Characterization of (Aminoethyl)chitin/DNA Nanoparticle for Gene Delivery

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Nonviral gene delivery systems have been increasingly proposed as a safer alternative to viral vehicles. In the present study, we synthesized water-soluble chitin by aminoalkylating onto chitin at the C-6 position, and its transfection efficiency was investigated. Aminoethyl-chitin (AEC) was complexed with DNA, and AEC/DNA nanoparticles were characterized. AEC/DNA nanoparticles showed good DNA binding ability, high protection of DNA from nuclease and serum, and low cytotoxicity. Mean particle size decreased from 367 to 290 nm and ζ potential increased from −4.58 to 22.87 mV when the AEC/DNA charge ratio (N/P) increased from 1.15 to 18.5. The transfection efficiency of AEC/DNA nanoparticles was investigated in a human embryonic kidney cell line (HEK293), and the results showed that AEC/DNA nanoparticles were much enhanced compare with naked DNA.

Introduction

The basic concept of fundamental gene therapy, the introduction of a gene into specific cells of a patient in order to treat a genetic disease, whether innate or acquired, is fascinating.[1] In many human diseases such as cancer, AIDS, and cardiovascular problems, gene therapy is now seen as a promising approach to the treatment of a broad spectrum of health problems.[2] Gene delivery systems used so far include viral and nonviral vehicles. Due to the high transfection efficiency of viral vehicles such as retroviruses, adenoviruses, and adeno-associated viruses compared to nonviral vehicles, they are the most commonly used transfection agents in current clinical trials.[3] However, their application to the human body is severely limited by several problems such as high immunogenicity, risk of replication, random DNA insertion, and difficulty of sterilization, characterization, and mass production.[3,4] Although nonviral vehicles show low transfection efficiency compared to viral vehicles, nonviral delivery systems for gene therapy have been increasingly proposed as safer alternatives to the viral vehicles.[5] Among nonviral-based gene delivery vehicles, cationic lipids and cationic polymers are the two major types currently investigated. Cationic lipids have several advantages over viral gene transfer, but toxicity and relatively low transfection efficiency compared to viral vehicles are main disadvantages.[1,6] Therefore, cationic polymers have gained much attention because they can interact electrostatically with the negatively charged plasmids to form self-assembling polyelectrolyte polymers.[7,8]

Chitin is a naturally abundant mucopolysaccharide distributed in the shell of crustaceans, in the cuticle of insects, and also in the cell wall of some fungi and microorganisms. Chitin consists of 2-acetamido-2-deoxy-(1→4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units) that has intra- and intermolecular hydrogen bonds and is a water-insoluble material. Chitosan is derived from chitin consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amino-D-glucose units. Due to physiological characteristics such as biocompatibility, biodegradability, and low toxicity, it has drawn much attention for its potential application in medical devices or drug delivery systems.[9] Chitosan is also considered to be an attractive gene vehicle because, at acidic pH, chitosan carries a strong positive surface charge due to the primary amines, which could bind to negatively charged DNA. In spite of these characters, water-insolubility and low transfection efficiency of chitosan are major disadvantages. Several researchers have reported water-soluble chitosan derivatives and high transfection efficiency of chitosan-based gene delivery systems.[10−12] However, little information on nonviral vehicles of chitin and chitin derivatives has been available until now.

In the present study, we prepared a water-soluble chitin derivative by grafting aminofunctionality, and its DNA complexation, protection ability, cytotoxicity, and transfection efficiency was investigated.

Experimental Section

Materials. Chitin (MW ∼310 000, degree of deacetylation 10%) prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). 2-Chloroethylamino hydrochloride, DNase I (12.7 units/μg), sodium sulfate, poly(ethylenimine) (PEI, 25 kDa), ethidium bromide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma−Aldrich (St. Louis, MO). Cell culture media and all other materials required for culturing were obtained from Gibco (Grand Island, NY).

Preparation of Amino-Derivatized Chitin. Amino-derivatized chitin was prepared according to our previous method.[9] In the Fourier transform infrared (FT-IR) spectrum of the substituted aminoethyl group, a new peak observed at 2950 cm^{-1} due to C−H stretching, supporting the occurrence of the substitution. In the ^1H NMR spectrum of AEC in D_2O, a new chemical shift that appeared for the aminoethyl chitin derivative at 2.8 ppm was assigned to protons of $-CH_2N$. The peak at 2.05 ppm was a residual acetyl peak, and protons of the pyranose unit are superimposed on the $-NH_2$ and $-OCH_2$ of aminoethyl group (2.9−3.6 ppm).[9] Degree of substitution of AEC was 1.01 by elemental analysis and ^1H NMR data. The product was freeze-dried to give the aminoethyl-chitin (AEC; 0.403 g).

