N/P Ratio Significantly Influences the Transfection Efficiency and Cytotoxicity of a Polyethylenimine/Chitosan/DNA Complex

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For designing a complex vector that has the advantages of both polyethylenimine (PEI) and chitosan for gene delivery, a PEI/chitosan/DNA complex was constructed at various N/P ratios (the ratios of moles of the amine groups of cationic polymers to those of the phosphate ones of DNA) and both the cytotoxicity and the transfection efficiency of the vector were evaluated. The results demonstrated that the chitosan/DNA binding degree was depended on the N/P ratio. The mean size of the complex vector was between 100 nm and 150 nm. Compared with PEI/DNA, the complex vector (PEI/chitosan/DNA with chitosan/DNA N/P=4, PEI/DNA N/P=10) appeared to have low cytotoxicity, which maintained the cell survival rate at greater than 80%, and showed higher transfection efficiency of nearly 1000 fold compared with that using chitosan/DNA alone. Furthermore, the expression efficiency of the complex vector carrying enhanced green fluorescent protein was not inhibited in the presence of serum in both HeLa cells and A549 cells. The PEI/chitosan complex may be a promising gene carrier that has high transfection efficiency as well as low cytotoxicity.

Key words chitosan; polyethylenimine; gene delivery; cytotoxicity; transfection efficiency

Gene therapy can be defined as the transfer of genetic materials to specific cells in order to exert a therapeutic effect. It is a promising approach to the treatment of a wide range of diseases by compensating for defective genes or producing beneficial proteins.1) Gene vectors have much important roles in gene therapy. Recently, nonviral vectors have been increasingly proposed as safer alternatives to viral vectors because of their potential advantages such as ease of synthesis, cell/tissue targeting, low immune response, and unrestricted plasmid size.2) Among nonviral systems, cationic polymers have attracted increasing attention because they can easily form self-assembling polyelectrolyte complexes between plasmid DNA and cationic polymers, and mediate transfection via condensing DNA into nanoparticles, protecting DNA from enzymatic degradation, and facilitating the cell uptake and endolysosomal escape.3) Among cationic polymers, polyethylenimine (PEI) and chitosan are widely used as nonviral vectors for gene delivery. They have the same ability to enter cells by binding to proteoglycans on cell surfaces and undergoing endocytosis.4–6) However, after uptake, they have quite different transfection efficiency. PEI is considered to be the most effective cationic polymer for gene delivery.7) Its high proton-buffering capacity results in rapid osmolysis of the endosomes, and the PEI–DNA complexes escape into the cytosol and are subsequently transported into the nucleus.8) However, PEI is also associated with dose-dependent toxicity, especially at high molecular weight, which probably explains why it has not yet been used in human studies.9) On the other hand, chitosan does not have high proton-buffering capacity, making it unable to escape from the endosomes in time. Chitosan is degraded in the endosome and the material is then released into the cytoplasm after hyperosmotic rupture of the cell membrane caused by the accumulation of degradation products. The material is then transported to the nucleus.5) Therefore chitosan is generally considered less effective in gene delivery systems than PEI in vitro and in vivo. But it is well known as a biocompatible, biodegradable, and relatively non toxic material with high cationic potential.10) Therefore to design a complex that has both the advantages of PEI and chitosan, in other words, both high transfection efficiency and low toxicity, would be promising.

Several groups have reported the pilot research in this area. Kim et al.11) demonstrated that when PEI (10k) was combined with a water-soluble chitosan (WSC)/DNA complex, the transfection efficiency was enhanced via the proton sponge effect, and cell survival was not markedly decreased. Another group designed a chitosan-graft-PEI (CHI-g-PEI) copolymer using an imine reaction between periodateoxidized chitosan and low molecular weight PEI and obtained good results.12) However, the influence of the N/P ratio (the ratios of moles of the amine groups of cationic polymers to those of the phosphate ones of DNA), which might be an important factor affecting the cytotoxicity and transfection efficiency remains to be unclear.11) Moreover, the PEI which was used in the previous studies had small molecular weight and was demonstrated to have low cytotoxicity alone. Generally, both the gene transfer ability and cytotoxicity are increased with the increasing of molecular weight of PEI.13) Therefore there is no evidence that combination with chitosan can reduce the toxicity of the complex.

In this work, we investigated the influence of the N/P ratio on the characteristics of the PEI/chitosan/DNA complex in which 25K PEI was used. Gene transfection efficiency with or without serum and the cytotoxicity of the complex vector with the optimal N/P ratio were evaluated.

