Articles

Hydrophobic Modification of Polyethyleneimine for Gene Transfectants

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A new gene transfer system was developed by using polylipoplexes, which were prepared by hydrophobic modification of polyethyleneimine (PEI, MW 2000). PEI 25kDa is well known for its excellent transfection efficiency but it has extreme cytotoxicity; therefore, its application for medical use is strictly limited. PEI 2kDa is able to form complexes with DNA and has low cytotoxicity. However, unfortunately, it shows no transfection efficiency so it can not be a candidate carrier for gene therapy. We designed novel polycationic amphilphiles by conjugating hydrophobic moieties, such as cholesterol and myristate, to PEI 2kDa. Cholesterol-conjugated PEI (PEI-Chol: P10C, P17C and P30C) and myristate-conjugated PEI (PEI-Myr: P10M, P16M and P26M) are different from the other cationic lipids in that they can form lipopolyplexes with plasmid DNA that have extra multi-positive charges in their hydrophilic parts. From a different point of view, they are also considered to be PEI derivatives with a small proportion of hydrophobic moiety. As a result of the modification, PEI-Chol and PEI-Myr showed much enhanced transfection activity but somewhat increased cytotoxicity. We also examined the effect of the amount of hydrophobic moiety on lipopolyplex-mediated gene transfer and observed that P17C and P26M are the most effective carriers in the series of two groups. MTT assay indicated that the more myristyl groups were attached to PEI, the more injurious results were observed. In the case of PEI-Chol, however, the opposite tendency was observed.

Keywords : Polyethylenimine, Liposome, Cytotoxicity, Gene therapy.

Introduction

Efficient and safe gene transfer systems are the fundamental requirement for gene therapy, as well as for laboratory applications. Viral systems are, in general, quite effective for gene transfer, although there are arguments about their safety and immunogenicity.[1] Gene therapy is an attractive approach for the treatment of genetic defects, as well as disease such as cancer and chronic viral infection.[2-4] Therefore, a number of nonviral systems, especially cationic liposomes, have been developed. Cationic liposomes form a complex with polyanionic DNA molecules and are thought to deliver DNA through endosomes after endocytosis of the complex, although the precise mechanism of gene transfer mediated by cationic liposomes is still unclear. The cationic liposomal system, however, has some disadvantages such as low transfection efficiency due to DNA degradation in lysosomes and strong cytotoxicity. Various polycationic polymers have also been introduced for nonviral systems.[5,6]

Among them, polethyleneimine (PEI) has been revealed to be effective in the gene delivery, whereby genes may be delivered to the cytoplasm *via* endosomes due to the 'proton-sponge' effect of PEI.[7] However, the transfection efficiency of PEI is molecular weight-dependent and small molecular-weight PEI is reported not to be efficient. We developed a novel nonviral gene transfer system by combining the advantages of both liposomes and polycations. Various kinds of polycationic lipids were prepared by hydrophobic modification of small molecular-weight polyethyleneimine (PEI, M.W. 2000) that is inactive for transfection. Cholesterol and myristic acid are commonly used as competent lipid moieties that enhances transfection efficiency when they are properly conjugated.[8,9] The complexes of DNA and lipid form aggregates due to ionic interactions between the positively charged cationic lipid and the negatively charged phosphate groups of the DNA. The mechanism involved in the transfection pathway is not well understood, but it is believed that DNA/lipid complexes enter the cell through the plasma membrane or *via* an intermediate endosome.[10,11] Two kinds of novel gene transfer system, PEI-Chol and PEI-Myr, were invented by simple one-step organic synthesis. Polycationic lipids and polycationic liposomes were tested in the absence of FBS in *in vitro* experiments. The shape and particle size of plasmid DNA/polycationic liposome complexes were examined by atomic force microscopy. The transfection efficiency was remarkably enhanced by hydrophobic modification and cholesterol is likely to be less toxic than myristic acid when it is conjugated to PEI. In this study, we report the characterization of its physical properties and the effect of hydophobically modified PEIs on transfection activity and cytotoxicity.

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Table 1.