Preparation of pSV40-β-Galactosidase Plasmid. The pSV40βgal plasmid (denoted as DNA) was propagated in *Escherichia coli* and purified by a Qiagen kit (Chatsworth, CA) according to the supplier's protocol. The concentration of DNA was determined by measuring UV absorbance at 260 and 280 nm, and the purity of DNA was checked by electrophoresis on a 0.8% agarose gel.

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Preparation of AEC/DNA Nanoparticle. AEC solution dissolved in phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, and 2.7 mM KCl, pH 7.4) and a DNA solution in 50 mM sodium sulfate solution were heated to 55 °C separately. Equal volumes of both solutions were quickly mixed under high vortexing for 30 s, and the nanoparticle was allowed to stand at room temperature (25 °C) for 30 min. Nanoparticle was used in all studies without further purification. The AEC/DNA charge ratio (N/P) was expressed as the ratio of moles of amine groups of AEC to those of phosphate groups of DNA. The formation of AEC/DNA complex was tested by electrophoresis on a 0.8% agarose gel with Tris-acetate (TAE) buffer at 100 V for 40 min, and DNA was visualized with ethidium bromide.

Protection of AEC/DNA Nanoparticle against DNase I and Serum. AEC/DNA nanoparticle and free DNA (0.57 μg) were separately incubated with 5 μL of DNase I solution (10 μg/mL in DNase/Mg^{2+} digestion buffer, which consisted of 50 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$) at 37 °C for 1 h, and degradation of plasmid DNA was analyzed by electrophoresis on a 0.8% agarose gel. For DNA release studies, nanoparticles were incubated with an equal volume of cell culture medium containing 10% fetal bovine serum (FBS) for 24 h.

Particle Size and ζ Potential Measurement. The nanoparticle sizes and surface charges were analyzed by use of an electrophoretic light-scattering spectrophotometer (ELS 8000, Otuska Electronics Co.) with 90° and 20° scattering angles at room temperature. Nanoparticles were prepared in water at charge ratios (N/P) 1.15−18.5.

Cell Line and Cell Culture. Human embryonic kidney 293 cells (HEK293) were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37 °C under a humidified atmosphere with 5% CO_2.

Evaluation of Cytotoxicity. Cytotoxicity of AEC in comparison with PEI was evaluated via MTT assay. Cells were seeded at 1.3×10^4 cells/well in 96-well microtiter plates in complete medium, DMEM with 10% FBS for HEK293 cells. After 24 h of incubation in a humidified 5% (v/v) CO_2/air environment at 37 °C, 20 μL of AEC and PEI in sterile DW were transferred to the well to give final concentration ranging from 1 to 400 μg/mL. Following 24 h of incubation with polymers, the culture medium was aspirated and 200 μL of MTT dye solution (0.5 mg/mL) was added to each well. After 4 h of incubation, the medium was aspirated and the purple color crystals were dissolved with dimethyl sulfoxide (DMSO). The absorbance in each well was measured at 540 nm by use of a Genios Multifunction microplate reader (Tecan).

Assay for β-Galactosidase Activity. For the transfection studies, HEK293 cells were seeded at a density of 1×10^5 cells/well in 24-well plates and incubated for 24 h before transfection. An hour prior to transfection, the medium was removed and replaced with DMEM alone; following this, AEC/DNA nanoparticles were added slowly to each well to give 2 μg of DNA/well, and plates were incubated for 6 h. Nanoparticles were then removed and replaced with cell culture after this time and further incubated for 48 h to allow protein expression.

In order to quantify the transgene expression in HEK293 cells exposed to naked DNA and nanoparticles, an enzyme assay was carried out. At the end of the experiments, the cells were lysed with 100 μL of lysis buffer, and centrifuged at 10 000 rpm for 15 min. The activity of β-galactosidase in the supernatant was measured by the conversion of a colorless *o*-nitrophenyl β-galactoside (ONPG) solution into yellow-colored *o*-nitrophenol (ONP). The supernatant (50 μL) was pipetted into a 96-well plate and the same volume of ONPG solution was added. After incubation at 37 °C, the reaction was stopped with 1 M Na_2CO_3. The visible absorbance of the formed ONP was measured at 410 nm on a microplate reader, and β-galactosidase activity was determined as milliunitsper milligram of protein. Total protein content of the samples was measured by the Bradford method.