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MATERIALS AND METHODS

Materials Chitosan (MW 100 kDa) was purchased from Yuhuan Biochemistry Co., Ltd. (Yuhuan, China). PEI (MW 25 kDa) was purchased from Aldrich Chemical Co., Ltd. (Milwaukee, WI, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM) and trypsin were obtained from Gibco BRL (Gaithersberg, MD, U.S.A.). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co., Ltd. (Hangzhou, China). All other chemicals were of analytical grade.

Cell Lines Human cervical adenocarcinoma cell line (HeLa) cells and A549 human lung carcinoma cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in DMEM medium containing 10% FBS at 37 °C in a humidified 5% CO₂ atmosphere.

Plasmid DNA Plasmid DNA (pGL3 and pEGFP-N1) was kindly provided by the Institute of Infectious Diseases, Zhejiang University (Hangzhou, China). The plasmid DNA was amplified as unified Escherichia coli DH5α and purified using an AxyPrep Maxi Plasmid Kit (Axygene Biotechnology Limited, Hangzhou, China). The purity of the plasmids consisting of supercoiled and open circular forms was determined using electrophoresis on 1.0% agarose gel, and the concentration of DNA was determined by measuring UV absorbance at 260 nm and 280 nm. DNA from protamine was used for the measurement of particle size.

Preparation of Chitosan/DNA Complex N/P ratios of chitosan/DNA complexes were the ratios of moles of the amine groups of chitosan to those of the phosphate ones of DNA. For the calculation of N/P ratios, 330 Da was used as the average mass per charge for DNA. The molecular weight of chitosan is 100 kDa. Chitosan was dissolved in 0.5% HAc solution and stirred overnight. Then it was extruded through 0.8 μm filter. Chitosan solution was added to the DNA solution at different N/P ratios (0.5, 1, 2, 4, 6) and incubated at room temperature for 30 min.

Preparation of PEI/Chitosan/DNA Complex PEI was dissolved in distilled water. Chitosan/DNA complexes with different N/P ratios were mixed with the PEI solution at N/P = 1, 5 and 10 (PEI/DNA). The mixtures were incubated at room temperature for 30 min.

Gel Retardation Assay Different amounts of chitosan (N/P ratio from 0.5 to 6) were combined with DNA (0.5 μg of pGL3), and the effect of chitosan on the condensation of DNA was investigated using electrophoresis on 1% agarose gel with Tris–acetate (TAE) running buffer at 100 V for 30 min. DNA was visualized with ethidium bromide (0.2 μg/ml).

Measurement of Particle Sizes and Zeta Potential Particle sizes and zeta potentials of the chitosan/DNA and PEI/chitosan/DNA complexes with different N/P ratios were determined using laser diffraction spectrometry (Malvern Zetasizer 3000HS, Malvern, U.K.). The volume of the samples was 1 ml containing a final DNA concentration of 50 μg/ml.

MTT Assay The DNA solution, chitosan solution and PEI solution were filtered through a 0.22 μm aseptic filter membrane before mixing. Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-

mide (MTT) assay method. HeLa cells were seeded at a density of 5×10⁴ cells/well in 100 μl of growth medium in 96-well plate and incubated for 24 h. Chitosan/DNA complex was prepared by mixing 0.2 μg of control pGL3 with appropriate amounts of chitosan at N/P ratios between 0.5 and 6 and incubated for 30 min at room temperature. Different amounts of PEI (N/P = 5, 10, weight per 0.2 μg of DNA) were added to complex-dispersed solution, and the final volume was adjusted to 100 μl with fresh serum-free DMEM. Cells were incubated with vectors at 37 °C in a humidified 5% CO₂-containing atmosphere for 6 h. Then fresh DMEM with 10% fetal bovine serum was added. After incubation at 37 °C in a humidified 5% CO₂-containing atmosphere for 42 h, 20 μl of MTT solution (5 mg/ml) was added to each well and measured according to the manufacturer’s instructions using the following equation:

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\text{cell viability} \% = \frac{OD595\text{(sample)}}{OD595\text{(control)}} \times 100
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Where OD595(sample) represents a measurement from a well treated with vectors and OD595(control) represents a measurement from a well treated with PBS buffer alone. All experiments were carried out triplicate to ascertain reproducibility.

