 s, respectively. Use of PVA, a nonionic polymer, as a stabilizer of NS produced a negative zeta potential for non-PLGA NSs, due to dissociation of the carboxyl group of PLGA. The zeta potential of CS-PLGA NSs was markedly higher than that of the non-PLGA NSs, due to protonation of the amino group. These observations are consistent with coating of the NSs by CS.

### 3.2. Cellular uptake of PLGA NSs

The 6-coumarin fluorescent dye from NSs in the cell lysates was extracted by incubating each cell lysate sample with methanol/chloroform. Davda and Labhasetwar (2002) reported that free 6-coumarin released from NSs accounts for only about 3% of the dye, indicating that the 6-coumarin detected in the cells is mainly associated with the NSs. The uptake of 400 nm PLGA NSs was also dependent on the incubation time (Fig. 1). Uptake was evident even at 1 h, and increased gradually with incubation time. As shown in Fig. 2, the rate of uptake of dye-loaded PLGA NSs 400 nm in diameter increased in a concentration-dependent manner when incubated at 37 °C for 4 h. The plot of uptake rate ratio against NS concentration indicates that the cellular uptake followed saturation kinetics. The uptake rate of NSs reached a plateau at higher NS concentrations (>500 μg/ml). The uptake rate of 400 nm diameter PLGA NSs increased in a concentration-dependent manner when incubated at 37 °C and followed the Michaelis–Menten equation ($R^2 = 0.9897$) with a $V_{max}$ of 39.84 μg/mg cell protein and a $K_m$ of 44 μg/ml (Fig. 3).

### 3.3. Effect of chitosan coating on cellular uptake

Cellular uptake of CS-PLGA NSs was found for 200, 400, and 1000 nm particle sizes using confocal laser scanning microscopy (Fig. 4). F-actin in the A549 cells was stained with TRITC-conjugated phalloidin (Red). Fluorescence of 6-coumarin (Green)-loaded PLGA NSs was observed in the cytoplasm of all particles irrespective of particle size, suggesting that PLGA NSs were internalized by the A549 cells. When the particle size was controlled to below the sub-

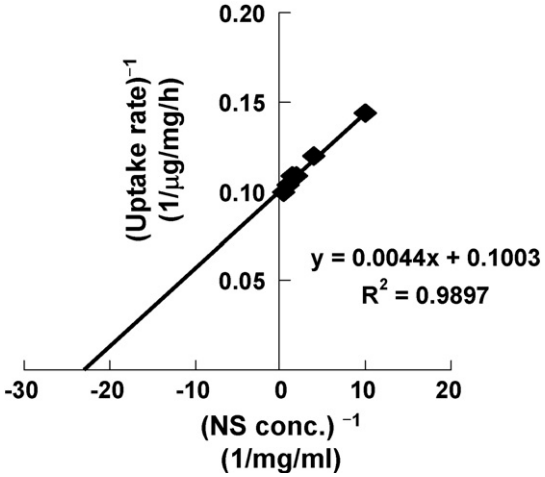


**Fig. 3.** Lineweaver–Burk plots of the rate of uptake data from Fig. 2. Reciprocal values of the mean rate of uptake of PLGA NS are plotted against the reciprocal of the corresponding PLGA NS concentrations. $V_{max}$ and $K_m$ values were calculated from the $x$- and $y$-intercepts, respectively. The $V_{max}$ and $K_m$ values were 39.84 μg/mg cell protein and 44 μg/ml, respectively.