Statistics. Data were expressed as mean ± standard error of the mean ($n = 3$). Student's t test was used to determine the level of significance at $p < 0.05$.

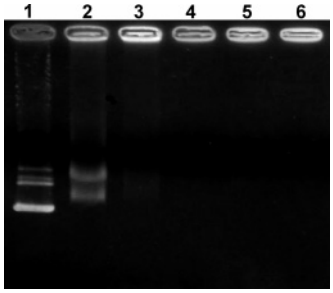

Figure 1. Electrophoretic mobility assay of AEC/DNA nanoparticles to determine DNA binding efficacy. Lane 1 contains plasmid DNA (0.57 μg) and lanes 2−6 show AEC/DNA nanoparticle formulations (DNA 0.57 μg) with N/P ratios increasing incrementally from 1.15 to 18.5. All samples were run on a 0.8% agarose gel and stained with ethidium bromide.

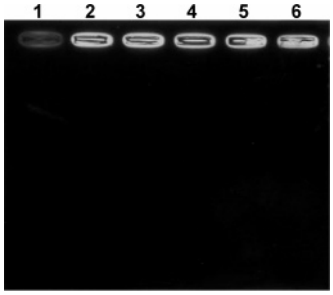

Figure 2. Protection assay of AEC/DNA nanoparticles by DNase I. Lane 1 contains plasmid DNA and lanes 2−6 show AEC/DNA nanoparticle formulations with N/P ratios increasing incrementally from 1.15 to 18.5. AEC/DNA nanoparticle and free DNA (0.57 μg) were separately incubated with 5 μL of DNase I solution (10 μg/mL in DNase/Mg^{2+} digestion buffer, which consisted of 50 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$) at 37 °C for 30 min, and degradation of plasmid DNA was analyzed by electrophoresis on a 0.8% agarose gel.

Results and Discussion

Complex Formation of AEC with DNA. To evaluate the ability of AEC to bind DNA, nanoparticle solutions were electrophoresed on agarose gels and visualized with ethidium bromide. As shown in Figure 1, AEC effectively condensed to DNA. The electrophoretic mobility of DNA was prevented by condensation with AEC and remained at the top of the gel at N/P ratios 2.3−18.5, indicating that AEC formed complexes with DNA at those ranges. At N/P ratio 1.15, free DNA band was found in the gel.

Protection of Nanoparticles against DNase I and Serum. The protection effect of AEC/DNA nanoparticles against DNase I degradation was investigated by gel electrophoresis, and the result is shown in Figure 2. When incubated with DNase I at 37 °C, naked DNA (lane 1) was completely degraded after 1 h and a trailing band at N/P ratio 1.15 (Figure 1) had also completely disappeared compared with Figure 2 (lane 2), whereas condensed DNA was protected at this N/P ratio.

The stability of AEC/DNA nanoparticles in the presence of serum proteins was investigated. The nanoparticles were incubated at room temperature with an equal volume of complete cell culture medium containing 10% FBS for 24 h, and the nanoparticles after incubation were analyzed by gel electrophoresis on an 0.8% agarose gel. As shown in Figure 3, nanoparticles appeared to be stable against serum proteins after 24 h of incubation compared with the result of Figure 1.

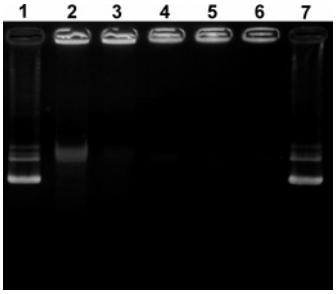

Figure 3. Electrophoretic mobility assay of AEC/DNA nanoparticles following incubation with serum-containing medium. Lanes 1 and 7 contain plasmid DNA and lanes 2−6 show AEC/DNA nanoparticle formulations with N/P ratios increasing incrementally from 1.15 to 18.5. All samples were run on a 0.8% agarose gel and stained with ethidium bromide.

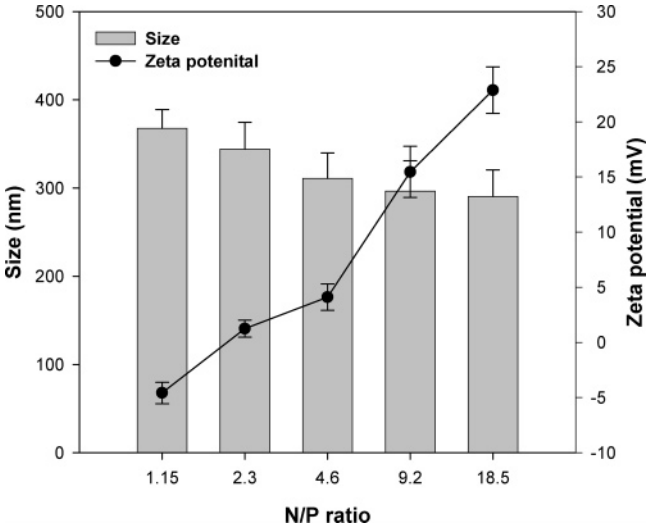

Figure 4. Particle sizes and ζ potential of AEC/DNA nanoparticles plotted against N/P ratio. Values represent means ± SE ($n = 3$).

