Chitosan-Based Vector/DNA Complexes for Gene Delivery: Biophysical Characteristics and Transfection Ability

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Purpose. Chitosan, a natural cationic polysaccharide, is a candidate non-viral vector for gene delivery. With the aim of developing this system, various biophysical characteristics of chitosan-condensed DNA complexes were measured, and transfections were performed.

Methods. Transmission electronic microscopy (TEM) visualizations, sedimentation experiments, dynamic light scattering (DLS), and zeta potential measurements were realized. Transfections were made by using the luciferase reporter gene.

Results. In defined conditions, plasmid DNA formulated with chitosan produced homogenous populations of complexes which were stable and had a diameter of approximately 50–100 nm. Discrete particles of nicely condensed DNA had a donut, rod, or even pretzel shape. Chitosan/DNA complexes efficiently transfected HeLa cells, independently of the presence of 10% serum, and did not require an added endosomolytic agent. In addition, gene expression gradually increased over time, from 24 to 96 hours, whereas in the same conditions the efficacy of polyethylenimine-mediated transfection dropped by two orders of magnitude. At 96 hours, chitosan was found to be 10 times more efficient than PEI. However, chitosan-mediated transfection depended on the cell type. This dependency is discussed here.

Conclusions. Chitosan presents some characteristics favorable for gene delivery, such as the ability to condense DNA and form small discrete particles in defined conditions.

KEY WORDS: gene therapy; gene transfer; cationic polymer; chitosan; polyethylenimine.

INTRODUCTION

Pharmacological approaches to gene therapy based on non-viral DNA vectors are in progress. Some future advances in this field require more information about structure, as well as colloidal and surface properties of vector/DNA condensed particles (1).

Cationic polymer-mediated transfection has become a well-established system. Macromolecules such as polylysine (2), cationic dendrimers (3), and polyethylenimine (4), interact electrostatically with plasmids to form toroidal structures (5), and are the most commonly used polycationic vectors. In general, synthetic vector/DNA complexes are efficient in gene transfer when the particles are positively charged (6), leading to cell binding and spontaneous endocytosis mediated by anionic cell surface proteoglycans (7–8).

Chitosan is an unacetylated derivative of a natural cationic polysaccharide consisting of repeated glucosamine units. This polycation is able to form polyelectrolyte complexes with DNA. Therefore, formation of DNA particles with chitosan (9) or chemically modified chitosan (10), such as N,N,N-trimethylchitosan, have been investigated for gene transfer. Successful chitosan-mediated transfection has been reported for a few cell types, such as HEK 293 cells (9). Nevertheless, no particular advantage of this system in comparison to other well-known polycationic vectors, i.e., polyethylenimine, dendrimer, or polylysine, has been clearly demonstrated.

We found chitosan presents some characteristics favorable for gene delivery, such as the ability to condense DNA and to form homogenous populations of complexes, smaller than 100 nm, in defined conditions. Moreover, chitosan could easily be chemically modified by coupling ligands, such as lactose, in order to target cells expressing a galactose-binding membrane lectin. With the aim of developing a chitosan-based vector system, various biophysical characteristics of chitosan-condensed DNA complexes were measured, and several transfection experiments mediated by chitosan and lactosylated chitosans were performed.

MATERIALS AND METHODS

Preparation of Lactosylated Chitosan Conjugates

Chitosan (average molecular weight of 70000, average degree of polymerization of 430) was obtained from Fluka. One gram of chitosan was solubilized in 50 ml of a 1% (v/v) acetic acid aqueous solution, giving a 0.123 M stock solution (expressed in nitrogen concentration). Chitosan was partially substituted with various amounts of lactosyl residues, by reductive amination in the presence of sodium cyanoborohydride. α-lactose (Sigma, Saint-Quentin-Fallavier, France) (7, 14 or 28 μmol) and sodium cyanoborohydride (Aldrich, Saint-Quentin-Fallavier, France) (35, 70 or 140 μmol, respectively) were added to chitosan (70 μmol, 0.6 ml stock solution) in 1 ml of 0.2 M sodium acetate and reacted for 96 h at 20°C. The glycosylated chitosan was precipitated by adding 10 volumes of 0.1 N NaOH and spun down (10 000 g for 10 min) 15 min later. The pellet was washed in 0.1 N NaOH, collected by centrifugation, and solubilized in 4 ml of 1% (v/v) acetic acid in water. The average number of lactosyl residues bound per chitosan molecule was determined by using the resorcinol sulfuric acid micromethod (11). Chitosan was substituted with 14, 43, and 86 lactosyl residues, giving yields of 33, 50, and 50%, respectively. The substitution level was also expressed as the percentage of lactosyl residue per amino group of chitosan and was 3.3, 10, and 20%, respectively. Lactosylation of PEI 25 kDa was obtained by reductive amination in the presence of sodium cyanoborohydride as previously described (12).

Electrophoretic Analysis

Complexes were prepared by adding (dropwise and under constant stirring) 0, 1, 2, 4, 6, 8, or 10 equivalents of polymer

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ABBREVIATIONS: Lact, lactosyl residue; PEI, polyethylenimine.