Assay of Luciferase Activity For evaluating transfection efficiency, HeLa cells were seeded in 24-well plates at a density of 1×10⁴ cells/well and cultured for 18 h before transfection. A chitosan/DNA complex was prepared by mixing 1 μg of control pGL3 with appropriate amounts of chitosan at N/P ratios between 0.5 and 6 and incubated for 30 min at room temperature. Different amounts of PEI (N/P = 5, 10, weight per 1 μg of DNA) were added to complex-dispersed solution (20 μl), and the final volume was adjusted to 500 μl with serum-free DMEM medium. All the solutions were filtered through a 0.22 μm aseptic filter before mixing, and all were mixed at the same volume. The vector/DNA complexes were added to 24-well plates and incubated for 6 h at 37 °C under a 5% CO₂ atmosphere. Then 1 ml of serum-free medium was replaced with fresh medium containing serum and incubated for 24 h at 37 °C under a 5% CO₂ atmosphere. The luciferase assay was carried out according to the manufacturer’s instructions (Promega, Madison, WI, U.S.A.). Light units (LUs) due to luciferase activity were measured with a chemiluminometer (Autolumat LB953, EG&G Detrhold, Germany). All the experiments were carried out triplicate to ascertain reproducibility.

Green Fluorescent Protein (GFP) Transfection The procedure for green fluorescent protein was almost the same as for the luciferase assay, except that the plasmid was pEGFP-N1 and a new array in which medium containing 10% serum as added when incubated with the vector/DNA complex in the phase of transfection. After 48 h, arrays were observed with a fluorescence inverted microscope. Both HeLa cells and A549 cells were used.

Statistical Analysis Data are expressed as means with standard deviations. Statistical analysis was performed using the unpaired Student’s t-test. Differences were considered statistically significant at a p value of less than 0.05.

RESULTS

Gel Retardation Assay The results of the gel retarda-
tion assay indicated that when the N/P ratio of chitosan/DNA was greater than 4, chitosan and DNA bound tightly and completely. In contrast, there a free DNA band appeared when N/P<4, suggesting that binding was not complete (Fig. 1). When N/P was equal to 2, the band was fainter than the 1/10 a mount of free DNA added (Fig. 1, lane 8), indicating that escaped DNA was less than 10% of the DNA added. The gel retardation assay suggested that chitosan has the ability to compact DNA and the N/P ratio affects the binding degree.

**Particle Sizes and Zeta Potentials of the Complex**

It can be seen in Fig. 2 that with the increase in the N/P ratio, the particle sizes first increased and then decreased. When the N/P of chitosan and DNA was equal to 1, particles had the largest size. The size became smaller with the addition of cation. After the formation of the PEI/chitosan/DNA complex, the results showed that when the N/P ratio of chitosan and DNA was less than 1, PEI was markedly compressed. However, the compression effect was not conspicuous when the N/P ratio increased further. The zeta potentials of complexes are listed in Table 1. The results showed that the potential was negative at the N/P ratio of chitosan/DNA of 0.5, and then increased to a positive charge with the addition of chitosan. The PEI/DNA complex induced a potential of about 30 mV. However, after incubation with chitosan, the potential of the PEI/chitosan/DNA complex was decreased.

**Complexation Reduced the Cytotoxicity of PEI**

After incubation with HeLa cells for 48 h, chitosan/DNA with all N/P ratios showed low cytotoxicity. Cell survival rates were greater than 85% (Fig. 3). On the other hand, DNA combined with 25 kDa PEI appeared to have significant cytotoxicity, and cell survival rates were less than 60%. However, it is interesting that when the N/P ratio of chitosan and DNA was greater than 4, the addition of PEI did not induce cytotoxicity and cell survival was similar to that when using chitosan alone. The cell survival rate with the PEI/chitosan/DNA at N/P ratio of 5 (PEI to DNA) was slightly higher than that at N/P=10, but there was no significant difference, indicating that not the N/P of PEI/DNA but the N/P of chitosan/DNA is the main factor affecting cytotoxicity.

**Complexation Enhanced Gene Expression**

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**Fig. 1. Gel Retardation Assay of Chitosan/DNA Nanoparticles**

Lane 1: marker; 2: N/P=0.25; 3: N/P=0.5; 4: N/P=1; 5: N/P=2; 6: N/P=4; 7: N/P=8; 8: 0.05 µg DNA; 9: 0.5 µg DNA. Chitosan as combined with DNA at different N/P ratios. The effects of chitosan on the condensation of DNA were investigated using electrophoresis on 1% agarose gel with Tris–acetate (TAE) running buffer at 100 V for 30 min. DNA was visualized with ethidium bromide (0.2 µg/ml).