Carrier name	Area of characteristic peak[a]			Ratio[b]
	PEI[c]	Cholesteryl group[d]	Myristyl group[e]	
P10C	41.748	3.398		10.5/1
P17C	74.233	3.2710		17/1
P30C	119.70	3.03		29.6/1
P10M	2.1508		1	10.5/1
P16M	3.2001		1	16/1
P26M	5.2327		1	26.2/1

[a]Considered as reference to calculate ratio. PEI: $-CH_2CH_2N-$ (2.3-3.0 ppm), cholesteryl group: $-CH_3$ (0.5-0.7 ppm), myristyl group: $-(CH_2)_{10}-$ (1.0-1.5 ppm). [b] # of PEI units/# of cholesteryl (or myristyl) group. [c]Peaks of PEI ranged 2.1-3.0 ppm. [d]Peaks of cholesteryl group ranged 0.5-2.0 ppm. [e]Peaks of myristyl group ranged 0.7-1.8 ppm.

Experimental Section

Materials. Polyethyleneimine with an average molecular weight of about 2000 (PEI 2 kDa), PEI 25 kDa, Ethyl myristate and cholesteryl chloroformate were purchased from Aldrich Chemical Company, Inc. *N*-[2-hydroxythyl] piperazine-*N*'-[2-ethanesulfonic acid] (HEPES), ethidium bromide (EtBr), *o*-nitrophenyl-*b*-D-galactopyranoside (ONPG) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD). Minimal essential medium (MEM) was from Hyclone (Logan, UT). 5xReporter lysis buffer, Lipofectin™ and pSV-β-gal plasmid DNA (6821bp, 4.33×10^6 Da) were from Promega (Madison, WI). All other chemicals were purchased and used without further purification.

Synthesis of PEI-Chol (P10C, P17C and P30C). For the preparation of P17C as a sample, 90 mg of cholesteryl chloroformate (Mw: 449.12) was dissolved in mixed solution of 15 mL of chloroform and 15 mL of methanol. PEI 2 kDa 200 mg was also dissolved in the same solution. The former was added dropwise to the latter for 2 hours. The reaction was allowed to proceed at 37 °C for 48 hrs. The final product was dissolved in water and unreacted cholesterol particles were identified as aggregates. Filtration was performed to eliminate these aggregates and then the solution was dialyzed against water at 4 °C for 1 day to exclude insoluble cholesteryl chloroformate. After the dialysis, the aqueous solution was lyophilized. The integration of the proton magnetic resonance (^{1}H NMR) spectrum of the product in MeOD indicated that the final compound contains one cholesteryl group per 17 ethyleneimine units (Table 1). The modified polymers (P10C, P17C and P30C) may be represented by the ratio of PEI units to the cholesteryl group. This procedure is recapitulated in Figure 1.

Synthesis of PEI-Myr (P10M, P16M and P26M). For the preparation of P26M as a sample, 30 μL of ethyl myristate (Fw: 256.43) was added to PEI 2 kDa in 4 mL of methanol. The reaction was allowed to proceed at 57 °C for 3 days. The final product was dissolved in water and centrifuged at 13000 rpm. The supernatant was discarded and the precipitate dialyzed against water at 4 °C for 1 day to exclude remaining ethyl myristate. After the dialysis, the aqueous solution was lyophilized. The integration of the proton magnetic resonance (^{1}H NMR) spectrum of the product in MeOD indicated that the final compound contains one myristate group per 16 ethylenimine units (Table 1). The modified polymers (P10M, P16M and P26M) may be represented by the ratio of PEI units to the myristyl group. This procedure is recapitulated in Figure 1.

Atomic Force Microscopy (AFM). The shape and particle size of plasmid DNA/P17C polylipoplex and plasmid DNA/P26M polylipoplex were examined by atomic force microscopy (NanoScope IIIa system, Digital Instruments, Inc., Santa Barbara, CA) under the same conditions as reported previously with some modification.[12,13] Complexes were formed by mixing DNA and liposome or polycationic lipid in water and incubated for 30 min at room temperature. About 1 μL of the complex solution was deposited onto the surface of a freshly split mica disk. After allowing 1 to 2 min for adsorption, the excess solution was removed with filter paper and washed with pure water. After blowing N_2 gas on the surface of mica, it was dried at room temperature and prepared for imaging.

Preparation of Liposomes. Liposomes were prepared by the following method. Polycationic lipid and dioleoylphosphatidyl ethanolamine (DOPE) were dissolved in chloroform in a weight ratio of 1 : 2, dried with N_2 gas and reduced under pressure to thin film. Then it was hydrated in distilled water, suspended by vortexing and stored overnight at 4 °C. The liposomes were formed by sonication for 10-15 min with a bath-type sonicator.