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nitrogen per DNA phosphate in 50 μl of 0.15 M NaCl to 2 μg (0.6 pmol) of pCMVLuc plasmid in 50 μl of 0.15 M NaCl. After incubation for 15 min at 20°C 20 μl of each sample were analyzed in a Tris-borate-EDTA (95 mM, 89 mM, and 2.5 mM, respectively) buffer (TBE) pH 8.6 by agarose gel electrophoresis (1 hour at 6V/cm; 1% agarose). DNA was stained in TBE containing ethidium bromide.

DNA Labeling Protocol and Sedimentation Experiments

pCMVLuc (25 ng) was radiolabeled by random priming with a Megaprime DNA labeling kit (Amersham, Les Ulis, France) and [α-^{32}P] dCTP (3000 Ci/mmol, Amersham) giving 2.4×10^9 cpm/μg DNA. The DNA probe was then added to 250 μg of unlabeled plasmid giving a final activity of 9.6×10^6 cpm/μg of DNA. To make complexes, 2 μg of DNA (pCMVLuc, kindly given by B. Demeneix, Muséum d'Histoire Naturelle, Paris, France) and various amounts of chitosan were diluted separately in 100 μl of 0.15 M NaCl, in prelubricated microcentrifuge tubes (Costar, Cambridge, MA). After 10 min, the vector was added to the DNA; the resulting solution was homogenized and left for 15 min at room temperature. The complexes were then centrifuged at various speed (17, 150, 420, 1620, 3770, 6700, 11340 g) for 10 min. Supernatants (180 μl) and pellets (20 μl) were counted separately in a scintillation counter, using the Cerenkov procedure. The percentage of sedimented DNA/polymer complexes was calculated according to the following formula:

$$[(CPM_{pellet} - 0.1 \times CPM_{supernatant})/(CPM_{pellet} + CPM_{supernatant})] \times 100$$

Measurement of Zeta Potential and Particle Size by Dynamic Light Scattering

Preparation of DNA/chitosan or lactosylated chitosan complexes: 20 μg DNA and various amounts of chitosan were diluted separately in 500 μl of 0.15 M NaCl in a microcentrifuge tube. After 10 min vector was added to DNA; the resulting solution was homogenized and left for 15 min at room temperature. Particle size was determined by light scattering using a Zetamaster (Malvern Instrument, Orsay, France) with the following specifications: sampling time, 90 s; 3 measurements per sample; medium viscosity, 1.014 cP; refractive index (RI) medium, 1.34; RI particle, 1.45; temperature, 20°C. Particle zeta potential was measured with the following specifications: sampling time, 30 s; 3 measurements per sample; viscosity, 1.014 cP; dielectric constant, 79; temperature, 20°C; beam mode F(Ka) = 1.50 (Smoluchowsky).

Cells and Cell Culture

HeLa human cervix epitheloid carcinoma cells were kindly given by Dr L. Monaco (Milan, Italy) and grown in Minimum Essential Medium (MEM) with Earle's salt (PolyLabo, Strasbourg, France). Hep G2 human hepatoma cells were purchased from ATCC (Rockville, MA, USA) and grown in Dubelcco's modified Eagle medium (DMEM, Gibco BRL, Paris, France). BNL CL.2 murine hepatocytes were kindly given by Dr. E. Wagner (Bender Co, Vienna, Austria) and grown in DMEM high glucose (4.5 g/l). All cell culture media were supplemented with 10% fetal calf serum (FCS, D. Dutcher, Brumath, France), 2 mM L-glutamine (Gibco BRL), 100 units/ml penicillin (Gibco BRL) and 100 μg/ml streptomycin (Gibco BRL). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Preparation of Complexes for Transfection

6 μg of DNA (pCMVLuc) and various amounts of chitosan were diluted separately in 150 μl of 0.150 M NaCl. After 10 min, vector was added to DNA; the resulting solution was homogenized and left for 10 min at room temperature.

Transfection Protocol

Twenty-four hours before transfection, $5–6 \times 10^4$ cells were seeded in 24 multi-well tissue culture plates. Prior to adherent cell transfection, cells were supplemented with 1 ml of fresh complete medium, with or without 100 μM chloroquine (Sigma). Then, 100 μl of polycation/DNA complexes were added in each well, as well as 100 μM chloroquine, when needed, and plates were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 2 h of incubation, the medium was replaced by 10% FCS supplemented medium, when chloroquine was present. After another 24 h of incubation, cells were lysed and the luciferase gene expression was quantified using a commercial kit (Promega) and a Biolumat LB 9500 luminometer (Berthold, Paris, France). Results were expressed as light units integrated over 10 s, per mg of cell protein using the BCA assay (Pierce, Paris, France).

For transfection in suspension, cells were detached by trypsin (2.5 μg/ml, Gibco BRL) and washed by centrifugation at 107 g. Cells were resuspended with serum free DMEM, diluted to 10^5 cells per ml, in 3 ml containing polycarbonate tubes. Complexes (300 μl) were added to the tubes (as well as 100 μM chloroquine when needed). The cells were incubated at 37°C for one hour and kept suspended by gentle stirring every ten minutes. Cells were gently homogenized several times in tubes, then seeded into plates (1 ml/well) and medium was supplemented with 10% FCS (when chloroquine was used, the medium was first replaced with complete fresh DMEM).