**Fig. 2. Particle Size of Complexes Composed of Chitosan/DNA, PEI/DNA, or PEI/Chitosan/DNA**

**Fig. 3. Cytotoxicity of Chitosan/DNA, PEI/DNA, and PEI/Chitosan/DNA Complexes**

HeLa cells were seeded at a density of 5×10⁴ cells/well in 96-well plates. Different complexes were added and cells were incubated with vectors for 6 h. After the medium was changed and incubation for another 48 h, 20 µl of MTT solution (5 mg/ml) was added and manipulated according to the manufacturer’s instructions.

**Fig. 4. Effects of PEI on the Transfection Efficiency of Complex Vector in HeLa Cells**

Luciferase activity of the chitosan/DNA complex combined with PEI was compared with that of the PEI/DNA complex and chitosan/DNA complex. The chitosan/DNA complex was prepared at N/P=0.5, 1, 2, 4, 6 and the PEI/DNA complex was prepared with the same amount used for the combination with the chitosan/DNA complex. Transfection was performed at a dose of 1 µg of pGL3 plasmid for all groups and the amount of PEI used was N/P=5 or 10. Cells were incubated in the transfection solution for 6 h and then changed to growth medium for 48 h before the luciferase assay. These data are expressed as relative light units (RLU) of luciferase activities per mg of total cell protein (mean±S.D., n=3).
the transfection efficiency of PEI/DNA was higher than that of the PEI/chitosan/DNA complex, when chitosan increased, the transfection efficiency decreased, and it can be concluded that they were in the same order of magnitude (Fig. 4). However, the complex vector with the optimal N/P ratio (chitosan/DNA N/P = 4, PEI/DNA N/P = 10) significantly enhanced the transfection efficiency to almost 1000-fold higher than that induced using chitosan alone (Fig. 4).

**Gene Expression of Complex Was Not Inhibited by the Presence of Serum** The effect of PEI on transfection of the chitosan complex was observed using fluorescence microscopy. To evaluate the effects of serum on transfection, the transfection efficiency of PEI/chitosan/DNA in the absence or presence of serum was investigated in both HeLa and A549 cells. As shown in Fig. 5, the transfection efficiency of PEI/chitosan/DNA was not inhibited by the presence of serum.

**DISCUSSION**

Transfection efficiency and cytotoxicity are most important standards in gene delivery systems. It is known that chitosan is a material with low cytotoxicity when used as a gene carrier. However, the low transfection efficiency limits its application. One of the primary causes of the poor gene delivery of chitosan is the inefficient release from endosomes into the cytoplasm. Therefore, to enhance the gene transfer ability of chitosan, PEI known to be an efficient nonviral gene carrier with high pH-buffering capacity believed to enhance escape from the endosomal compartment, was combined with chitosan in this study to construct the complex vector.

Recently, it has been reported that low molecular-weight PEI when combined with cationic or anionic liposomes, delivers genes into cells effectively due to the synergistic advantages of both PEI and cationic liposomes. Our previous study also demonstrated that polycation modified liposomes with PEI-cholesterol showed equivalent transfection efficiency with significantly low cytotoxicity compared with Lipofectamine 2000.

In constructing gene complex carriers, the N/P ratio may play an important role in influencing the degree of complexation particle diameter, transfection efficiency, and cytotoxicity of carriers. We first developed complexes of PEI/chitosan/DNA with different N/P ratios of chitosan/DNA and PEI/DNA. The ability of chitosan to combine with DNA was studied in the gel retardation assay. After the addition of chitosan at an N/P ratio of 4 or 6, the DNA band almost disappeared (Fig. 1). This loss in fluorescence can be attributed to the polycation/DNA complex, which could result in the displacement of the intercalated ethidium bromide in the DNA. Complexes with an N/P ratio of 4 were observed with the transmission electric microscope and indicated that the morphology of the nanoparticles was rounded (data not shown). Zeta potentials also demonstrated that different complexes have different values, suggesting different construction of the complexes (Table 1).

Particle size is an important factor in particulate delivery systems. It is closely associated with the targeted lymphatic system and cell uptake. Particle sizes of complexes were measured, and listed in Fig. 2. It demonstrated that when the N/P ratio of chitosan and DNA was less than 2, with PEI added, the particle diameter first increased and then decreased. In contrast, when the N/P ratio of chitosan/DNA was greater than 4, the change in diameter can be ignored. This can be explained combined with the data obtained from the gel retardation assay. Free DNA remained when N/P < 2 (Fig. 1), therefore the compression effect of PEI was conspicuous. A pilot study also indicated that PEI might coat the surface of chitosan/DNA complex through ionic interaction.