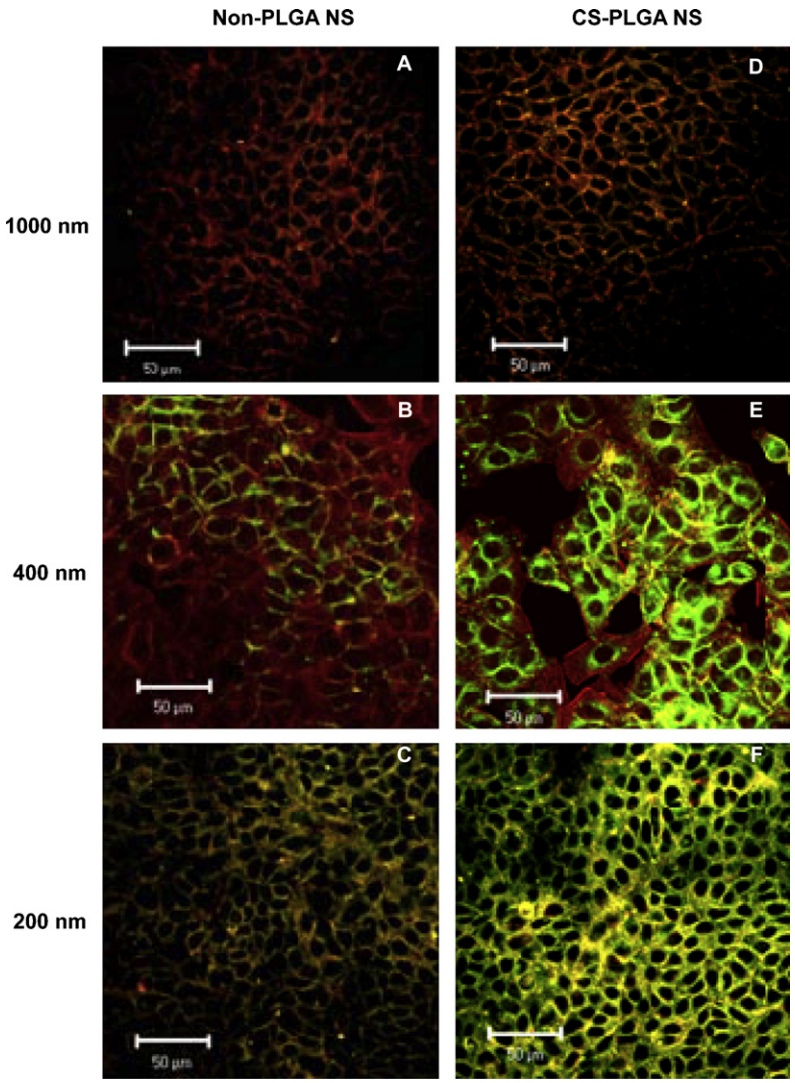


**Fig. 4.** Confocal laser microscopy images of A549 cells incubated with surface-modified PLGA NSs (magnification 40×). After 4-h incubation with a suspension of 6-coumarin (Green)-containing PLGA NSs (2.5 mg/ml) at 37 °C, cellular actin of A549 cells were stained with TRITC-conjugated phalloidin (Red) and examined using a confocal microscope. (A) 1000-nm non-PLGA NS, (B) 400-nm non-PLGA NS, (C) 200-nm non-PLGA NS, (D) 1000-nm CS-PLGA NS, (E) 400-nm CS-PLGA NS, and (F) 200-nm CS-PLGA NS. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

micron region (<400 nm), cellular uptake of PLGA NS increased. In contrast, significantly less intense fluorescence was observed in cells incubated with micron-sized PLGA NSs (1000 nm). These results suggest that the cellular uptake of PLGA NSs is dependent on particle size.

As shown in Fig. 5, serial z-sections of cells, each 1 μm in thickness, demonstrated fluorescence activity of 200-nm dye-loaded PLGA NSs in all sections from the cell surfaces. These results indicate that most of the PLGA NS interacting with cells was inside the cells, with some on the surface, and was mostly localized in the cytoplasm.

The effects of CS coating on cellular uptake were examined by quantitative analysis of the uptake, as shown in Fig. 6. Consistent with the confocal microscopy studies, cellular uptake of PLGA NSs was increased by CS modification, irrespective of particle size. Cellular uptake of CS-PLGA NSs was also concentration-dependent and saturable.