Transmission Electron Microscopy (TEM) of Chitosan/DNA Complexes

A carbon film was prepared on freshly cleaved mica, by evaporation of a carbon rod under vacuum. The flotation technique was then used to cover electron microscope copper/rhodium grids (300 Mesh, Touzard et Matignon, Courtaboeuf, France) with carbon film. After drying overnight, the grids were kept on blotting paper placed in a Petri dish. Just prior to adding the samples, the grids were glow discharged (110 V, 1 mbar, 25 μA, 25 s). Chitosan/DNA complexes were prepared according to the aforementioned procedure. A drop (5) μl of the solution was then added to the grid for one minute. Complexes were negatively stained with 30 μl of an aqueous uranyl acetate solution (1%, w/w) for 20 s and then the excess liquid was removed with blotting paper. Observations were done at 80 kV on a Philips EM 410 transmission electron microscope (Amsterdam, The Netherlands). Chitosan/DNA complexes (10 μg DNA; 100 μl) were added to each well (24 multi-well tissue culture plate), containing 1×10^5 HeLa or BNL CL.2 cells cultured in 1 ml serum-supplemented medium, and the plate

was incubated at 37°C in a 5% CO_2 humidified atmosphere. After 24 h of incubation, the medium was removed, and the cells were washed with 10 mM phosphate buffered saline. Then the cells were fixed *in situ* and, ultrathin (50-nm) sections were prepared as previously described (8). Sections were stained with 5% uranyl acetate and 0.15 M lead citrate and examined on a Philips EM 410 transmission electron microscope.

RESULTS

Chitosan/DNA Complexes: Biophysical Results

Chitosan is a polymer made of repeated glucosamine units with 430 as an average degree of polymerization. This polycation has a high density of amino groups along its backbone. We can suppose that not all of the amino groups are protonated at physiological pH. We can therefore express the N/P ratio as the number polymer nitrogen per DNA phosphate and not as a charge ratio. Chitosan requires aqueous acetic acid (1%, v/v) to be soluble; it precipitates out above pH 7. When complete DNA complexation is obtained, electrophoresis does not induce DNA migration. Chitosan is able to complex nucleic acids and full DNA retardation is obtained for a N/P of or greater than 2 (data not shown).

In order to obtain information about the formation of chitosan/DNA complexes, as well as their apparent density, sedimentation experiments were performed with radiolabeled DNA (Fig. 1). The sedimentation profile of DNA/chitosan complexes, vs. the centrifugation speed, depended on the N/P ratio. At N/P = 0.5, the DNA was not fully condensed and not spun down. At N/P = 1 and 2, the amount of sedimented DNA was increasing proportional to the centrifugation speed, meaning there was a distribution of particle density and/or particle size. Particles were probably heterogeneous in size. At N/P = 3 and 5, less DNA sedimented; the particles were smaller and/or had

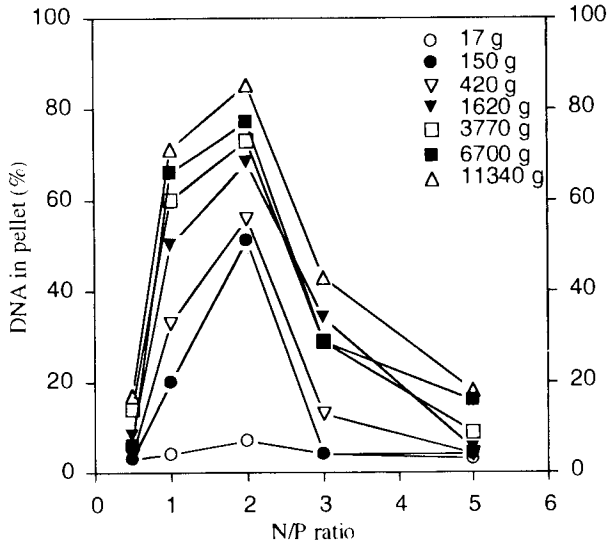

Fig. 1. Sedimentation profile of chitosan/DNA complexes related to the N/P ratio. Ten minutes after the formation of complexes with radiolabeled DNA, complexes were centrifuged at various speed, for 10 min. Radioactivity present in the supernatant and in the pellet was measured and the results were expressed as percentage of DNA found in the pellet.

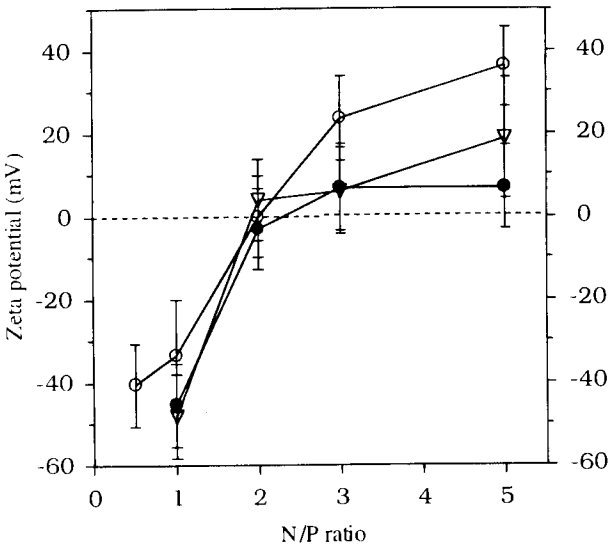

Fig. 2. Zeta potential measurements of chitosan/DNA (○), chitosan-Lact 10%/DNA (●) and chitosan-Lact 20%/DNA (▽). Measurements were performed with 20 μg of DNA in 1 ml of 0.15 M NaCl.

a lower density. After 5 h of incubation, particles made at N/P = 5 were stable and the sedimentation profile was left unchanged (data not shown). Sedimentation profiles obtained with lactosylated chitosan were similar to those obtained with the unmodified polymer (data not shown) and showed the same dependency on the N/P ratio.