Preparation of Liposome-DNA Complexes. pSV-β-gal plasmid DNA (6821bp, 4.33×10^6 Da) from Promega (Madison, WI) was amplified in E.coli JM109 and purified by Qiagen Plasmid Maxi Kit (Quiagen, Germany). The purity of the plasmid was confirmed by UV spectrum and agarose gel electrophoresis. Plasmids were dissolved in water and the liposome/DNA complexes were prepared as follows: liposomal solution was added to an appropriate amount of media and then plasmid DNA was slowly added to the solution. After the addition of the plasmid DNA, the mixture was incubated for 30 min.

Determination of Cytotoxicity. Evaluation of cytotoxicity was performed by MTT assay.[14,15] MTT was dissolved in 1×PBS at 2 mg/mL, filtered through 0.22 μm polycarbonate membrane filters for sterilization and removal of a small amount of insoluble residue present in some batches of MTT. At the end of the transfection experiment, the transfection mixture was replaced with 100 μL of fresh medium, containing 10% FBS. Twenty-six microliters of 2 mg/mL MTT solution in 1×PBS was then added. The plates were incubated for an additional 4 hr at 37 °C in a tissue culture incubator, then MTT-containing medium was aspirated off and 150 mL of DMSO was added to dissolve the formazan crystal formed by living cells. Cells were seeded in 96 well (2×10^4 cells per well) plates and incubated for one day prior