Particle Size and ζ Potentials. Figure 4 shows the sizes and ζ potentials of the complexes with different N/P ratios. The size of the complexes was decreased with increasing N/P ratio while their ζ potential was increased with decreasing N/P ratio. Mean particle size decreased from 367 to 290 nm and ζ potential increased from −4.58 to 22.87 mV when the N/P ratio increased from 1.15 to 18.5.

Size and ζ potential are necessary to ensure nanoparticle uptake by cells. Illum et al.[13] have synthesized chitosan/DNA nanoparticles ranging from 20 to 500 nm, and other polymeric systems have also been reported ranging from 100 to 700 nm.[12,14,15] In the present study, our nanoparticle size is similar to those in the literature. However, most polycation/DNA nanoparticles enter mammalian cells by endocytosis, which is limited to particles size less than about 150 nm in diameter.[16] The ζ potential of the complexes is known to be one of the major factors affecting their biodistribution[17] and transfection efficiency[18] because a positive surface charge allows an electrostatic interaction between negatively charged cellular membranes and positively charged complexes.[19] As shown in Figure 4, the positive values of ζ potential were obtained in the present study except at N/P ratio of 1.15.

Evaluation of Cytotoxicity. In order to assess the cytotoxicity of AEC used in transfection experiments on HEK293 cells, an MTT assay was carried out. AEC was applied to the cells at concentrations ranging from 1 to 400 μg/mL, and the effect on cell viability was measured. Figure 5 shows the cytotoxicity of AEC and PEI at various concentrations against HEK293 cells.

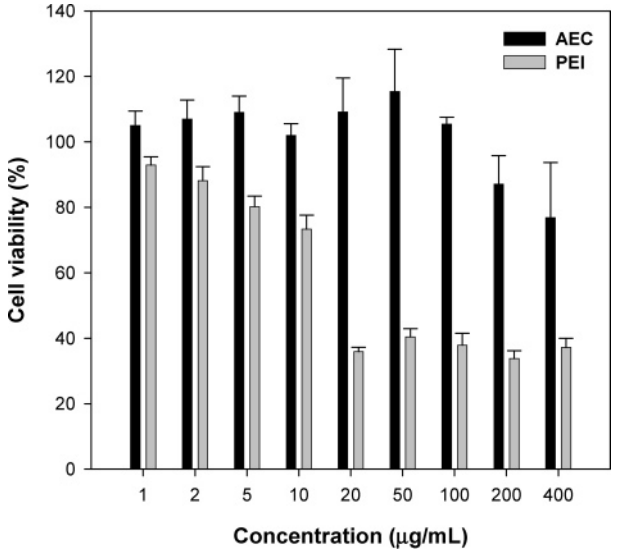

Figure 5. Cytotoxicity of AEC and PEI in HEK293 cells. HEK293 cells were seeded at a density of 1.3×10^4 cells/well in 96-well microtiter plates. Nanoparticles were added and the samples were incubated for 24 h at 37 °C. The mixture was replaced with 200 μL of MTT dye solution, and after 4 h of incubation, crystals were dissolved with DMSO. The absorbance was read at 540 nm by use of a microplate reader. Values represent means ± SE ($n = 3$).

AEC exhibited no effect on cell viability at the concentration of 100 μg/mL, and at higher concentrations cell viability was decreased. PEI, which is commonly used in gene delivery systems, was also evaluated for comparison, and the results showed that PEI exhibited higher cytotoxicity than AEC at tested concentrations. The major factor affecting cytotoxicity of cationic polymers was polymer aggregation on cell surfaces, which impaired important membrane functions.[20] Especially, electrostatic interaction between cationic charged primary amines and negatively charged groups on the cell surface may disrupt membrane functions of cells. In spite of a higher amine constitution for AEC than PEI (25 kDa), due to its molecular weight and degree of substitution, AEC showed a less toxic effect than PEI. According to the literature, low molecular weight PEI showed lower cytotoxicity than high molecular weight PEI (25 kDa).[21] AEC, which is derived from chitin having biocompatibility, biodegradability, and low toxicity, may degrade in cells, producing chitin units with lower amine content. Therefore, low molecular weight and low amine content of degraded AEC should improve its biocompatibility and safety. Generally, cationic polymers condensed with DNA used for gene delivery showed less toxicity than pure polymers.[3,22]