The zeta potential of DNA/chitosan complexes was measured (Fig. 2). The particles had a negative surface charge when the complexes were made with chitosan at N/P below 2. At N/P = 2, the particles were neutral and became positively charged at N/P above 2, up to +35 mV at N/P = 5. Chemical grafting of lactosyl residues to chitosan decreased the zeta potential of complexes made with a N/P ratio above 2 in comparison with the unmodified polymer. This result is in agreement with the expected effect of amino group substitution on zeta potential.

The size range of particles made with chitosan was determined by dynamic light scattering (Fig. 3A). A relatively homogenous size distribution was observed within a range of 80–500 nm for a N/P ratio varying from 0.5 to 10; in class, a major population of complexes showed a size near 100–200 nm. However, in conditions where zeta potential was close to 0 mV (N/P = 2), a different size range was obtained (1 to 5 μm). Complexes prepared with chitosan-Lact 3% had essentially the same size range than those prepared with chitosan (data not shown). The particles made with chitosan-Lact 10% (Fig. 3B) and 20% (data not shown) showed a size range below 1 μm at N/P = 2 and presented the same size distribution described above for the other ratio, but with more heterogeneous sizes, as shown by the presence of two populations. In general, increasing the N/P ratio decreased the size range, except for the conditions where neutral particles were formed.

Transmission electron microscopy (TEM) observation of chitosan/DNA complexes was performed. At N/P = 0.5, DNA complexation and condensation by chitosan did occur to some extent, but uncondensed DNA loops were still clearly visible (Fig. 4). The complexes had donut and rod shapes, the latter resulting probably from a donut collapse. The size of complexes

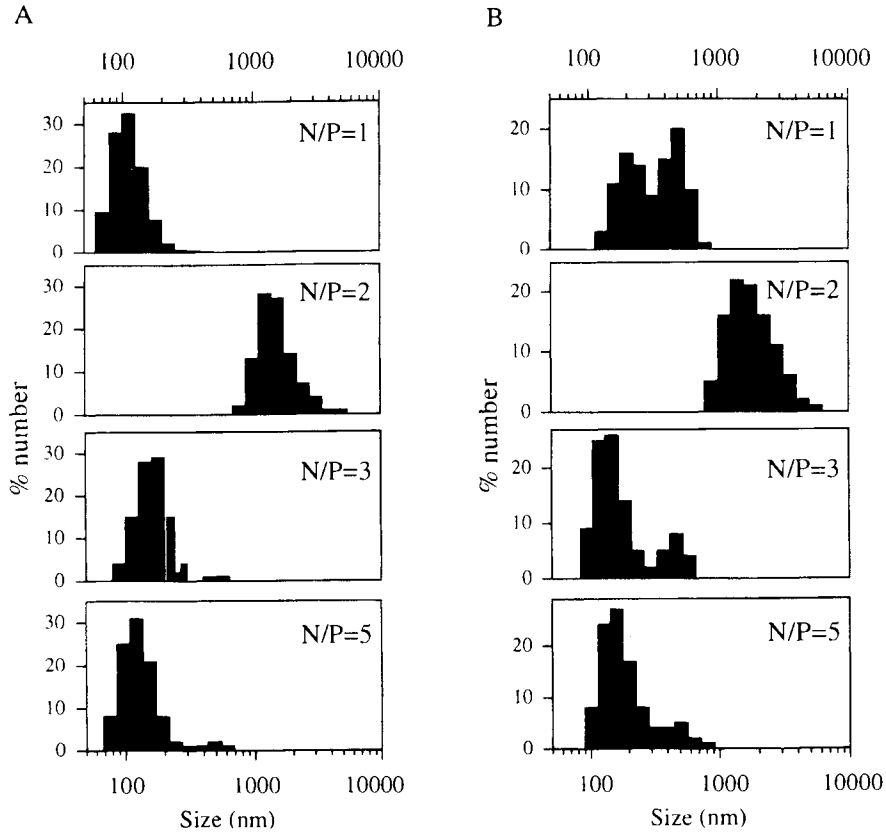

Fig. 3. Size range determination by laser light scattering of chitosan/DNA (A) and chitosan-Lact 10%/ DNA (B) complexes. Measurements were done with 20 μg of DNA in 1ml of 0.15M NaCl.

