

Mechanism of cell transfection with plasmid/chitosan complexes

Tsuyoshi Ishii ^a, Yoshio Okahata ^a, Toshinori Sato ^{b,*}

^a Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

^b Department of Applied Chemistry, Keio University, 3-14-1 Hiyoshi, Kouhoku-ku, Yokohama 223-8522, Japan

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Abstract

Chitosan is useful as a non-viral vector for gene delivery. Although there are several reports supporting the use of chitosan for gene delivery, studies regarding effects on transfection and the chitosan-specific transfection mechanism remain insufficient. In this report, the level of expression with plasmid/chitosan was observed to be no less than that with plasmid/lipofectin complexes in SOJ cells. The transfection mechanism of plasmid/chitosan complexes as well as the relationship between transfection activity and cell uptake was analyzed by using fluorescein isothiocyanate-labeled plasmid and Texas Red-labeled chitosan. In regard to effects on transfection, there were several factors to affect transfection activity and cell uptake, for example: the molecular mass of chitosan, stoichiometry of complex, as well as serum concentration and pH of transfection medium. The level of transfection with plasmid/chitosan complexes was found to be highest when the molecular mass of chitosan was 40 or 84 kDa, ratio of chitosan nitrogen to DNA phosphate (N/P ratio) was 5, and transfection medium contained 10% serum at pH 7.0. We also investigated the transfection mechanism, and found that plasmid/chitosan complexes most likely condense to form large aggregates (5–8 μm), which absorb to the cell surface. After this, plasmid/chitosan complexes are endocytosed, and possibly released from endosomes due to swelling of lysosomal in addition to swelling of plasmid/chitosan complex, causing the endosome to rupture. Finally, complexes were also observed to accumulate in the nucleus using a confocal laser scanning microscope. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Chitosan; Transfection; Subcellular distribution; DNA complex; Plasmid

1. Introduction

Gene therapy is a powerful treatment for curing inborn and acquired diseases. There are primarily

two types of gene carriers: viral and non-viral vectors. Viral vectors, such as retrovirus, adenovirus, etc., have been utilized for approximately 80% of approved phase I clinical trials due to their ability to transfect [1]. However, they have limitations in vivo such as wild-type reversion and immunogenicity [2]. In contrast, the non-viral delivery system has several potential advantages including low cost, non-infectivity, absence of immunogenicity, good compliance, and the possibility of repeated clinical administration [3]. Therefore, the research and development of non-viral vectors is a very important undertaking.

Abbreviations: pGalN, polygalactosamine; PLL, poly-L-lysine; N/P ratio, chitosan nitrogen to DNA phosphate ratio; DEAE-dextran, diethylaminoethyl-dextran; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate

* Corresponding author. Fax: +81-45-566-1447.

E-mail address: sato@appc.keio.ac.jp (T. Sato).

There are several options available for exploration, such as cationic liposomes [4,5], cationic lipids [6–10], polymers [11]. Cationic lipids, such as lipofectamine [12], have a high transfection efficiency in vitro, but in vivo their use is limited by general toxicity [13]. Recently, polyamines such as chitosan [14–25] and polyethyleneimine [4,26–30] have received much attention as gene carriers. We previously reported that plasmid/chitosan complexes produced better gene expression than plasmid/lipofectin complexes in three cell lines: A549 cells, B16 cells, and HeLa cells. Moreover, the molecular mass of chitosan, stoichiometry of complex, serum concentration and pH of the transfection medium were all found to affect the expression of plasmid/chitosan complexes [22,25].

Chitosan is a naturally occurring polysaccharide consisting of two subunits, D-glucosamine and N-acetyl-D-glucosamine linked together by $\beta(1,4)$ glycosidic bonds. Chitosan has several advantageous qualities in comparison with other non-viral vectors in that it is relatively non-toxic [13] and has high transfection efficiency [14,17,22]. To develop chitosan as a gene carrier, several studies have been conducted to address the preparation of plasmid/chitosan complexes, such as nanospheres [17], self-aggregates [16], and chitosan complexes chemically modified by coupling ligand [14,19]. Recently, Roy et al. reported that oral administration of DNA nanoparticles prepared by complexing plasmid DNA with chitosan resulted in transduced gene expression in the intestinal epithelium in vivo [20]. Although there are several such reports supporting the use of chitosan for gene delivery, studies regarding the transfection mechanism remain insufficient. Erbacher et al. [14] and MacLaughlin et al. [18] attempted to study the transfection mechanism, however, Erbacher et al. did not describe problems with the nuclear import of plasmid/chitosan complexes while MacLaughlin et al. performed transfection at a very high DNA concentration [18].

In this work, FITC-labeled plasmid and Texas Red-labeled chitosan were synthesized for further analyzing the transfection mechanism of plasmid/chitosan complexes. Transfection efficiency, cell uptake, and subcellular distribution were evaluated using these labeled materials. We found that several factors, such as molecular mass of chitosan, plasmid concentration, stoichiometry of complex, and serum

concentration and pH of transfection medium, influenced transfection activity and cell uptake. Moreover, plasmid/chitosan complexes appeared to have the ability to overcome the three major obstacles for transfection, i.e., cell uptake, endosomal release, and nuclear localization.

2. Materials and methods

2.1. Materials

Chitosan hydroacetate was obtained from Yaizu Suisankagaku Industry (Shizuoka, Japan). PLL (9.6 kDa and 22.6 kDa) and DEAE-dextran (500 kDa) were obtained from Sigma (St. Louis, MO). Polygalactosamine (pGalN) hydroacetate (10–30 kDa) was obtained from Higeta Shoyu (Tokyo, Japan). Lipofectin was obtained from Gibco BRL (Grand Island, NY). Chitosan and pGalN hydrochloride were prepared according to the following procedure. The polymers were placed in a bottle equipped with a magnetic stirrer and 12 N HCl was added until the solution became transparent; next a powder was prepared by freeze-drying. The average molecular masses of the chitosan hydrochloride were 40, 84, and 110 kDa. The degrees of deacetylation were above 85%. The average molecular mass of pGalN hydrochloride was 4.6 kDa and the degree of deacetylation was 100%.