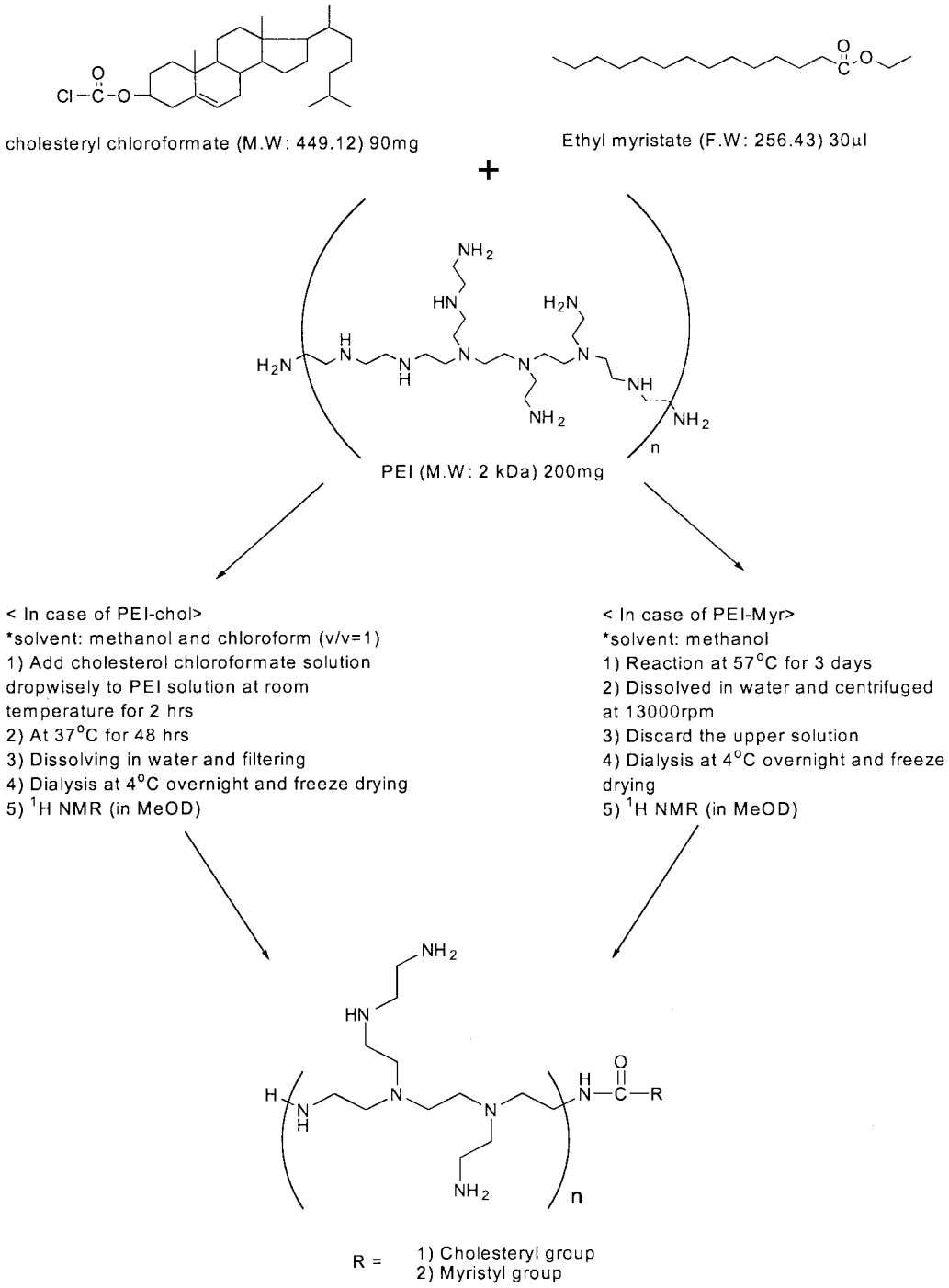

Figure 1. Summary of P17C and P26M synthesis and schematic structure of hydrophobically modified PEI (2 kDa) with cholesterol and myristic acid.

to experiment. PEI 25 kDa, PEI-Chol or PEI-Myr was introduced to the cells and incubated for 48 hours at 37 °C. The cytotoxicity was determined by comparing the amount of MTT reduced by cells treated with carriers to that reduced by control cells.[16] Absorbance was measured at 570 nm using a microplate reader at the end of 1 hr after adding dimethyl sulfoxide (DMSO). The cell viability (%) was calculated by the following equation:

$$\text{Cell viability (\%)} = (OD_{570(\text{sample})}/OD_{570(\text{control})}) \times 100$$

Cell Culture. Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cells were routinely maintained on plastic tissue culture dishes (Falcon) at 37 °C in a humidified 5% CO_2/95% air-containing atmosphere.

Transfection Procedures. Cells were seeded at a density of 1.0×10^5 cells/well in 24 well plates one day prior to

transfection experiments, and grown in the appropriate medium with 10% fetal bovine serum. The cell lines were 60-90% confluent at the time of transfection. Liposome/DNA complexes were prepared in FBS-free media. Each complex solution was further incubated for 30 min at room temperature and added to the cells. Transfection was performed in serum-free medium for 4 hours. The medium was replaced with a fresh complete medium and gene expression was assayed 48 hours post-transfection. Control transfections were performed by using commercially available transfection reagents such as DC-chol and PEI 25 kDa.

Transfection Assay. The expressed β-galactosidase activity was measured by the standard method recommended by the manufacturer (Promega Corp.).[17] Briefly, each cell in a 24-well plate was washed with DPBS and lysed with Reporter lysis buffer. The cell lysates were analyzed using the colorimetric ONPG assay in a 96-well plate format. β-galactosidase activity is expressed in total milliunits per well with 10^5 cells for 293 cells. One milliunit is defined as the amount of β-galactosidase that hydrolyzes 1 nmole of ONPG per min at pH 7.5 at 37 °C.

Agarose Gel Electrophoresis. Solutions of liposome and pSV-β-gal plasmid DNA were prepared in distilled water and mixed together in different weight ratios. The lipopolyplexes were formed by incubating for 30 min at room temperature in 20 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl. The sample buffer for gel electrophoresis was added to each sample, and it was electrophoresed on 0.7% (w/v) agarose gel for 90 min. at 100 V. TBE (45 mM Tris-Borate, 1 mM EDTA, pH 8.0) solution was used as electrophoresis buffer. The gel was stained with ethidium bromide (0.5 μg/mL) for 30 min and illuminated with an ultraviolet (UV) illuminator to show the location of the DNA.

Results

Synthesis and Characterization of PEI-Chol and PEI-Myr. The new polycationic lipids, PEI-Chol and PEI-Myr, were prepared in 50-70% yields by simple organic synthesis. The scheme for synthesis of PEI-Chol and PEI-Myr is summarized in Figure 1. The identity of these compounds was confirmed by ^{1}H NMR spectroscopy (Table 1). Since PEI 2 kDa is a polymer having high polydispersity in its molecular weight and the products do not have an exact molecular weight and shape, we denoted the compounds by average ratio of PEI units versus hydrophobic moiety. For example, P17C denotes that it has an average of 17 PEI units per cholesteryl group.