was ranging from 50 nm to 100 nm, for donuts and rods respectively. The low input of cationic polymer explained the presence of many DNA loops. Moreover, the shearing force due to the electric potential may be able to disrupt complexes that were only partially condensed, as evidenced by the agarose gel electrophoretic analysis. At N/P = 1 and 3 equivalents, two populations of complexes were seen. The major population was made of nicely condensed DNA plasmids having mostly a rod shape and sometimes looking like donuts. The width and length of these rods were 20 ± 2 nm and 50 to 100 nm, respectively. Their width indicates that several DNA double helixes were aligned and stuck together. The minor population is composed of large aggregates of 500 nm to 1 μm. These results are in agreement with those obtained by light scattering. TEM showed a majority of large aggregates at N/P = 2 (data not shown). Zeta potential measurements clearly showed that complexes are neutral at exactly N/P = 2, thus having the tendency to aggregate. Above N/P = 3, complexes were homogenous in size (50–100 nm) and had a donut, rod, or even pretzel shape (Fig. 4.) There was a two-fold discrepancy in size between light scattering and TEM measurements, probably reflecting the assumption of spherical particles, in the former case.

TEM observation of complexes made with lactosylated chitosan (data not shown) showed no differences in shape as compared to chitosan itself, except for the presence of large aggregates. Indeed, at a neutral charge ratio, the proportion of aggregated complexes was slightly increased. These results are in agreement with light scattering measurements. Both TEM, and dynamic light scattering indicated that complexes containing lactosylated chitosan might be even bigger than chitosan/DNA complexes.

Transfection Mediated by Chitosan and Lactosylated Chitosan Vectors

Transfection of HeLa cells using chitosan/DNA complexes was made in the presence of 10% serum, and luciferase activities were monitored over a period of 24 to 96 h upon the transfection step. In HeLa cells, the luciferase activity was clearly depending on the post-transfection time, with maximal gene expression reached at 72–96 h (Fig. 5). Transfections made with PEI gave more transient expression, with maximal luciferase activity at 24 h, followed by a drop to a low level at 96 h. The results showed that chitosan/DNA complexes made with a N/P ratio of 3 gave the highest transfection activity. 100 μM chloroquine did not improve transfection efficiency of chitosan/DNA complexes in HeLa cells (a ten-fold decrease in luciferase activity was even obtained). In contrast, chitosan was inefficient at transfecting BNL CL.2 or Hep G2 cells, whether in the presence or in the absence of chloroquine, and in serum containing medium (data not shown). Luciferase activities obtained 24, 48 or 72 h after transfection were below 10^5 RLU/mg of protein, although a ten-fold increase was shown in the case of Hep G2 cells with chloroquine. Under the same conditions, PEI-mediated transfection gave luciferase activity up to 10^8 RLU/mg of protein in BNL CL.2, and Hep G2 cells (data not shown).