A potential cytotoxic effect of PEI after combination with chitosan/DNA complexes was then investigated in HeLa cells using the MTT assay. The results showed that no cytotoxicity toward HeLa cells appeared in both groups treated with chitosan/DNA complex and PEI/chitosan/DNA at N/P ratio of chitosan/DNA of 4 or 6 (Fig. 3). In contrast, the PEI/DNA-treated group demonstrated obvious cytotoxicity. The results suggest that when high molecular-weight PEI combined with the chitosan/DNA complex at an N/P ratio greater than 4 increases the safety of PEI compared with that of the PEI/DNA complex alone even though the same amount of PEI was added in both experiments. Our previous study also demonstrated that PEI linked with cholesterol decreased the cytotoxicity. Another study also showed that PEI combined with the galactosylated chitosan decreased the cytotoxicity compared with the PEI/DNA complex and was assumed that the steric hindrance and charge shielding effect were the main reasons for the decrease in cytotoxicity. Furthermore, the change in zeta potentials between PEI/DNA and PEI/chitosan/DNA also contributed to the decrease in cytotoxicity. However, at the N/P ratio of chitosan/DNA equal to 2, the cytotoxicity did not decrease notably. It is hypothesized that when PEI is added, it combines with the free DNA remaining due to the ionic effect and forms PEI/DNA but not the complex of PEI/chitosan/DNA, which impairs its safety.

When transfection efficiency was evaluated, chitosan alone did not efficiently transfect HeLa cells (Fig. 4). The lu-
ciferase activity of chitosan was very low. However, the transfection efficiency was dramatically enhanced when PEI was combined with the chitosan/DNA complexes. It is known that a key cellular barrier impeding the transfection efficiency of nonviral gene vectors is the inefficient release of endosomally trapped DNA into the cell cytosol. The use of chitosan for gene delivery is limited due to the low transfection efficiency and difficulty in transfecting into a variety of cell types. It is believed that PEI can escape from endosome through the proton-sponge mechanism and facilitate gene entry into the nucleus. Therefore adding PEI enhanced the escape of chitosan/DNA nanoparticles from the acidic endosomes and subsequently induced high gene transfection. A previous study also showed that bafilomycin, which can inhibit the endo-/lysosomal proton pump and act as a specific inhibitor of vacuolar type H^+ ATPase, decreased PEI-mediated transfection, suggesting the synergistic effect of PEI with chitosan. Our results shown in Fig. 4 also indicated that there was no significant difference between PEI/DNA and PEI/chitosan/DNA with the ratio of PEI/DNA at 10. However, with the ratio of PEI/DNA at 5, the gene expression degree was decreased especially at a N/P ratio of chitosan/DNA greater than 4, suggesting that a balanced quantity of PEI, chitosan, and DNA is needed, and it is assumed that the more compactly chitosan binds to DNA, the less space remains for PEI to be inserted. Previous research also demonstrated that the combination of PEI and cationic liposomes enhanced the transfection efficiency, and the synergism appeared when the plasmid DNA was partially complexed but not fully condensed with PEI before adding cationic liposome.

Among nonviral vectors, cationic lipids such as Lipofect-Aminek and Lipofectamine have high transfection efficiency in vitro but their high toxicity and instability in the presence of serum limit applications in vivo. It is reported that cationic polymers are able to condense more DNA than lipids. The results shown in Fig. 4 suggest that serum did not impair the transfection efficiency of the complex carrier developed in the present study using both HeLa and A549 cells. The GFP gene was still expressed after 7 d (data not shown). The transfection results of PEI/chitosan/DNA in the presence of serum revealed that PEI modification of the surface of chitosan/DNA nanoparticles might increase complex stability. These results were in agreement with those reported by Yamazaki et al. and Garcia et al. although the exact mechanism was still not clear.

CONCLUSIONS

We developed an efficient method of transfection by combining chitosan and PEI and showed that the N/P ratio of chitosan/DNA and PEI/DNA may markedly influence the characteristics of the complex vector. The combination of PEI and chitosan resulted in the high gene transfection and low cytotoxicity. Our study suggested that the complex vector might be a promising gene carrier and can be considered for use in gene transfer in vivo. However, further understanding of the detailed mechanism of the synergism is necessary for improving the design of novel gene transfer carriers.

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