### 3.4. Mechanism of PLGA NS uptake

Uptake of PLGA NSs was energy dependent, as shown by the reduction in uptake at lower incubation temperatures (4 °C) irrespective of particle size (Fig. 7). There are several pathways of energy-dependent endocytosis. To determine the endocytosis uptake mechanism, the three kinds of endocytosis, clathrin- and caveola-coated pit endocytosis and macropinocytosis, were examined using appropriate inhibitors during uptake of 200-nm PLGA NS into the A549 cells (Fig. 8). Inhibition of clathrin-coated pit endocytosis by hypertonic growth medium (450 mM sucrose) decreased the intracellular uptake of 200-nm PLGA NSs, whereas inhibition of caveola-coated pit endocytosis by filipin and macropinocytosis by cytochalasin D did not decrease the uptake (Fig. 8). These results indicate that uptake of submicron-sized (200-nm) non- and CS-PLGA NSs is mediated predominantly by clathrin-mediated endocytosis, at least in the A549 cells.

### 3.5. Cytotoxic side effects of chitosan-modified PLGA NSs

Because PLGA is a biodegradable and biocompatible polymer, it is well tolerated by the cells. The MTS assay demonstrated that NSs over a concentration range of 0.3125–5.0000 mg/ml had no adverse effects on cell viability (Fig. 9). Neither non-PLGA NSs nor CS-PLGA NSs were cytotoxic to A549 cells.

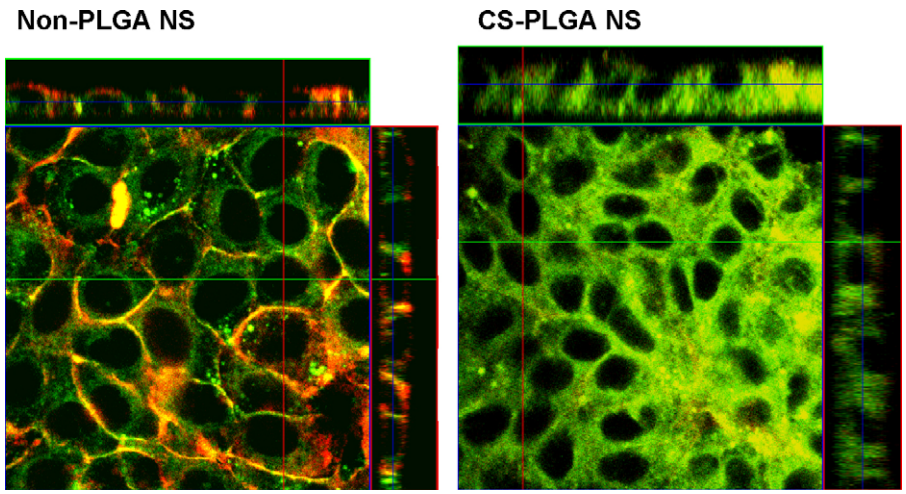


**Fig. 5.** Optical section of the ($x$, $y$) axis, with respective projections of the ($x$, $z$) and ($y$, $z$) axes, of A549 cells incubated for 4 h at 37 °C with 2.5 mg/ml of 200-nm non- and CS-PLGA NSs (magnification 100×).

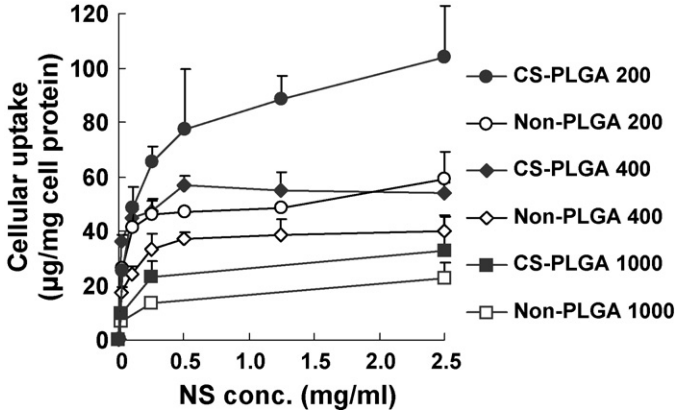


**Fig. 6.** Effects of CS modification and particle size on cellular uptake of PLGA NS at 37 °C by A549 cells. Various particle sizes with diameters of (A) 1000 nm, (B) 400 nm, and (C) 200 nm and different NS concentrations ranging from 0 to 2.5 mg/ml were applied to A549 cells for 4 h. Symbols represent mean ± SD ($n$ = 3).