2.2. Plasmid

The plasmid pGL3-Luc encoding luciferase (Promega, Madison, IL) was grown in Lo9 coli and purified by a Qiagen[®] kit (Qiagen, Chatsworth, CA). The purity of the plasmid was checked by electrophoresis on a 1% agarose gel and the DNA concentration was determined by measuring UV absorbance at 260 nm.

2.3. Cell culture

SOJ cells (kindly provided by Dr. Hironobu Yanagie, Tokyo University, Japan) were cultured in 75-cm² flasks in RPMI 1640 medium (Nissui, Japan) supplemented with 10% fetal bovine serum (FBS), 100 μ g/ml of streptomycin (Meiji Seika, Japan), 100 IU/ml of penicillin (Meiji Seika), 10 mM MOPS

(Gibco BRL) and 0.3 mg/l L-glutamine (Nissui) at 37°C and 5% CO₂.

2.4. Preparation of FITC-labeled plasmid and Texas Red-labeled chitosan

The preparation of FITC-labeled plasmid was described previously [31]. To prepare Texas Red chitosan, 154 mg of chitosan hydrochloride (40 kDa) and 5 mg of sulforhodamine 101 acid chloride (Texas Red, Dojindo, Laboratory, Japan) were dissolved separately in 1.5 ml of Milli-Q water adjusted to pH 7.2 with 0.1 N NaOH. The water solutions were mixed and the reaction was carried out overnight at 20°C under stirring. In order to purify the product, 5 ml of ethanol was added to the reaction mixtures. Next, a few drops of 1 N NaOH solution were added to the solution in order to precipitate the product. After centrifugation of the precipitated product at 6000 rpm for 10 min, the supernatant solution was discarded to recover the product. Five ml of Milli-Q water with a few drops of 1 N HCl was then added and the product was dissolved again. This purification process was repeated five times until fluorescence from Texas Red could not be detected in the supernatant. The Texas Red-labeled chitosan was then freeze-dried. The average number of Texas Red residues was determined to be 1/40 per chitosan molecule. The labeled chitosan and plasmid showed no significant differences in transfection efficiency from non-labeled chitosan and plasmid.

2.5. Preparation of plasmid/chitosan complexes for transfection

The solutions of plasmid and various amounts of chitosan were diluted separately in 50 µl of serum-free medium (ASF-104, Ajinomoto, Japan) at pH 6.5. After 20 min, the solution of chitosan was added to the solution of plasmid and the resulting solution was homogenized and left for 20 min at 20°C. The formation of plasmid/chitosan complexes was analyzed by electrophoresis.

2.6. Cell transfection

Cells were seeded at 5×10^4 cells per well in 24-well dishes and incubated for 19 h at 37°C under a 5%

CO₂ atmosphere. Immediately before transfection, cells were rinsed and supplemented with 900 µl of fresh culture medium with or without FBS at pH 6.5, 7.0, 7.5 or 8.0. Then, 100 µl of plasmid/polycation complexes were added in each well and the plates were incubated at 37°C under a 5% CO₂ atmosphere. After 4 h of incubation, the medium and complexes were removed, and the cells were rinsed once with serum-containing medium and cultured with 10% FBS-supplemented medium. After another 24 h of incubation, luciferase gene expression was monitored using a commercial kit (Promega) and photon counting (TD-20/20 Luminometer, Promega). Each transfection experiment was carried out in triplicate and transfection activity was expressed as relative light units. Transfection activity of plasmid was normalized to 100.

2.7. Cell uptake

Plasmid/polymer complexes were formulated following the procedure for transfection. However, cells were incubated with plasmid complexes for 4 h at 4°C or 37°C. After 4 h of incubation, the medium and complexes were removed and the cells were rinsed twice with PBS and harvested with 0.05% trypsin/0.01% EDTA before analysis on a flow cytometer (EPICS XL, Coulter). Each sample for cell uptake was measured in doublet and the quantities of cell uptake were expressed as relative fluorescence intensity versus that of cell uptake with naked plasmid. The value for cell uptake of naked plasmid was normalized to 100.

2.8. Particle size and zeta potential measurements

To study the size of plasmid/polymer complexes, 10 µg of plasmid and various amounts of polymers were diluted separately in 500 µl of RPMI 1640 medium (pH 6.5) containing 10 mM MOPS without phenol red. After 20 min, the solution of polymer was added to the solution of plasmid and the resulting solution was homogenized at room temperature. After 20 min, 0.1 N NaOH solution was added to the solution of complexes in order to adjust the medium at pH 7.0. The particle size of complexes was determined by means of dynamic laser light scattering using PAR-IIIIS (Otsuka Electric, Japan) at 25°C.

The zeta potential was determined with ELS 8000 (Otsuka Electric) at 25°C.

2.9. Resistance of plasmid/polymer complexes to SDS

Plasmid/polymer complexes were formulated as described in preparation of complexes, by incubating 1.5 µg of plasmid DNA with the appropriate amount of cationic polymer, such as chitosan (M_r 45 kDa) or PLL (M_r 22.6 kDa), at N/P=5 in 300 µl of serum-free medium (ASF-104). After 20 min incubation, the complexes were divided into five parts. Each fraction (12 µl) was mixed with or without SDS dissolved in serum-free medium at a final ratio of 0.01%, 0.1%, 1%, and 5%. The final volume of the fraction was 20 µl. After 20 min, these fractions were analyzed by 1% (w/v) agarose gel electrophoresis for 40 min at 100 V and stained with Gel Star (FMC Co.).

2.10. Subcellular distribution of plasmid/chitosan complexes

Cell fluorescence was analyzed with a confocal laser scanning microscope (TCS-NT, Leica) equipped with a heating stage (tempcontrol 37-2, Leica). A Kr/Ar laser (488 and 568 nm) was used for FITC and Texas Red excitation. This microscope allowed the simultaneous monitoring of FITC and Texas Red emission and the simultaneous visualization of two different molecules. To observe the subcellular distribution of plasmid/chitosan complexes, cells were seeded at 5×10^4 in 35-mm-diameter glass-bottom

dishes and incubated for 19 h at 37°C under 5% CO₂. Fluorescent molecules, such as Texas Red-dextran (M_r 10 000, Molecular Probes), FITC-plasmid, and Texas Red-chitosan, were used for observation. Plasmid/polymer complexes were formulated according to the procedure for transfection and incubated with the cells. After 1 h of incubation, the cells were rinsed once with serum-containing medium to remove free complexes, and to the dishes 3 ml of 10% FBS-supplemented medium was added. A time course of subcellular distribution of plasmid/chitosan complex was observed at regular time intervals after removal of the complexes using a confocal scanning laser microscope.