PEI-Chol is a cholesterol derivative conjugated with PEI 2 kDa *via* carbamate ester linkage and PEI-Myr is a myristate derivative coupled with PEI 2 kDa *via* amide bond. PEI-Chol is structurally related to various cationic cholesterol derivatives previously reported, such as DC-chol, spermine-cholesterol, BGSC or BGTC and K-chol or O-chol.[14,15,18-21] The cholesterol is an abundant constituent of animal cell membranes with a major role in modulation of membrane fluidity. Myristic acid is one of the naturally occurring fatty acids in animals and is known for its excellent transfection efficiency when conjugated to appropriate materials. The carbamate ester bond is chemically unstable and easily broken down at high pH and elevated temperatures[19,22] and amide bond is also degradable by the action of peptidase in cellular matrix. Based on the result of MTT assay, we concluded that PEI 2 kDa is a nontoxic agent even though it has no transfection efficiency. Therefore, PEI-Chol and PEI-Myr, composed of naturally occurring lipids, are considered to be biodegradable after administration into animal cells. In addition, they are expected to reduce the potential toxic effects.

Formation of Liposome/DNA Complexes

Preparation of liposomes: Two kinds of liposomes, PEI-Chol/DOPE and PEI-Myr/DOPE, were formed by sonication. PEI is very soluble in water, but when conjugated with hydrophobic group such as cholesterol or myristate, its solubility varies in proportion to the residue. P30C, P16M, and P26M were easily soluble in water and did not form any precipitates. P17C, P10C, and P10M were somewhat insoluble in water and white fine sediments were formed in small amounts just like DC-chol since the interaction between hydrophobic moieties aggregated the liposomes.

Agarose gel shift assay: To investigate the complex formation of PEI-Chol and PEI-Myr, agarose gel electrophoresis of liposome/plasmid DNA was performed at various weight ratios. As shown in Figure 2, the electrophoretic mobility of liposome/plasmid DNA complex was decreased gradually as increasing amounts of liposome. Complete retardation was observed when the weight ratio of the complex was between 0.5-1 (+/–) on PEI-Chol and between 0.25-0.5 (+/–) on PEI-Myr.

AFM images of liposome (or cationic lipid)/DNA complexes: We investigated the size and shape of PEI-Chol/DNA and PEI-Myr/DNA complexes at weight ratios of 1 (+/–) and 2 (+/–), respectively, using atomic force microscopy since the highest transfection efficiency was observed at these ratios. Images of carrier/DNA are presented in Figure

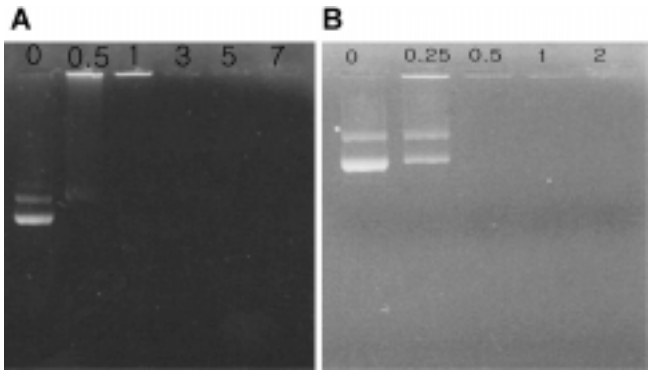

Figure 2. Electrophoretic Mobility Shift Assay (EMSA) of liposome/plasmid DNA complexes. Weight ratios are indicated above each lane and the first lane is control. (A) PEI-Chol (P17C) liposome/plasmid DNA complexes. (B) PEI-Myr (P26M) liposome/plasmid DNA complexes.