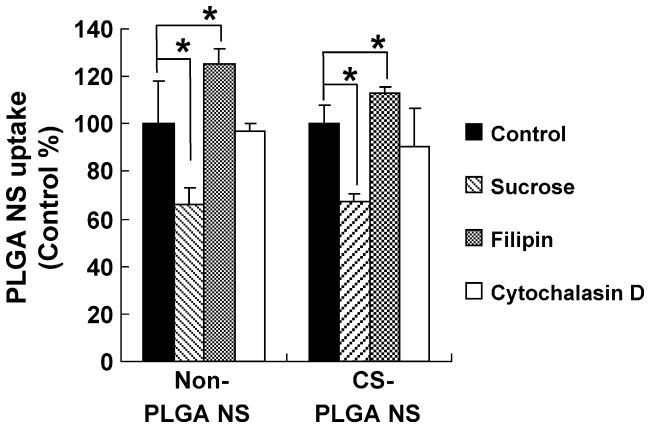


**Fig. 8.** Cellular uptake of PLGA NSs (200-nm) by A549 cells in the presence of hypertonic growth medium (450 mM sucrose) as an inhibitor of clathrin-mediated endocytosis, filipin (1 μg/ml) as an inhibitor of caveolae-mediated endocytosis, and cytochalasin D (30 μM) as an inhibitor of macropinocytosis. Data are shown as the mean ± SD ($n$ = 3), significantly different, $^{*}p < 0.05$ compared with control.

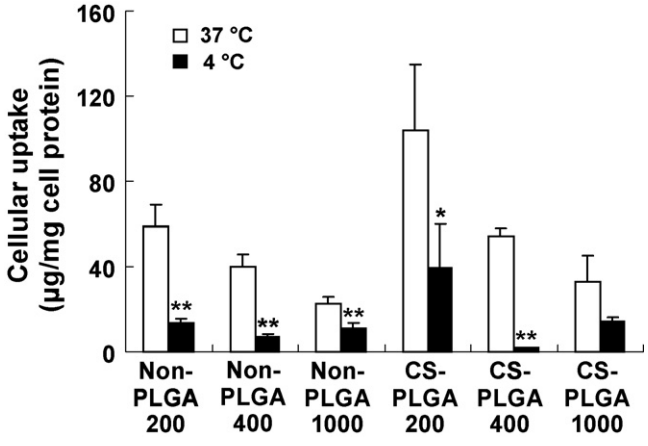


**Fig. 7.** Effects of incubation temperature on cellular uptake of PLGA NS by A549 cells. Two different temperatures (4 and 37 °C) were used to evaluate the uptake of various particle sizes of PLGA NS (1000, 400, and 200 nm). Data are shown as the mean ± SD ($n$ = 3), significantly different, $^{**}p < 0.01$, $^{*}p < 0.05$ compared with 37 °C.

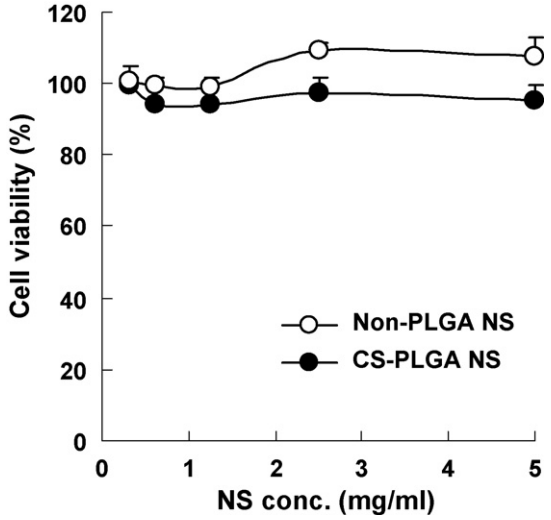


**Fig. 9.** Cytotoxic side effects of PLGA NS (200-nm) at concentrations ranging from 0 to 5 mg/ml on A549 cells at 37 °C. The cell viability of the treated cells was determined by MTS assay. Symbols represent mean ± SD ($n$ = 6).