2.11. Titration

DNA/polymer complexes were formulated by incubating DNA ($[DNA \text{ (monomer unit)}] = 2 \times 10^{-5}$ mol/l, salmon testes DNA; ca. 1000 b.p., Nichiro, Japan) with the appropriate amount of cationic polymer ($[polymer \text{ (monomer unit)}] = 1 \times 10^{-4}$ mol/l) at a 5 (N/P ratio) in 25 ml of Milli-Q water. After 20 min incubation, the solution of complexes was titrated with 0.01 N NaOH solution monitored with a pH meter (Delta320, Mettler). The percentage of proton release from plasmid/polymer complexes from pH 5.0 to 7.0 was calculated according to the following formula:

$$10^{(-pH)} \times a/1000 + b \times c/[M]$$

where a is volume of solvent of complexes (25 ml),

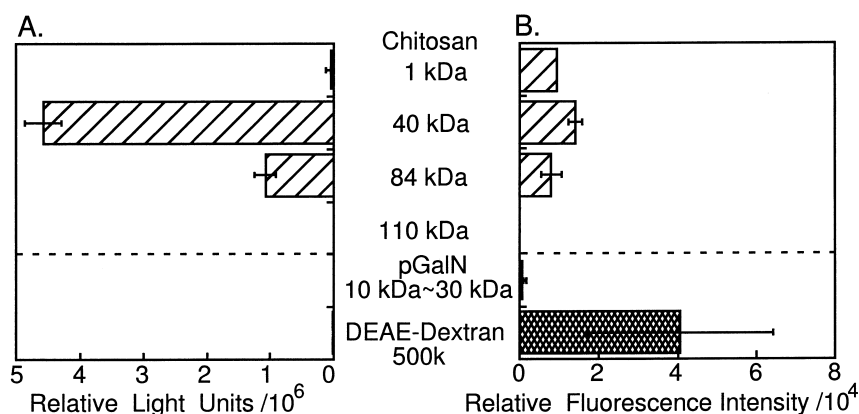


Fig. 1. Effect of various gene transfer reagents on luciferase activity (A) and cell uptake (B) for SOJ cells. Transfection using plasmid/cationic polymer complexes at N/P=5 was made in the presence of 10% FBS at a plasmid concentration of 1 µg/ml.

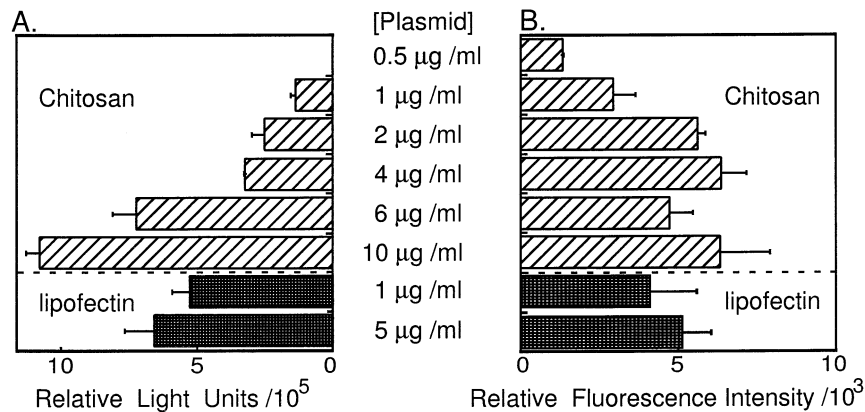


Fig. 2. Effect of plasmid concentration on luciferase activity (A) and cell uptake (B) in SOJ cells. Molecular mass of chitosan was 40 kDa. Transfection using plasmid/chitosan complexes at N/P = 5 was made in the presence of 10% FBS. The weight ratio of plasmid/lipofectin was 1:2.

b is volume of 0.01 N NaOH (μl), c is mole of NaOH per 1 μl (1×10^{-8} mol/l), and $[M]$ is [polymer (monomer unit)] = 1×10^{-4} mol/l.

3. Results

3.1. Transfection activity and cell uptake

This set of experiments demonstrates the effect of various factors on transfection activity and cell uptake, specifically the influence of changes in chitosan molecular mass, plasmid concentration, stoichiometry of complex, serum supplement, and pH of transfection medium were analyzed.

3.1.1. The effect of chitosan molecular mass

A plasmid concentration of 1 $\mu\text{g/ml}$ was employed and complexes (N/P ratio = 5) were formed using the various molecular masses for chitosan and cationic polymer. High levels of transfection were observed with the plasmid/chitosan complexes containing 40 and 84 kDa chitosan. In contrast, expression was not entirely detectable with complexes containing 1 kDa and 110 kDa chitosan, pGalN, and DEAE-dextran (Fig. 1A). The cell uptake of complexes, excluding those made with 1 kDa chitosan and DEAE-dextran, were correlated to the overall transfection activity. By contrast, although transfection activity was not observed with the 1 kDa chitosan and DEAE-dextran complexes, these complexes easily associated with the cell (Fig. 1B).

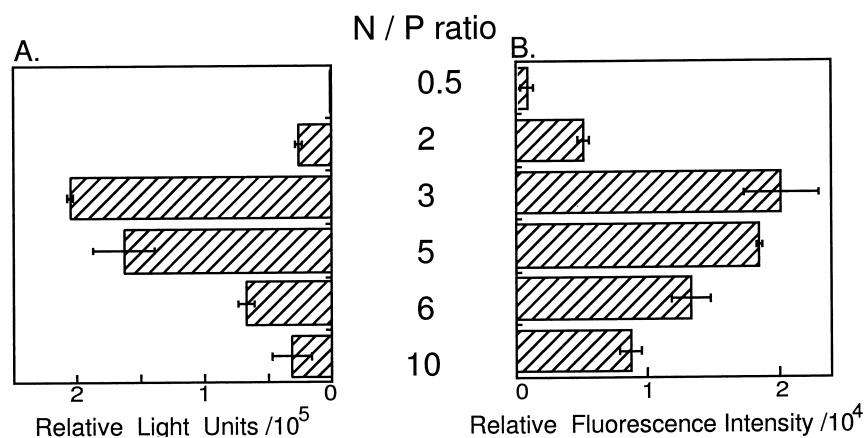


Fig. 3. Effect of N/P ratio of plasmid/chitosan complex on luciferase activity (A) and cell uptake (B) in SOJ cells. Molecular mass of chitosan was 40 kDa. Transfection using plasmid/chitosan complex was made in the presence of 10% FBS at a plasmid concentration of 1 $\mu\text{g/ml}$.

