

Low Molecular Weight Polyethylenimine-Mitochondrial Leader Peptide Conjugate for DNA Delivery to Mitochondria

Joon Sig Choi,¹ Min Ji Choi,² Kyung Soo Ko,³ Byoung Doo Rhee,³ Youngmi Kim Pak,⁴
In Seok Bang,⁵ and Minhyung Lee^{2,*}

¹Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon 151-742, Korea

²Department of Bioengineering, College of Engineering, Hanyang University, Seoul 133-791, Korea

*E-mail: minhyung@hanyang.ac.kr

³Department of Internal Medicine, Mitochondrial Research Group, College of Medicine, Inje University, Seoul 139-707, Korea

⁴Asan Institute for Life Science, University of Ulsan, Seoul 138-736, Korea

⁵MyGene Bioscience Institute, 202-16, Nonhyun-dong, Kangnam-gu, Seoul 135-010, Korea

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It has been found that a number of diseases are associated with mutations in the mitochondrial DNA. Therapeutic gene delivery to mitochondria has been suggested as a clinical option for these diseases. In this study, we developed a gene carrier to mitochondria by the conjugation of mitochondrial leader peptide (LP) to polyethylenimine (PEI). Mitochondrial LP conjugated PEI (PEI-LP) was synthesized with low molecular weight PEI (2,000 Da, PEI2K). Gel retardation assay showed that PEI2K-LP formed complexes at a 1.0/1 weight ratio. In addition, PEI2K-LP protected DNA from the enzymatic degradation for at least 60 min, while naked DNA was completely degraded within 20 min. PEI2K-LP was compared with LP conjugated high molecular weight PEI (25,000 Da, PEI25K) in terms of toxicity and delivery efficiency. MTT assay showed that PEI2K-LP had much lower cytotoxicity than PEI25K-LP to 293 cells. In addition, cell-free DNA delivery assay showed that PEI2K-LP delivered more DNA to mitochondria at a 1.8/1 weight ratio than naked DNA or PEI. This result suggests that PEI2K-LP may be useful for the development of mitochondrial gene therapy system with lower cytotoxicity.

Key Words : Gene delivery, Mitochondria, Leader peptide, Polyethylenimine

Introduction

Mitochondria are responsible for energy production in respiring cells by the oxidative phosphorylation (OXPHOS). Also, mitochondria contain the enzymes required for fatty acid metabolism and the citric acid cycle. It was suggested that mitochondria contain approximately 1,200 proteins.¹⁻⁴ Most of the proteins are nuclear encoding proteins. The nuclear encoded mRNAs of the mitochondrial proteins are translated in cytoplasm and imported to mitochondria. Besides the nuclear encoding proteins, mitochondria own their own DNA (mtDNA) which encodes 13 peptide subunits of the OXPHOS enzymes. The mtDNA also contains genes for two RNAs and 22 tRNAs which are required for the translation of the mitochondrial gene.⁵ It has been found that a growing number of diseases are associated with single-base mutation or large deletion of mtDNA.⁵ The diseases include blindness, diabetes, deafness, cardiac failure, and renal dysfunction.⁶⁻¹⁴ Various metabolic therapies such as bypass metabolic block and antioxidant therapy have been developed to treat these diseases. In addition, gene therapy has been suggested as a therapeutic option for these diseases.

For the efficient delivery of genetic materials to mitochondria, novel delivery methods or carriers should be developed. However, efficient carriers targeted to mitochondria are not currently available. Although many gene delivery carriers

including viral or nonviral vectors have been developed, most of them are targeting to the nucleus, not to mitochondria. Therefore, it was thought that direct DNA delivery to mitochondria is theoretical and speculative so far. However, a few reports proved the possibility of DNA delivery to mitochondria. In a previous report, mitochondrial leader-peptide (LP) was conjugated to DNA.¹⁵ The report showed that substantially large DNA-LP conjugate could be imported into mitochondrial matrix. In another research, a cationic carrier named as DQAsome was developed as a gene carrier to mitochondria.¹⁶⁻¹⁸ DQAsome bound and condensed DNA by electrostatic interaction. After transfection, some of DQAsome/plasmid DNA complexes were localized at mitochondria and DNA was released from the complex at mitochondrial sites.¹⁹

For gene delivery to the nucleus, various delivery vectors are available, which are viral or non-viral vectors. Non-viral vectors include cationic liposomes, cationic polymers, and their conjugates.^{20,21} Cationic polymers can easily be modified by the conjugation of functional molecules.^{21,22} Therefore, tissue- or cell-specific polymeric carriers have been developed by the conjugation of ligands to polymers. The sub-cellular distribution of polymer carriers can be achieved by the conjugation of targeting molecules. For example, nuclear targeting delivery carrier has been developed by the conjugation of nuclear localization signal peptide to polymeric carriers.^{23,24}

Polyethylenimine (PEI) has been widely investigated as a gene carrier, because of its high transfection efficiency.^{20,21} It was previously reported that low molecular weight PEI (PEI2K, 2,000 Da) had much lower cytotoxicity than high molecular PEI (PEI25K, 25,000 Da).²⁵ Therefore, PEI2K is more biocompatible than PEI25K for gene delivery. In this research, we synthesized and evaluated mitochondrial LP conjugated PEI2K (PEI2K-LP) as a DNA delivery carrier to mitochondria. The results showed that PEI2K-LP might be useful for the development of mitochondrial gene delivery with low cytotoxicity.