## 4. Discussion

Various preparation conditions and physicochemical properties of NSs play a key role in biological applications. Important factors that could influence cellular uptake include particle size and surface character of the NSs. CS-PLGA NSs can be produced using a PVA-CS blend with the emulsion solvent evaporation method (Ravi Kumar et al., 2004). Smaller droplets in the emulsion can be produced by long-term sonication, which results in submicron-sized NSs. Modification of the surface of PLGA NSs by CS was confirmed by the shift of the zeta potential of CS-PLGA NSs to zero compared with that of non-PLGA NSs with negative zeta potential. This finding suggests that the surface of PLGA NSs can be modified with CS bound to PLGA NS by electrostatic interaction. Coating with CS resulted in an increased particle size because the surface of the PLGA NS was surrounded by the adsorbed CS molecules.

Although the CS-PLGA NSs possessed a negative charge in the present study, it has been reported that CS-PLGA NSs can have a positive zeta potential (Kawashima et al., 2000; Ravi Kumar et al., 2004; Yang et al., 2009). When the concentration of CS in the preparation process was increased, the zeta potential of CS-PLGA NSs became positive, but the particle size of NS after freeze-drying was in the micron range due to aggregation (data not shown). Therefore, if the aggregation of NS after freeze-drying is prevented, it is necessary to add cryoprotectants to the suspension of PLGA NS before freeze-drying.

Intracellular uptake of NSs is affected by a number of factors, such as particle size, and surface characteristics, such as hydrophilicity and zeta potential (Lutsiak et al., 2002; Prabha et al., 2002; Qaddoumi et al., 2004). The plot of uptake ratio against NS concentration indicated that cellular uptake followed saturation kinetics. The total uptake of 400-nm PLGA NSs increased in a concentration-dependent manner when incubated at 37 °C, and followed the Michaelis–Menten equation ($R^2 = 0.99$) with a $V_{max}$ of 39.84 μg/mg cell protein and a $K_m$ of 44 μg/ml. Because the uptake of PLGA NS by A549 cells was temperature- and concentration-dependent saturable event, PLGA NSs are unlikely to be internalized by fluid-phase endocytosis, a constitutive process in cells (Huang et al., 2002). Of the two energy-dependent, saturable endocytic pathways, receptor-mediated endocytosis is initiated by ligand binding to specific cell membrane receptors, whereas adsorptive endocytosis is preceded by nonspecific interaction of ligands with the cell membrane. It has not been reported that PLGA NSs adsorbed to PVA are taken up via receptor-mediated endocytosis by A549 cells. Moreover, the affinity constant for the uptake of PLGA NSs was 44 μg/ml (8.8 μM), which supports the involvement of adsorptive endocytosis, because substrates transported by receptor-mediated endocytosis generally have higher affinities ($K_m$ in the nM range) than those taken up by adsorptive endocytosis ($K_m$ in the μM range) (Huang et al., 2002). Therefore, it was postulated that cellular uptake of PLGA NSs into A549 cells occurs through adsorptive-mediated endocytosis.

Fig. 6 shows that particle size significantly affected cellular uptake in A549 cells; only submicron-sized (200-nm) particles were taken up efficiently, and not the large-sized microparticles (1 μm). In the present study, NSs with a size of 200 nm showed ~2.5-fold greater uptake than those with a size of 1 μm by the A549 cell line.

The zeta potential of PLGA NSs increased upon CS modification. This finding suggests that CS-PLGA NSs have electrostatic interactions between positively charged CS amino groups and the negatively charged cell membrane. Therefore, the uptake of CS surface-modified PLGA NSs was greater than the non-PLGA NSs.

Cellular uptake of CS-PLGA NSs exhibited a saturable pattern similar to that of non-PLGA NSs. CS is known to interact with cell membranes through nonspecific attractive electrostatic forces (Schipper et al., 1997; Huang et al., 2002; Yang et al., 2009), and no receptor specific to CS has been reported to exist in A549 cell membranes. Therefore, it can be reasonably assumed that CS-PLGA NSs are also transported by the adsorptive endocytic pathway.