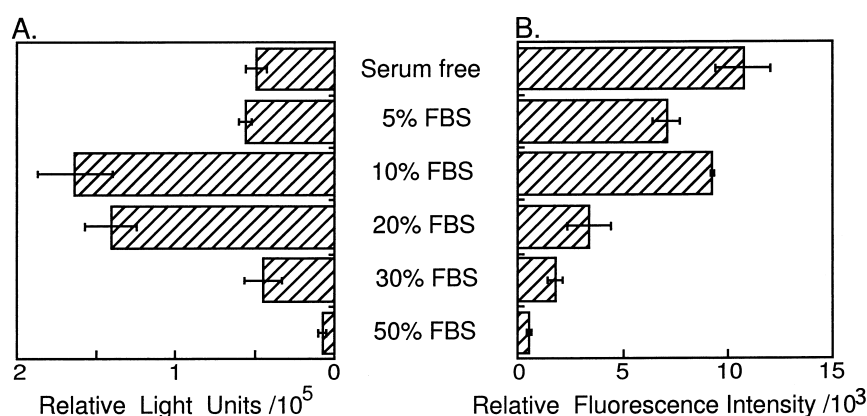


Fig. 4. Effect of serum content on luciferase activity (A) and cell uptake (B) in SOJ cells. Molecular mass of chitosan was 40 kDa. Transfection using plasmid/chitosan complex at N/P=5 was made with or without FBS at a plasmid concentration of 1 μ g/ml.

3.1.2. Effect of plasmid concentrations

Complexes used to examine the influence of plasmid concentration were formed with 40 kDa chitosan at a 5 (N/P ratio). As the plasmid concentration increased, the level of complex transfection increased (Fig. 2A). However, the level of cell uptake of complexes increased only up to a plasmid concentration of 2 μ g/ml, after which uptake leveled off at a range of 2–10 μ g/ml (Fig. 2B). The level of expression with the plasmid/chitosan complexes at a plasmid concentration of 5–10 μ g/ml was no less than that observed with the plasmid/lipofectin complexes.

3.1.3. Effect of stoichiometry of complex

The N/P ratio is the stoichiometry of chitosan's nitrogen and DNA's phosphate. The transfection activities of the complexes increased at a stoichiometry

of complex between 3 and 5 (N/P ratio), and decreased at higher stoichiometries of complex (Fig. 3A). The cell uptake efficiencies of the complexes were found to be in correlation with transfection activities (Fig. 3B). The complete complexes were obtained at a chitosan nitrogen to DNA phosphate ratio (N/P ratio) of greater than 2, analyzed by electrophoresis (data not shown).

3.1.4. Effect of serum

The level of transfection increased up to 10% or 20% serum-containing medium, however, largely decreased at serum concentrations from a 20–50% (Fig. 4A). With respect to the cell uptake of the complexes, the level of cell uptake gradually declined moving from serum-free medium to 50%-serum medium (Fig. 4B).

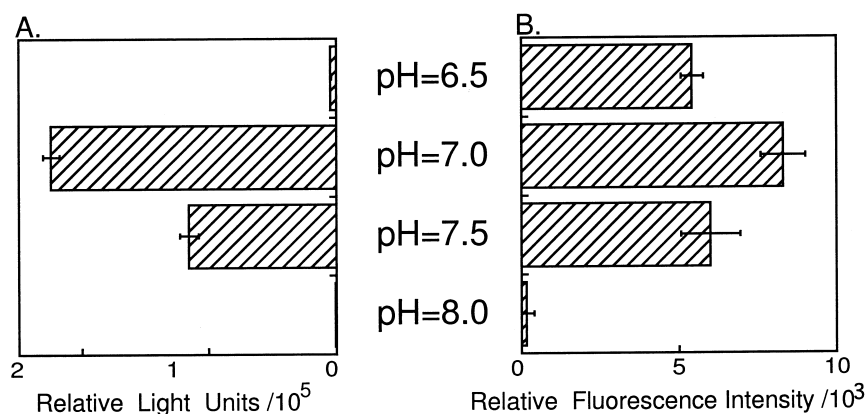


Fig. 5. Effect of pH of transfection medium on luciferase activity (A) and cell uptake (B) in SOJ cells. Molecular mass of chitosan was 40 kDa. Transfection using plasmid/chitosan complex at N/P=5 was made in the presence of 10% FBS at a plasmid concentration of 1 μ g/ml.

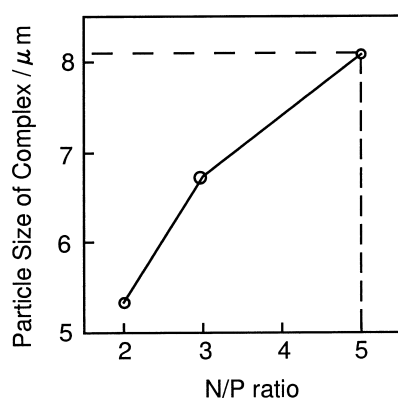


Fig. 6. Particle size of plasmid/chitosan complex (N/P = 5). Molecular mass of chitosan was 40 kDa. Measurements were performed at a plasmid concentration of 10 $\mu\text{g/ml}$ in RPMI-1640.

3.1.5. Effect of culture medium pH

The rise of transfection activity continued from pH 6.5 to 7.0, and fell at higher pH levels (Fig. 5A). The amount of cell uptake was correlated to the level of transfection activity except for medium of pH 6.5. The amount of cell uptake at pH 6.5 was found to be the same as at pH 7.5 (Fig. 5B).