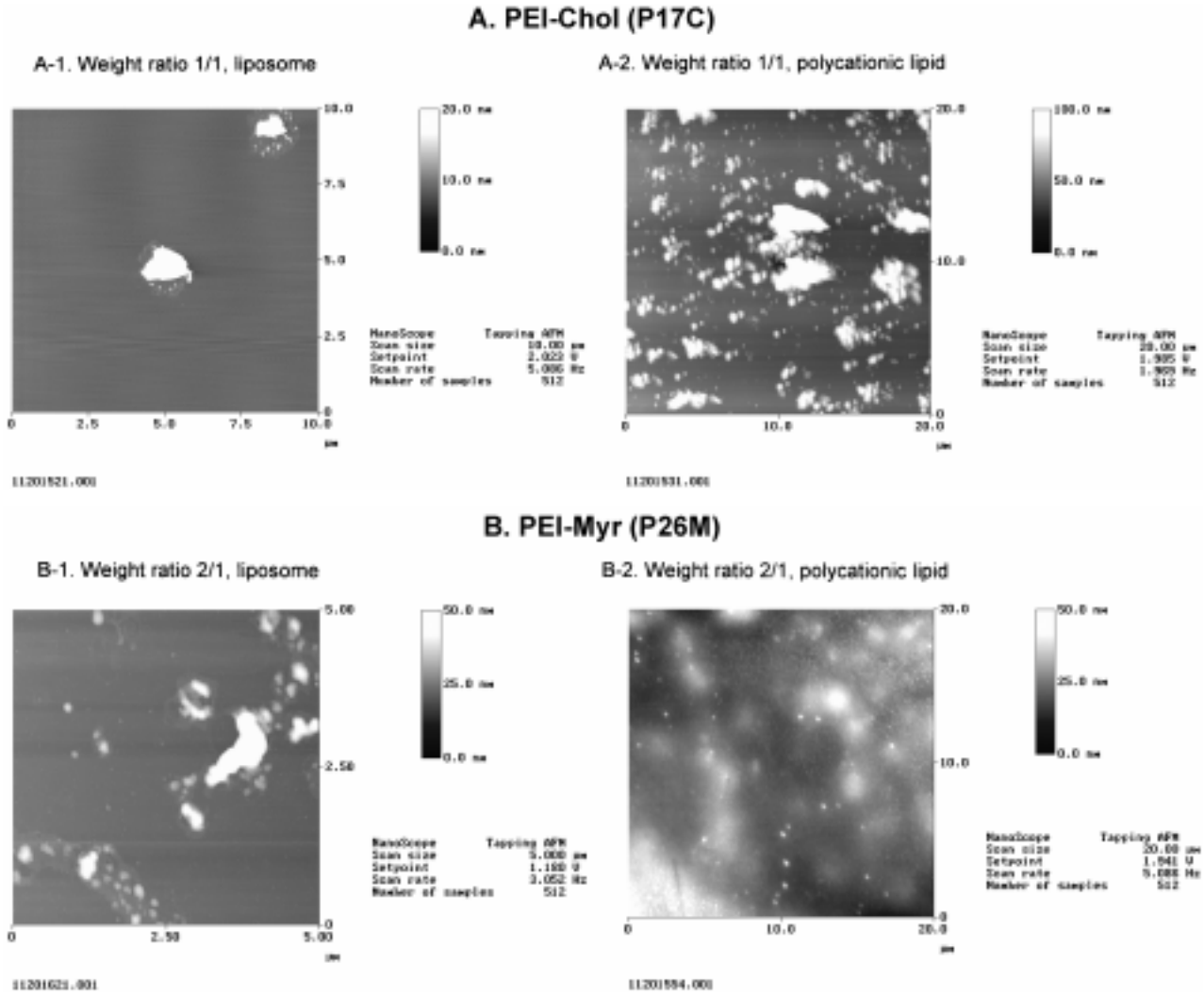

Figure 3. AFM images of liposome (or cationic lipid without DOPE)/plasmid DNA complexes. Weight ratio was set according to its best transfection range.

3. Each complex showed irregular morphology and a broad size distribution.

Transfection Efficiency of PEI-Chol and PEI-Myr. The ability to promote the uptake of a β-galactosidase reporter plasmid was evaluated by measuring the β-galactosidase activity in lysates of transfected cells. To optimize the transfection condition, we performed experiments by mixing a fixed amount (2.0 μg per well) of pSV-β-Gal plasmid with various amounts of carriers in absence of serum. Each carrier has dose-dependent transfection efficiency. PEI-Chol has its highest activity when weight ratio is around 1 but in the case of PEI-Myr, its optimal ratio is around 2. It was apparent that the transfection efficiency showed a bell-shape dependence on the lipid concentration (data not shown). The amount of DOPE was also critical to the transfection efficiency. Higher efficiency was observed when the weight ratio (DOPE/cationic lipid) was 2 than for a ratio of 1. Cells were treated with PEI-Chol/DNA and PEI-Myr/DNA complexes at its optimum transfection condition. After 4 hours, transfection media containing lipoplexes were replaced with normal growth media and incubated for an additional 48 hours in the presence of 10% FBS.