Several endocytic pathways that regulate cellular trafficking have been identified. The most widely studied, clathrin, forms coated membrane invaginations on plasma membranes that recruit cell-surface receptors. Plasmalemmal vesicles that use caveolae, macropinosomes, or other clathrin-independent pathways can also form at the cell surface to transport molecular cargo (Panyam et al., 2002).

Clathrin is important for the internalization of both non- and CS-PLGA NSs, and its inhibition by hypertonic growth medium prevents assembly of clathrin-coated pits or pinching-off of the plasma membrane (Heuser and Anderson, 1989), causing up to ~35% reduction in non- and CS-PLGA NSs uptake by the A549 cells. However, this condition did not completely eliminate the internalization of NS, suggesting that clathrin-independent pathways are likely also involved.

Inhibition of cholesterol depletion by filipin (5 μg/ml), a cholesterol-binding agent that selectively inhibits caveolae invagination without affecting the function of coated pits (Schnitzer et al., 1994), resulted in a 20% increase in NS uptake, indicating that cholesterol may be involved in controlling intracellular uptake of NS.

Another known clathrin-independent pathway is macropinocytosis, which is inhibited by cytochalasin D. In the macropinocytosis, large (up to 5-μm), irregular endocytic vesicles generated at the ruffling membrane domain are involved in non-selective fluid-phase endocytosis of macromolecules. PLGA NS uptake was not affected by cytochalasin D, a potent inhibitor of actin polymerization (Nabi and Le, 2003), suggesting that microfilaments do not play an important role in the uptake of NS, at least in this cell line.

In the present study, the mechanism of cellular uptake of PLGA NSs was identified as partially clathrin-mediated endocytosis. The mechanism of cellular uptake of NSs did not change through surface modification of CS. However, Panyam et al. (2002) reported that in vascular smooth muscle cells, internalization of non-PLGA NSs (100-nm) occurs partially through fluid phase pinocytosis and partially through clathrin-coated pit endocytosis. The mechanism of cellular uptake of wheat germ agglutinin-conjugated PLGA NSs (200-nm) by A549 cells is mediated by the caveolae-dependent pathway (Mo and Lim, 2004). Therefore, the mechanism of cellular uptake is dependent on particle size, surface properties of NSs, and cell line type.

Both non- and CS-PLGA NSs had no cytotoxicity to the A549 cells. At higher concentrations, most cationic polymers and cationic lipids have toxic effects on cells (Choksakulnimitr et al., 1995). Therefore, it is possible for CS to cause cytotoxicity because CS is a cationic polymer. However, the CS-PLGA NS was not cytotoxic to A549 cells, as shown in Fig. 9. This result suggests that CS that is strongly adsorbed to the surface of PLGA NS is harmless to cells because the free CS was removed by centrifugation during the preparation process. CS-PLGA NSs are a suitable system for the intracytoplasmic delivery of drugs, proteins, or genes because both PLGA and CS are biodegradable and biocompatible.

## 5. Conclusions

The cellular uptake of non- and CS-PLGA NSs was found to be a time-, temperature-, and concentration-dependent saturable event mediated by clathrin-coated pit endocytic pathways. Cellular uptake of PLGA NSs increased with decreasing particle diameter to the submicron range. CS modification promoted cellular uptake of PLGA NSs through electrostatic interactions between the CS adsorbed to the NS surface and the negatively charged cell mem-

brane. Internalization of both non- and CS-PLGA NS (200-nm) by A549 cells appears to occur predominantly through adsorptive endocytosis initiated by nonspecific interactions between NS and cell membranes, and is partially mediated by a clathrin-mediated process. Moreover, CS-PLGA NSs did not show cytotoxicity to A549 cells. Nanoparticles have been investigated for the delivery of various types of therapeutic agents, including proteins, peptides, and DNA. In addition to providing sustained release, nanoparticles can protect the encapsulated agent from enzymatic degradation. CS is suitable as a material for surface modification of PLGA NSs for intracellular targeting because CS-PLGA NSs increased the interaction between the cell membrane and NSs without showing cytotoxicity. Thus, CS-PLGA NSs are highly recommended as a preferable drug carrier because of high cellular uptake resulting from their strong interaction with cells and safety with respect to cytotoxicity.

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