3.2. Biophysical characteristics of plasmid/chitosan complexes

3.2.1. Analysis of particle size

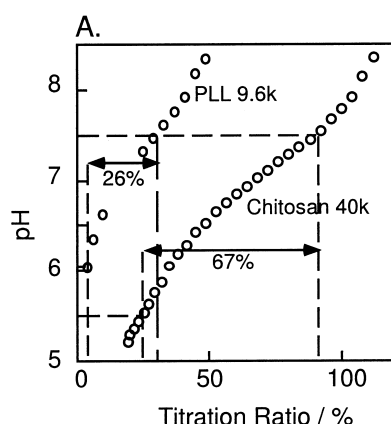
The size of plasmid/chitosan complexes was analyzed by means of dynamic light scattering (Fig. 6A). An increase in size was observed with increasing N/P ratio and a size distribution from 5 μm to 8 μm was observed for N/P ratios from 2 to 5.

In order to obtain information about the size of complexes which produce gene expression, 2 types of solution, filtrated (0.22 μm) and non-filtrated, were prepared. Filtration was designed to exclude aggregate complexes before incubation. There was a high level of transfection with non-filtrated complexes; however, no transfection activity was detected with filtrated complexes.

3.2.2. Proton release from plasmid/cationic polymer complexes

To further investigate proton release from DNA/polymer complexes from pH 5.0 to 7.0, solutions were titrated with NaOH solution (Fig. 7A). In this experiment, we confirmed that the amount of proton release from DNA/polymer complexes was nearly equal to that of the proton absorption to complexes ranging from pH 5.0 to 7.0 (data not shown). There was high proton release from the 40 kDa and 110 kDa chitosan complexes (approximately 60–70%). The amount of proton release from the chitosan complexes was approximately 1.5-fold larger than that observed with pGalN and DEAE-dextran complexes and moreover, approximately 2.5-fold larger than that observed with PLL complexes (Fig. 7B).

To better examine the high amount of proton release from plasmid/chitosan complexes, the zeta potential of complexes was measured (Fig. 8). At pH 7.0, the plasmid/chitosan complexes had a slightly positive surface charge and there was a marked increase in the zeta potential of the complexes from pH 7.0 (+2 mV) to 6.0 (+21 mV). However, there was a



B.

Polymer	Proton Release from pH 5.0 to 7.0
Chitosan 40 kDa	67%
Chitosan 110 kDa	60%
pGalN 4.6 kDa	44%
DEAE-Dextran 500 kDa	40%
PLL 9.6 kDa	26%

Fig. 7. Proton release from DNA/(cationic polymer) complexes (N/P = 5). Measurements were performed at a plasmid concentration of 6.7 $\mu\text{g/ml}$ in Milli-Q water and were titrated with 0.01 N NaOH. Molecular mass of chitosan was 40 kDa.

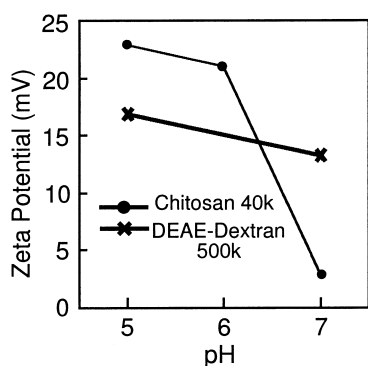


Fig. 8. Zeta potential of plasmid/(cationic polymer) complexes (N/P=5). Measurements were performed at a plasmid concentration of 10 μ g/ml in RPMI-1640. Chitosan 40 kDa (circle), DEAE-dextran 500 kDa (cross).

slight increase in the surface charge of plasmid/DEAE-dextran complexes from pH 7.0 (+13 mV) to 5.0 (+16 mV).

3.2.3. Exchange with anionic surfactants

The resistance of plasmid/chitosan complexes to anionic surfactants, exposed to different concentrations of SDS (0.01%, 0.1%, 1% and 5% (w/w)), is presented in Fig. 9. Binding of a cationic polymer to plasmid DNA results in neutralization of negative charges in the phosphate backbone of DNA. This interaction was evaluated by existence of the bands of uncomplexed plasmid DNA. As shown in Fig. 9, the release of plasmid DNA from chitosan complexes was observed at concentrations of SDS above 0.1%. However, the release of plasmid DNA from PLL complexes was observed at low concentrations of SDS around 0.01%. This result confirms that plasmid/chitosan complexes are highly resistant to anionic surfactants.

3.3. Cellular localization of plasmid/chitosan complexes

The mechanism of cell uptake of plasmid/chitosan complexes was observed with a confocal laser scanning microscope using FITC-plasmid and Texas Red-dextran. Texas Red-dextran was added to transfection medium for use as an endosome marker. By recognizing that fluorescence images of FITC-plasmid (Fig. 10A) were identical to those taken with Texas Red-dextran (Fig. 10B) at 1 h post-incubation,

we were able to confirm that plasmid/chitosan complexes had been endocytosed.

To ascertain the cellular localization of plasmid/chitosan complexes, cells were incubated with plasmid/chitosan complexes formulated with FITC-plasmid and Texas Red-chitosan. After 1 h post-incubation, FITC-plasmid and Texas Red-chitosan (Fig. 11A) displayed the same cytoplasmic location. At 4 h post-incubation, the fluorescence of the two molecules had accumulated in the nucleus (Fig. 11B) and distinct nuclear staining was observed at 24 h post-incubation (Fig. 11C).