To determine the transfection efficiency of PEI-Chol and PEI-Myr, we have prepared PEI 25 kDa and compared its transfection activity in 293 cell lines. PEI 25 kDa is widely accepted as the most efficient reagent for transfection of mammalian cells among all PEI derivatives.[23-26] The ratio of PEI 25 kDa to DNA was adjusted by using standard conditions recommended by the manufacturer. PEI was tested at its optimal condition in this and other experiments as previously reported.[27,28] Figure 4 indicated that PEI-Chol and PEI-Myr had improved transfection efficiency considering that the cells treated with them displayed remarkable β-galactosidae activity which was not shown in the case of PEI 25kDa. P17C and P26M showed the highest transfection efficiencies among series of PEI-Chol and PEI-Myr, respectively.

Cytotoxicity Studies. As shown in Figure 5, PEI 2 kDa had a negligible cytotoxicity on the 293 cells. PEI 25 kDa was highly toxic to both cells above 8 μg/mL. Cholesterol

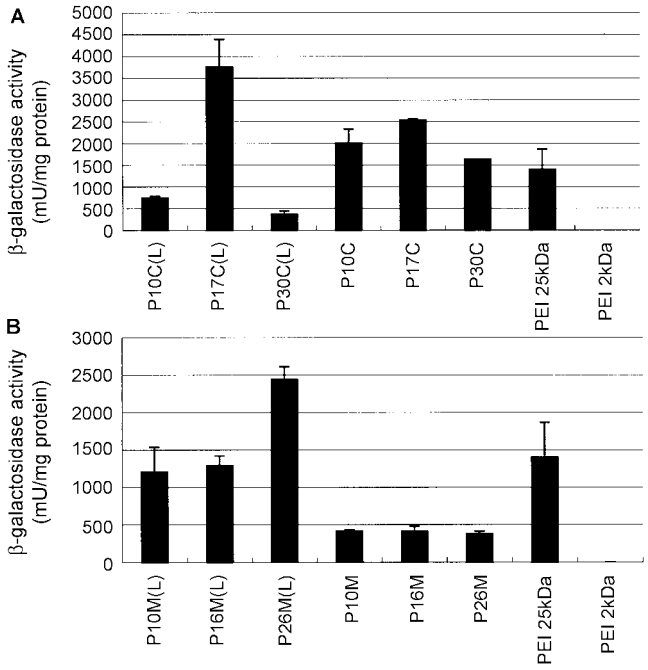

Figure 4. Comparison of transfection efficiency of PEI-Chol and PEI-Myr in 293 cell lines. The concentration of plasmid DNA was set to be 2.0 mg per well. 'L' indicates liposome with DOPE and the others are cationic lipids without DOPE.