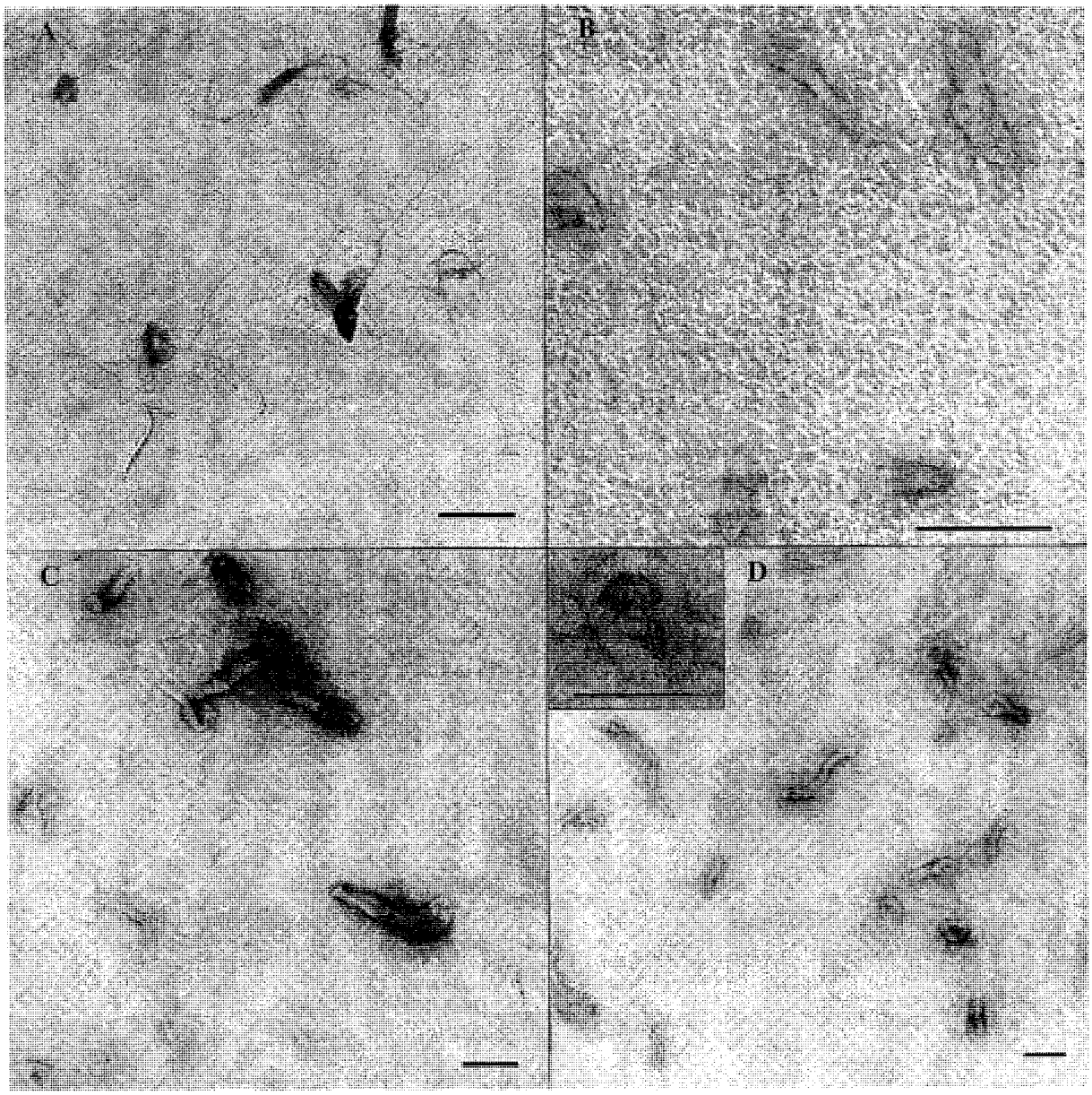

Fig. 4. Transmission electron micrographs of chitosan/DNA complexes at N/P = 0.5 (top left), 1 (top right), 3 (bottom left), and 5 (bottom right). Bars are 100 nm.

In the case of HeLa cells, chitosan mediated transfection more efficiently in the presence of 10% serum than in its absence (Fig. 6) and with no noticeable cellular toxicity. In contrast, PEI-mediated transfection was decreased by the presence of 10% serum and induced toxicity at high N/P ratios in the absence of serum.

In order to increase the transfection efficiency and to selectively deliver a gene, experiments were performed using lactosylated chitosan as a vector targeted for cells expressing a galactose-specific membrane lectin (BNL CL.2 and HepG2 cells). It was shown that lactosylated chitosans, substituted with 3, 10 or 20% lactose residues, were poorly efficient to transfect BNL CL2 or Hep G2 cells with or without 100 μM of chloroquine (luciferase activities below 10^5 RLU/mg of protein, data not shown). In comparison, lactosylated PEI worked a thousand-fold better (RLU/mg of protein up to 10^8) in the same experiment, indicating that the inefficiency of lactosylated chitosan was not due to a lack of receptor.

Considering the complexes formed by chitosan were small and did not sediment at 3 and 5 eq, they presumably stayed in suspension, thus resulting into inefficient contact with adherent cells, transfections were therefore performed with BNL CL.2 cells in suspension (data not shown). No improvement was obtained for chitosan-mediated transfection whereas lactosylated PEI remained efficient (RLU/mg of protein up to 10^7).

Cell Binding and Entry of Chitosan/DNA Complexes

Cell binding and entry was monitored on HeLa and BNL CL.2 cells. TEM observations showed that chitosan/DNA complexes (N/P = 5) were taken up by spontaneous endocytosis, as shown by plasma membrane invaginations of HeLa cells

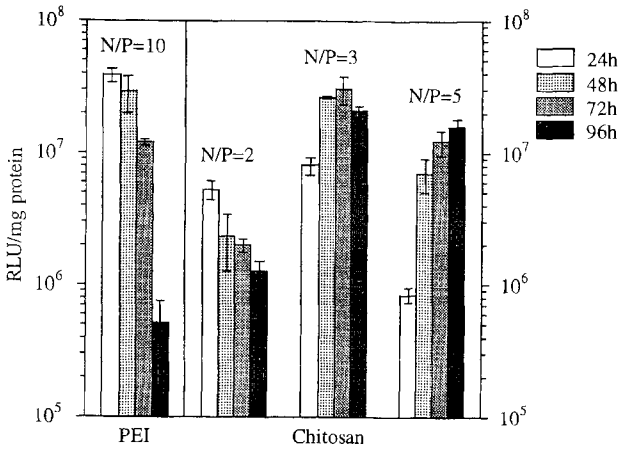

Fig. 5. Time course of luciferase gene expression. HeLa cells were transfected in the presence of 10% serum with pCMVLuc plasmid (2 μg/well) complexed with PEI or with chitosan. Gene expression was determined by luciferase assay and expressed as RLU/mg of protein (luciferase activity per mg of protein in cell lysates).

surrounding the contours of particles (Fig. 7). The complexes were internalized by HeLa cells, and were located inside small vesicles and large endosome-like compartments. In contrast, chitosan or lactosylated derivatives/DNA complexes were hardly seen associated to the plasma membrane or internalized by BNL CL.2 cells (data not shown).

DISCUSSION

The development of optimized non-viral gene transfer vectors requires vector/DNA particles to meet several characteristics. The present biophysical study shows that chitosan perfectly fulfils some of them: chitosan/DNA particles are small, with a size range below than or equal to 100 nm. These particles are homogenous and stable over time (at least several hours).