4. Discussion

4.1. Effect of experimental conditions on transfection activity and cell uptake

There are several reports stating successful chitosan-mediated transfection. However, the relationship between transfection activity and cell uptake of plasmid/chitosan complexes has not to be clearly illustrated. Our study fully demonstrates this relationship

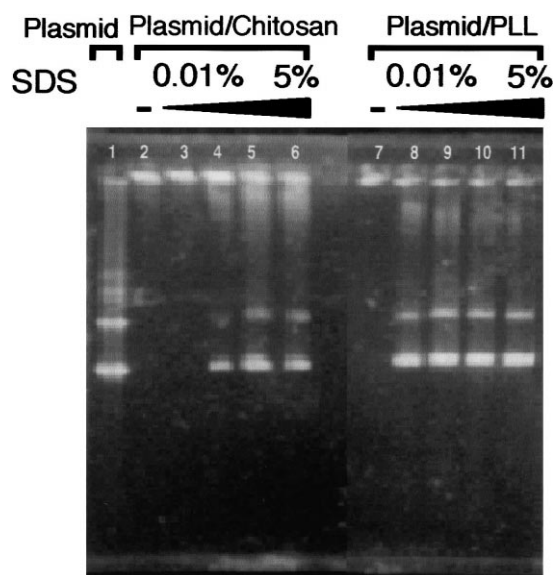


Fig. 9. Resistance of plasmid/(cationic polymer) complexes (N/P=5) to anionic surfactant. Lane 1, plasmid alone; lanes 2–6, plasmid/chitosan complexes with progressively increasing proportions of SDS (0%, 0.01%, 0.1%, 1%, and 5%); lanes 7–11, plasmid/PLL complexes with progressively increasing proportions of SDS (0%, 0.01%, 0.1%, 1%, and 5%). Molecular mass of chitosan was 40 kDa.

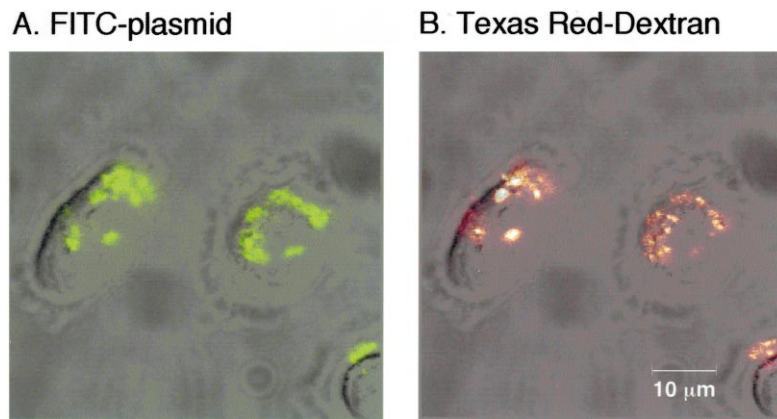


Fig. 10. Intracellular distribution of FITC-plasmid/chitosan complexes (A, green) and Texas Red-dextran (B, red) were observed with a confocal fluorescence microscope at 1 h post-incubation in SOJ cells. The confocal images show overlaid images of the fluorescence probe and the phase contrast. Molecular mass of chitosan was 40 kDa. Bars = 10 μm .

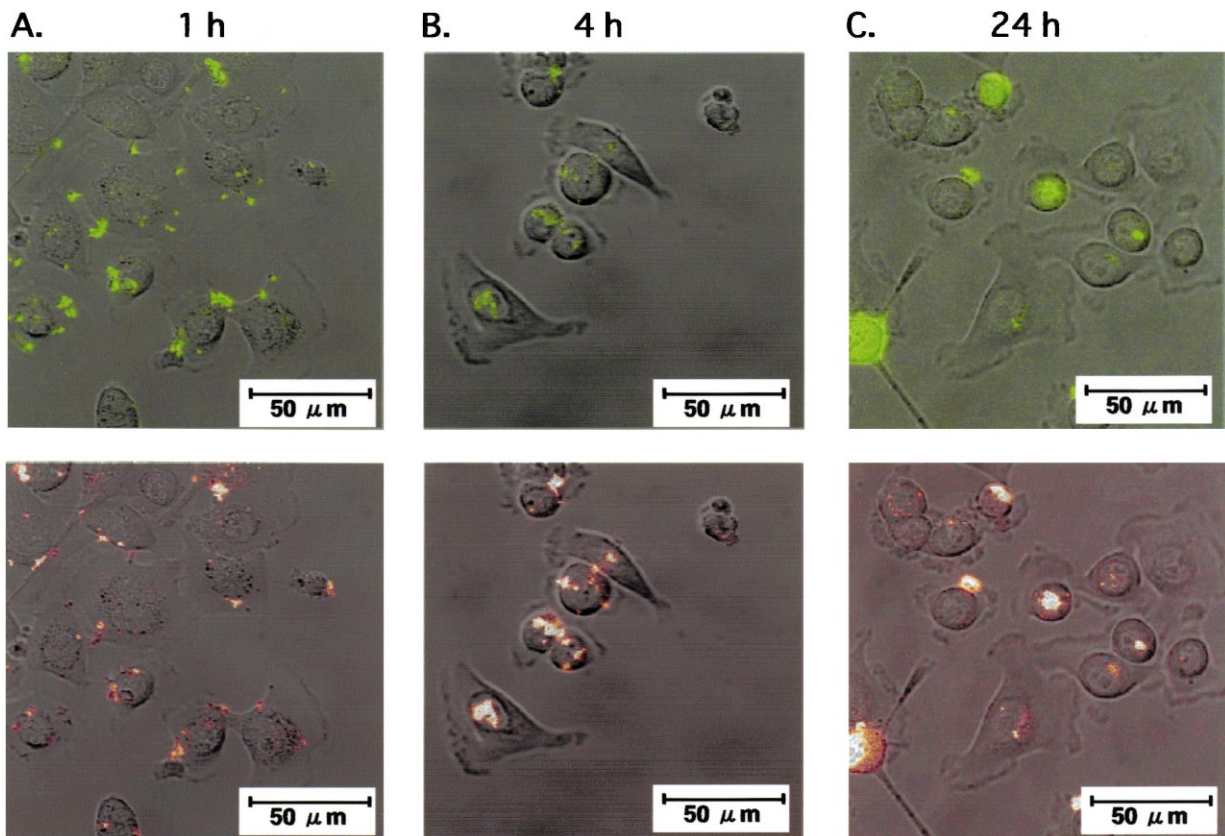


Fig. 11. Intracellular distributions of FITC-plasmid/Texas Red-chitosan complexes were observed with a confocal fluorescence microscope in SOJ cells. (A) One hour post-incubation; (B) 4 h post-incubation; (C) 24 h post-incubation. The confocal images show overlaid images of the fluorescence probe and the phase contrast. The top panel shows the FITC-plasmid (green) and the bottom panel shows the Texas Red-chitosan (red). Molecular mass of chitosan was 40 kDa. Bars = 50 μm .

by means of complexes formed with an FITC-labeled plasmid. There were several factors found to influence transfection activity and cell uptake, in particular: the molecular mass of chitosan, plasmid concentration, stoichiometry of complex, and serum concentration, and pH of the transfection medium.