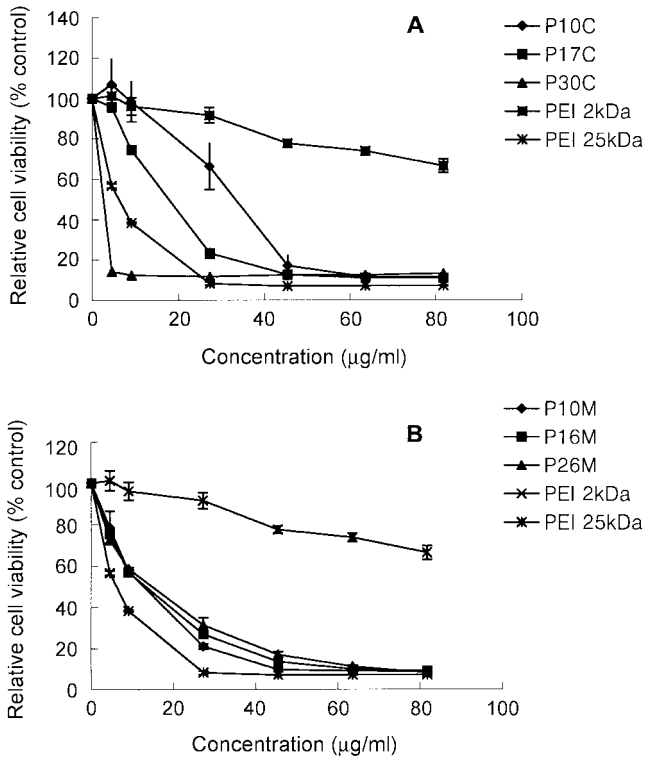

Figure 5. Effect of PEI-Chol, PEI-Myr, PEI 2 kDa and PEI 25 kDa on cell viability. 293 cells were seeded 2×10^4 cells per well in a 96 well plate. After 24 hours, cells were exposed to the indicated concentrations of each carrier for 2 days. Relative cell viability was calculated, regarding the absorbance at 570 nm of intact 293 cells as 100%. The absorbance is directly proportional to the number of living cells. (A) PEI-Chol (B) PEI-Myr

and myristate are materials found ubiquitously in animals and are not expected to have harmful effects on cell viability. Our strategy was that PEI-Chol, joined through a carbamate linkage, and PEI-Myr, conjugated by amide bond, are expected to be biodegradable and nontoxic. In order to test this, the cytotoxic effects of PEI-Chol and PEI-Myr on cell proliferation were assayed. PEI-Chol and PEI-Myr were less toxic than PEI 25 kDa but they had much increased toxicity compared to PEI 2 kDa. The PEI-Chol series had increased viability as the amount of cholesterol in their composition increased. On the other hand, PEI-Myr showed the opposite tendency.

Discussion

Among the candidates for gene therapy, gene delivery systems using cationic lipids have been rapidly developed since 1990's. This non-viral gene transfer vehicle has some disadvantages such as low transfection efficiency and stability but it is free from concerns regarding immunogenicity and can be stably obtained by simple organic synthesis. The aim of a gene delivery system is to construct a vehicle that delivers DNA into cells efficiently and safely, and finally to make it applicable for treatment of human disease. We designed a new series of synthetic vectors using low molecular weight PEI, cholesterol, and myristate. PEI 2 kDa is a cationic polymer similar to PEI 25 kDa, which forms a stable complex with DNA through charge interaction and has no transfection efficiency and negligible cytotoxiciy. Cholesterol and myristate are also found in the human body, that is, they are safe to be used in gene therapy. Our strategy was that PEI-Chol, joined through a carbamate linkage, and PEI-Myr, conjugated by amide bond, were expected to be biodegradable and nontoxic, and to have enhanced transfection efficiency due to characteristics altered by hydrophobic modification.

Two series of gene delivery systems, PEI-Chol and PEI-Myr, were easily prepared by simple organic reaction. They could form complexes with DNA molecules producing compact unity and the size of the complexes was not fixed in certain range as shown in Figure 2 and 3. PEI-Chol and PEI-Myr, especially P17C and P26M, had much improved transfection efficiency compared with PEI 2 kDa. Their efficiency level in the presence of serum was comparable to that of PEI 25 kDa (data not shown). Even though the exact mechanism underlying the phenomena could not be explained, one possible theory is based on the fact that hydrophobic modification of hydrophilic PEI changed its characteristics such as method of interaction with cell membranes or channels and nuclear pores. In addition, liposome/plasmid DNA complex is generally more efficient than cationic lipid/plasmid DNA.

Figure 5 indicated that the result of MTT assay did not match our expectations. PEI-Chol and PEI-Myr were less toxic than PEI 25 kDa but the sharp increase in toxicity compared to PEI 2 kDa remains a subject for further investigation before they can be practically applied. Even

though the compounds should be biodegraded into relatively nontoxic constituents the altered physical and chemical properties of the carriers had a serious effect on the cell viability. The PEI-Chol series showed greater cell viability with increasing the amount of cholesterol in their composition. It could have reduced cytotoxity if the proportion of cholesteryl group was increased. PEI-Myr showed the same tendency by diminishing the amount of myristate.

In this paper, we reported another amphiphilic gene transfer system which has a polycationic PEI head and lipophilic moiety. It could not be a satisfactory transfection agent and needs further development to be used as a promising candidate for gene therapy. Nevertheless, the result demonstrated the possible effects of chemical modification on the properties of cationic polymers and guided us towards the use of novel polymer derivatives as vehicles for gene delivery.

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