When chitosan mediates efficient *in vitro* gene transfer, the transfecting particles, at N/P = 3 and 5, are small (50–100 nm, TEM observations) and positively charged (+25 and +37 mV, respectively), underlying the commonly admitted ionic cell membrane transfection process (6–8). Moreover, no cytotoxicity has been noticed even at high N/P ratios. This extremely low toxicity of chitosan on mammalian cells in culture has been previously observed (10). Chitosan showed significantly lower toxicity than poly-L-lysine (9), and in our hands, to PEI. In addition, chitosan-mediated transfection is resistant to 10% serum inhibition, and even so, an increased efficacy is obtained. This result suggests an *in vivo* potentiality of this vector. Demonstration of a successful gene transfer after direct intracranial tumor injection, mediated by the APL PolyCat 57 vector, a synthetic polyamino polymer with a glucose backbone, probably similar to chitosan, has been recently reported (13). In contrast to chitosan, PEI 25 kDa-mediated transfection was reduced by an order of magnitude in the presence of serum, this independently of the cell line. Even so, PEI remains one of the most active polymers for delivering genes.

What is remarkable is the late luciferase gene expression (72–96 h) obtained on HeLa cells, which seems to be characteristic of the chitosan vector. In comparison, a very transient gene expression is seen with the PEI-based system. This delay may be attributed to a slow release of the plasmid, to a slow endosomal and/or nuclear passage, or to a slow cellular uptake. This question remains to be addressed.

Chitosan can be chemically modified with ligands, such as lactose. As lactosylated chitosans bear a galactose residue, they have the potency to target cells expressing galactose membrane lectins, such as hepatocytes. Lactosylated-chitosan/DNA complexes still have a size compatible with either vascular extravasation through fenestrated endothelium or receptor-mediated endocytosis, although they are larger than their unmodified counterparts. This may reflect two phenomena: i) lactosylation of chitosan reduces the surface charge of the complexes, as shown by zeta potential measurements. As the surface potential is reduced, the tendency toward aggregation is raised. ii) The affinity of chitosan for DNA is presumably reduced when the extent of lactosylation is increased, together with steric hindrance enhancement. In our interpretation, the volume occupied by a single polymer molecule is probably outstretched, thus giving the tendency to cross-link several complexes and, to finally end with aggregation.

However, *in vitro* chitosan-mediated transfection depends on the cell type. HeLa cells were efficiently transfected by this system even in the presence of 10% serum but neither chitosan nor lactosylated chitosans have been able to efficiently transfect HepG2 and BNL CL.2 cells (luciferase activities $< 10^6$ RLU/mg of protein), both well-known cell lines and commonly used in targeting and gene transfer experiments. Chloroquine, which is known to enhance poly-L-lysine mediated transfection (14–15), has no enhancing effect when used with chitosan, and even decreased its efficacy on HeLa cells. Several hypotheses can explain the low transfection activity observed for BNL CL.2 and HepG2 cells:

(i) Complexes may not reach the cell surface and/or may not stick to the plasma membrane. Others (16) have shown that particles having a size of 100 nm or below have a fast, brownian

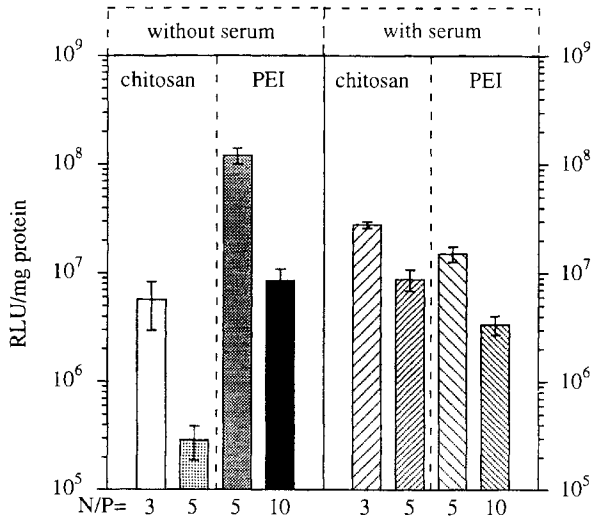

Fig. 6. Effect of serum on gene transfer efficiency. HeLa cells were incubated in the presence or in the absence of 10% serum with pCMVLuc plasmid (2 μg/well) complexed with PEI or with chitosan. After 2 h of incubation, 10% serum was added to the wells. Cells were lysed 72h later for luciferase activity quantitation. Gene expression was expressed as RLU/mg of protein.