In Vitro Transfection. Up to now, several systems have been used to transfer genetic material into the human body. For direct injection of naked DNA into certain tissues, the overall gene expression is much lower than with either viral or nonviral vehicles. Furthermore, naked DNA is also unsuitable for systemic administration due to the presence of serum nucleases as a potential obstacle for functional delivery to the target cell.[23] To overcome these problems, nonviral delivery systems for gene therapy have been increasingly proposed as safer alternatives to viral vehicles.

In the present study, transfection efficiency of AEC/DNA nanoparticles as nonviral vehicles with various N/P ratios was evaluated against HEK293 cells. Cells were incubated with the nanoparticles for 6 h and assayed for β-galactosidase activity after transfection. The transfection of HEK293 cells is shown in Figure 6. At N/P ratio 4.6, transfection efficiency was increased around 2.5-fold compared with naked DNA. PEI,

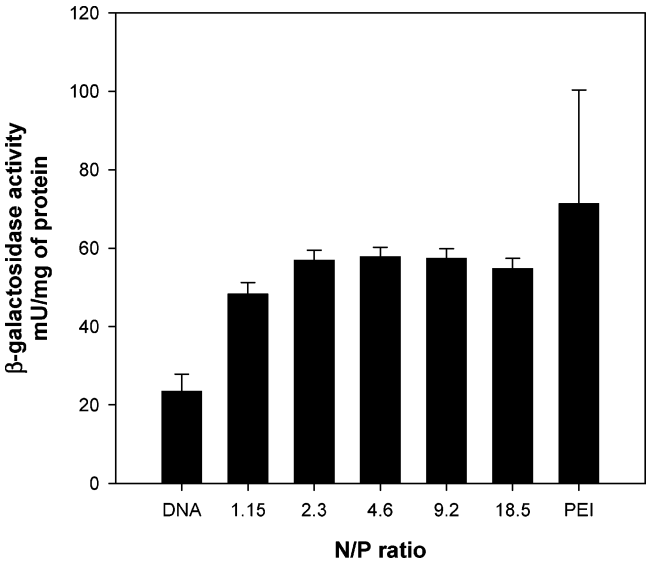

Figure 6. Transfection of HEK293 cells by AEC/DNA naanoparticles prepared at various N/P ratios. N/P ratio of PEI is 5. Average transfection efficiencies are expressed as β-galactosidase activity in milliunits per milligram of protein. Values represent means ± SE (n = 3).

widely applied in transfection studies, was used as a positive control. Compared with transfection efficiency of AEC/DNA nanoparticles, PEI showed slightly higher efficiency than that of nanoparticles at N/P ratio 4.6. We also transfected the AEC/DNA nanoparticles against HeLa human cervix cells; however, enhanced transfection efficiency of nanoparticles was not obtained (data not shown). This result indicated that transfection efficiency may be dependent on cell type. Several reports showed similar results of such cell-type dependence of chitosan/DNA nanoparticles.[12,24,25]

Whatever the mechanism, the ideal gene delivery system must be capable of protecting the DNA until it reaches its target. To achieve this goal, the system must be small enough to allow internalization into cells and delivery to the nucleus, which requires uptake of the complex into intracellular endosomes by endocytosis, complex release from the endosome into cytoplasm, and uptake of the complex in the nucleus.[1,4]

In this study, we used PEI as a positive control; its superior transfection efficiency, as compared with chitin and chitosan, is thought to be due to ionization at acidic pH because of its tertiary amine that increases endosomal pH and destabilizes the endosome, which is related to higher buffering effect[26] and also related to the proton sponge effect.[27,28] Transfection efficiency of PEI against HEK293 cells was higher than that of AEC. Although PEI transfection is superior to AEC, it is more toxic on HEK293 cells than AEC, and this property is disadvantageous for use in the human body.

Conclusions

In this study, we evaluated (aminoethyl)chitin nanoparticles as ligands for DNA for nonviral vehicles as alternatives to viral vehicles. AEC showed great ability to form complexes with DNA and to protect DNA against DNase I and serum. This polymer also has low cytotoxicity and exhibited much greater gene transfer efficiency than naked DNA. Therefore, AEC has much potential for use as a safe and efficient gene carrier.

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