In the case of the molecular mass of chitosan, the effect on transfection activity of plasmid/chitosan complexes made with different chitosan molecular mass (7–540 kDa) had been previously observed [18]; nonetheless, there exist several problems. For example, their transfection experiments were performed at a plasmid concentration of several hundred $\mu\text{g/ml}$ [18]. Moreover, the level of expression was 250-fold less than that observed with the plasmid/Lipofectamine at a plasmid concentration of 2.5 $\mu\text{g/ml}$. However, our study was performed at a plasmid concentration of 5 $\mu\text{g/ml}$ and the level of expression with plasmid/chitosan complexes was no less than that of expression with plasmid/lipofectin complexes at 2 or 5 $\mu\text{g/ml}$ (Fig. 2A). The cell uptake of chitosan complexes made with 40, 84, and 110 kDa chitosan were correlated to plasmid expression (Fig. 1A). Nevertheless, plasmid complexes made with 1 kDa chitosan and DEAE-dextran were not found to correlate with the cell uptake.

With respect to the effect of plasmid concentration, the level of cell uptake of plasmid/chitosan complexes leveled off as the plasmid concentration increased, while the level of expression was enhanced (Fig. 2A,B). We confirmed 1.3-fold enhancement of cell viability with plasmid/chitosan complexes at a plasmid concentration of 1–5 $\mu\text{g/ml}$ analyzed by MTT assay (data not shown). Moreover, chitosan (>10 kDa) was found to improve the viability of L132 human embryonic lung cells and CCRF-CFM human lymphoblastic leukemia cells, compared to control cells at concentrations of 0.1–1000 $\mu\text{g/ml}$ [13]. Thus, the enhancement of expression might be attributed to that of cell viability, as the plasmid concentration increased.

Erbacher et al. have shown that plasmid/chitosan complexes made with a N/P ratio of 3 give the highest transfection activity in 10% serum [14], in agreement with our observations. However, there exists no study that attempts to further prove this phenomenon. Therefore, we analyzed the relationship between

the level of expression and the amount of cell uptake by using FITC-plasmid/chitosan complexes. Our results exhibited that they were strongly correlated with respect to N/P ratio (Fig. 3A,B). In regard to serum concentration, however, there was no relationship observed between transfection activity and cell uptake (Fig. 4A,B). This result indicates that the cell uptake of the complexes declines due to non-specific interaction between the complexes and serum components. Nevertheless, there was the highest transfection activity in the presence of 10% or 20% serum, due to cell viability being optimal for transfection under this circumstance.

In addition, plasmid/chitosan complexes exhibited a unique distinctive feature in that transfection activity and cell uptake were affected by the pH of transfection medium. Especially the transfection activity and cell uptake decreased at pH 7.5 and 8.0. Moreover, although the level of cell uptake at pH 6.5 was the same as that of cell uptake at pH 7.5, the plasmid/chitosan complexes were unable to transfect (Fig. 5A,B). The effect of pH (6.5, 7.0, 7.5, 8.0) on plasmid/chitosan complex formation, when complex ratio was 5, was analyzed by agarose gel electrophoresis. Free plasmids dissociated from the complexes at pH 7.5 and 8.0 were detected (data not shown). This result is quite similar to that of DNA complex formed with hydrophobically modified chitosan [16]. It follows from what has been described thus far that the release of plasmid from plasmid/chitosan complexes might be attributed to the low amount of cell uptake at pH 7.5 and 8.0.

Concerning the low expression at pH 6.5, it is likely that this result was due to cytotoxicity or changed characteristics of plasmid/chitosan complex at pH 6.5. However, plasmid/lipofectin complexes showed high transfection efficiency at pH 6.5 in comparison with other pHs (data not shown). This revealed that the level of transfection efficiency was not due to cytotoxicity at pH 6.5. Another possible reason may be subcellular distribution of the complexes after cell uptake. The FITC-plasmid/chitosan complexes showed no-accumulation in the nucleus but localization around nucleus when pH of culture medium was 6.5 (data not shown). This result suggested that the plasmid/chitosan complexes were not released from endosome. Release of the complexes from endosome is considered to be induced by the

buffering effect as described in Section 4.2.2. Based on the mechanism of buffering effect, it is considered that highly protonated chitosan cannot release from endosome. Indeed, as shown in Fig. 8, protonation of chitosan drastically occurred when pH of medium altered from pH 7 to 6. Thus, the transfection efficiency at pH 6.5 appeared to be based on the protonation of plasmid/chitosan complexes.

4.2. Transfection mechanism

To know the mechanisms of cell transfection with plasmid/chitosan complexes; following three processes were investigated: (1) cell uptake, (2) release from endosomes, and (3) nuclear transport.

4.2.1. Cell uptake

Several studies have been made on the particle size of plasmid/chitosan complexes and show that particle size ranges from 80 to 500 nm in 0.15 M NaCl [14,18]. Moreover, Erbacher et al. proposed that zeta potential was close to 0 mV ($N/P=2$) with a size range of 1–5 μm [14]. However, these experiments were carried out in 0.15 M NaCl solution and the pH effects of the measurement solution were not taken into consideration. Therefore, we performed an analysis of zeta potential at the adjusted pH. When pH was altered from 7.0 to 6.0, an analysis of zeta potential showed increase in the positive charge of the complexes (Fig. 8). This result suggested that pH is an important factor for complex-cell interaction.