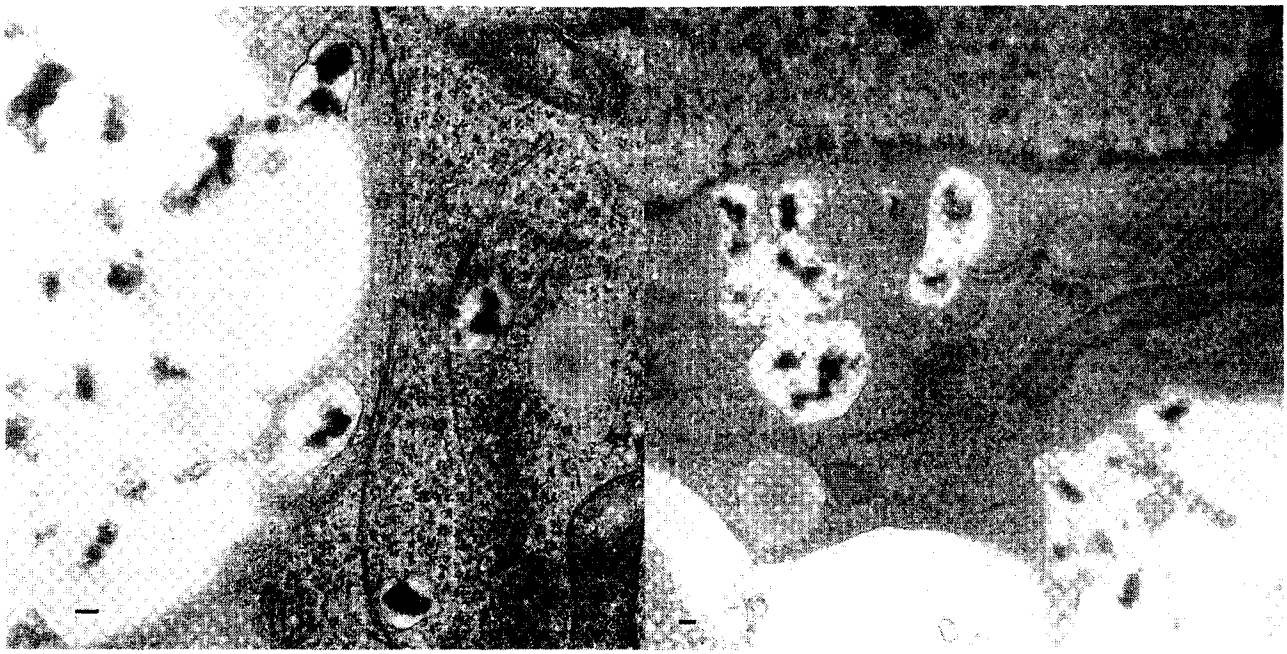

Fig. 7. TEM micrographs of HeLa cells 24h after transfection. Chitosan/DNA complexes (N/P = 5) appear as characteristic black condensed structures. Entry of complexes into cells, as shown by plasma membrane invaginations and endocytosis into small vesicles (left). Sequestration of complexes into large endosome-like compartments (right). For details, see Materials and Methods; bars are 100 nm.

motion which prevents them from reaching the cells. Moreover, we were unable to spin down chitosan/DNA complexes having the same size range. To answer this issue, we have performed transfections on suspended cells, these conditions allowing them to meet the particles. At N/P = 5, cell aggregation was clearly visible, implying that complexes not only reached the plasma membrane, but also sticked to it. Still, we can not rule out the possibility that particles may not reach the surface of adherent cells.

(ii) Complexes may not be endocytosed or may be sequestered in endocytotic vesicles, ending by DNA degradation in lysosomes. TEM observations showed that chitosan/DNA complexes were internalized into HeLa cells, and were located inside small vesicles and endosome-like compartments. In contrast, chitosan or lactosylated derivatives/DNA complexes were hardly seen associated to the plasma membrane or internalized by BNL CL.2 cells. However, data from flow cytometry experiments have shown that fluorescein-labeled chitosan/DNA complexes were internalized by BNL CL.2 cells but with a 5-fold lower extent than PEI/DNA complexes. After a post-incubation of BNL CL.2 cells incubated with fluorescein-labeled chitosan/DNA complexes with monensin, a drug allowing the neutralization of the acidic compartments and relieving fluorescein from acidic quenching, a 2-fold enhancement of the cell associated fluorescence was shown (P. Erbacher, personal results). This result indicated that chitosan/DNA complexes were internalized through the acidic compartments pathway (endosomes, lysosomes). Taken together, TEM observations and flow cytometry experiments show that only few chitosan/DNA complexes are taken up in acidic compartments by BNL CL.2 cells, leading to low transfection levels. Fully protonated polymers, such as poly-L-lysine, require an endosomolytic agent (17–20) to escape from degradation and to reach the cytosol. Partially protonated polymers, such as PEI, are doing this by themselves ('proton sponge' effect) (4,21). Chitosan does not appear to be fully protonated at physiological pH, since zeta potential measurements indicate that neutrality is reached at N/P = 2. However, its efficiency in HeLa cells or its low efficiency in BNL CL.2 and Hep G2 cells prevents us from concluding it has the ability to buffer the endosomes. We found that chloroquine did not improve chitosan-mediated transfection levels. In Hep G2 cells, chloroquine is known to raise endosomal pH up to 8 (20). At this pH value, chitosan is fairly insoluble. Precipitation of the complexes may then occur, preventing transmembrane passage and DNA release.

(iii) Complexes may not reach the cell nucleus and DNA may not be released from complexes, either before entering the nucleus, or at the transcription stage. None of these later issues has yet been clarified.

In conclusion, even if in our hands chitosan mediated transfection system was found to be dependent on the cell line, this system presented some characteristics required for *in vivo* gene delivery, such as the ability to condense DNA, and to form small and stable complexes. The resistance to serum inhibition in gene transfer and the low cytotoxicity of this system are also positive characteristics.

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