Next, having clarified this interaction between cell surface and plasmid/chitosan complexes, we examined the mechanism of cell uptake to ascertain how the complexes are internalized into the cell. Observations with a confocal laser scanning microscope showed that FITC-plasmid/chitosan complexes ($N/P=5$) were taken up by endocytosis in so far as the fluorescence of FITC-plasmid and Texas Red-dextran was observed at the same cellular localization after 1 h post-incubation (Fig. 10). In addition, the amount of cell uptake of plasmid/chitosan (40 kDa) complexes was monitored by a flow cytofluorometer. Cell uptake at 37°C was about 3-fold more than that measured at 4°C, although the amount of cell uptake of 1 kDa chitosan and DEEA-dextran complexes was virtually constant (data not shown). This result

implied that the cell uptake of plasmid/chitosan complexes might be thermal and energy dependent, and that plasmid/chitosan complexes might be endocytosed [32]. These results are in agreement with the transmission electron microscopy (TEM) observation of plasmid/chitosan complexes ($N/P=5$) in HeLa cells [14].

4.2.2. Release from endosome

The release of plasmid/chitosan complexes from the endosomal compartment may be another rate-limiting step in the transfection process [18]. Protons accumulate into the endosomal vesicles through a vesicular ATPase-driven proton pump [33], for example in BALB/c 3T3 cells, pH of the endosomal vesicles is reported to be 5.0 [34]. Partially protonated polymers such as polyethyleneimine (PEI) retain a substantial buffering capacity so that they can be protonated at virtually any pH [27]. There may exist two main effects of buffering capacity of cationic polymers in endosome. (1) Endosome buffering may protect plasmids from a lysosomal nuclease due to the fact that acidification of endosome is necessary for the activity of lysosomal enzyme [33]. (2) It may also perturb the trafficking of endosome for osmotic swelling and subsequent endosome disruption.

It has been reported that low molecular mass chitosan (M_r 1700) with pK_a 6.4 exhibited more effective inhibition of pH fall from pH 7.0 to 4.0 than aspartame, phosphate, and monofluorophosphate buffer [35]. Furthermore, we also demonstrated that the amount of proton adsorption of DNA/chitosan (M_r 40 and 110 kDa) complexes from pH 7.0 to 5.0 was approximately 2.5-fold larger than that of DNA/PLL complexes (Fig. 7B). The capacity of the DNA/chitosan complex for proton adsorption was also several hundred times larger than the estimated amount of proton influx into endosomes (hypothesis: pH 7.0→5.0, endosome diameter 50 μm). Accordingly, the surface charge of plasmid/chitosan complexes increased from pH 7.0 (+2 mV) to pH 5.0 (+21 mV) (Fig. 9).

These features about proton adsorption may be a cause of pH-dependent transfection efficiency of plasmid/chitosan complexes. Therefore, it is considered that the transfection efficiency was not observed at a pH 6.5 of transfection medium, although a certain

cell uptake of plasmid/chitosan complexes was observed at the same pH (Fig. 5). These results are also attributable to the low buffering effect at a pH 6.5 of the transfection medium. Moreover, we found that lysosomal agents such as monensin and chloroquine, which increase the pH of endocytic vesicles, decreased transfection levels with plasmid/chitosan complexes in SOJ cells. However, these agents improved transfection levels with plasmid/PLL complexes (data not shown) and this result was correlated to that of Erbacher et al. [14]. It was found from these results that plasmid/chitosan complexes have a high ability for proton accumulation and that endosome buffering of plasmid/chitosan complexes may perturb the endosomal membrane and release complexes from endosomes as the pH of endosomes decline.

We further propose ‘hydrogel effect’ what plasmid/chitosan complexes are released from endosomes. Recently, a new drug delivery device based on a novel squeezing concept has been developed. The device utilizes the specific swelling–deswelling characteristics of temperature, temperature/pH, and pH-sensitive hydrogels. There have been several reports about the use of gels modified with chitosan in drug delivery [36–38]. If pH is lowered, the equilibrium ($-\text{NH}_2 + \text{H}^+ \rightarrow -\text{NH}_3^+$) is driven in the rightward direction, raising the internal osmotic pressure [39]. Thus chitosan/gelatin hybrid polymer networks are susceptible to swelling under acidic conditions, and the swelling degree at pH 5.0 was approximately 3 times as large as the degree at pH 7.0 [40]. Plasmid/chitosan complexes may thus serve as a type of hydrogel while in the endosomes; as pH in the endosome is lowered, the swelling degree of complexes increases, rupturing the endosomal membrane.

There is another factor that seems to limit the transfection activity of plasmid/chitosan complexes in regard to the endosome. To have transfection activity with plasmid/chitosan complexes, the complexes are released from endosomes. Next, the chitosan in the complexes are exchange with endosomal membranes consisting of anionic lipids in electric interaction. However, the release of DNA from DNA/chitosan complexes was observed at a concentration of SDS 10 times greater than that observed for DNA/PLL complexes. Thus plasmid/chitosan com-

plexes were not likely to exchange with endosomal membranes and to release the intact complexes from endosomes.

4.2.3. Nuclear import

Erbacher et al. utilized TEM to show that complexes were internalized by HeLa cells and were located inside small vesicles and large endosome-like compartments [14]. Nevertheless, nuclear import of complexes is still controversial; specifically the nuclear import of plasmid/chitosan complexes has never been fully examined. In this study, plasmid/chitosan complexes made with FITC-labeled plasmid and Texas Red-labeled chitosan were used to observe cellular localization. At 4 h post-incubation, the fluorescence of the two molecules had accumulated in the nucleus (Fig. 11B) and remained in the same localization at 24 h post-incubation (Fig. 11C). On the other hand, FITC-plasmid/(PLL or lipofectin) complexes did not display fluorescence images even at several hours post-incubation (data not shown).

These results illustrated that plasmid/chitosan complexes have high nuclear accumulation and resistance to digestion by cellular and nuclear enzymes. [^3H]Chitosan is conspicuously detectable within the plant nucleus only 15 min after applying chitosan [41]; however, there are no reports stating that plasmid/chitosan complexes were detected within animal nuclei.

5. Conclusion

There were several indications that the transfection activity of plasmid/chitosan complexes are dependent on cell line [14] and that the level of transfection is lower than that observed with complexes made from commercially available carriers [16,18]. However, in this work, several factors such as the molecular mass of chitosan, stoichiometry of complex, and serum concentration/pH of the transfection medium were shown to be important in enhancing the transfection efficiency. This unique characteristic of plasmid/chitosan complexes is the result of their ability to overcome the three steps of cellular transport; cell uptake, release from endosomes, and nuclear transport.

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