Experimental Section

Synthesis of PEI2K-LP. PEI (2 kD) was purchased from Sigma-Aldrich (Seoul, Korea). The ornithin transcarbamylase leader peptide (H₂N-MLS₂NL RILLN KAALR KAHTS MVRNF RYGKP VQC-COOH, MW 3,830)¹⁵ was obtained from Pepton (Daejeon, Korea). *N*-Succinimidyl 3-(2-pyridyl-dithio) propionate (SPDP) was purchased from Pierce (Rockford, IL, USA). The LP was conjugated to PEI by the disulfide linkage using SPDP as a cross-linking reagent (Fig. 1).²⁶ Briefly, 6.6 mg of PEI (2kD) in 0.5 mL phosphate buffer (pH 8.0) was mixed with 0.5 mL of DMSO solution containing 3.8 mg of SPDP, and was stirred overnight at room temperature. The reaction mixture was dialyzed against water at 4 °C overnight using a Spectra/Por dialysis membrane (MWCO 1000). After dialysis, the solution was freeze-dried (2.2 mg). To the PEI-SPDP obtained (0.9 mg), the leader peptide (2.0 mg) was added and solubilized in 1.0 mL of PBS. The reaction was proceeded overnight at room temperature. The reaction mixture was dialyzed overnight using a Spectra/Por membrane (MWCO 1000) and freeze-dried yielding a product. The product was solubilized in pure water and used for the following experiments.

Plasmid DNA preparation and agarose gel retardation assay. pCMV-Luc was transformed in JM109 and amplified in terrific broth media at 37 °C overnight. The amplified pCMV-Luc was purified by using a Maxi plasmid purification kit (Qiagen, Valencia, CA). The purity and concentration of the purified pCMV-Luc were determined

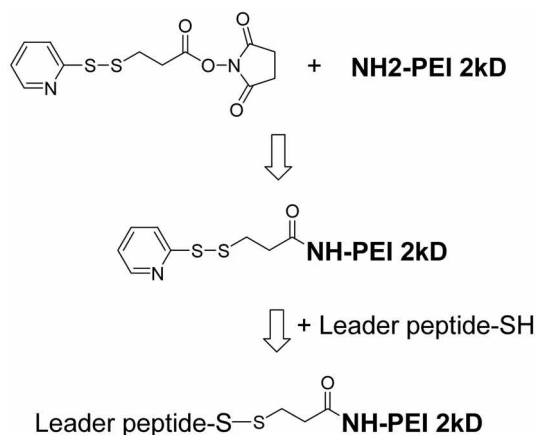


Figure 1. Synthesis scheme of PEI2K-LP.

by ultraviolet (UV) absorbance at 260 nm. The optical density ratio at 260 to 280 nm of the pCMV-Luc preparation was in the range of 1.7-1.8.

PEI25K-LP/pCMV-Luc and PEI2K-LP/pCMV-Luc complexes were prepared in 5% glucose solution at various weight ratios as described previously.²⁵ The complex solutions were incubated at room temperature for 30 min and subject to electrophoresis on 1% (w/v) agarose gel. The gel was stained with ethidium bromide (0.5 μg/mL) for 30 min and illuminated on a UV illuminator to identify the location of DNA.

DNase I protection assay. Ten micrograms of pCMV-Luc was mixed with 6, 12 or 18 mg of PEI2K-LP in 500 μL of PBS. After complex formation, DNase I (10 units, Promega, Madison, WI) was added to the complex solution and the reaction mixture was incubated at 37 °C. One hundred microliters of the sample was taken at 20, 40 or 60 min after incubation and mixed with 100 μL of 2 × stop solution (80 mM EDTA and 2% SDS) to dissociate pCMV-Luc from PEI2K-LP. The DNA was separated and analyzed by agarose gel electrophoresis.

Preparation of RITC-labeled pCMV-Luc. pCMV-Luc and rhodamine B isothiocyanate (RITC) (Sigma-Aldrich Korea) were mixed in 0.2 M sodium carbonated-buffered solution (pH 9.7) at 4 °C for 12 hrs at both the concentration of 1 mg/mL. The residual RITC was separated by gel filtration of a MicroSpin G-25 column (Sigma-Aldrich Korea, Seoul, Korea). The RITC-labeled pCMV-Luc was obtained by ethanol precipitation.

Isolation of mitochondria and cell free assay. 293 cells, human embryonic kidney cell lines, were grown in DMEM with 10% FBS. The cells were routinely maintained on plastic tissue culture dishes (Falcon Co., Becton Dickenson, Franklin Lakes, NJ) at 37 °C in an incubator with a humidified atmosphere containing 5% CO₂/95% air. All media routinely contained 1× antibiotic-antimycotic agent. For isolation of mitochondria, the cells were washed with ice-cold PBS. The cells were harvested in the buffer A (10 mM HEPES, pH 7.5, 200 mM mannitol, and 70 mM sucrose). The cells were homogenized in a glass homogenizer and the solution was centrifuged at 2,700 rpm. The supernatants were re-centrifuged at 8,500 rpm. The pellets were resuspended in the buffer A containing 1% Triton X-100.

Each PEI-LP/pCMV-Luc complex was prepared at the indicated weight ratios (polymer/pCMV-Luc). PEI25K/pCMV-Luc complex was formed at a 0.8/1 weight ratio, based on the previous reports.²⁵ The RITC-labeled pCMV-Luc plasmid DNA was used for all experiments. Each PEI25K-LP/pCMV-Luc, PEI2K-LP/pCMV-Luc, and PEI25K/pCMV-Luc complex was incubated with the isolated mitochondria for 30 min at 37 °C. After the incubation, the mitochondria were purified by centrifugation. The pellets were washed twice with the buffer A containing 1% Triton X-100 and re-suspended. The mitochondria were observed using a confocal microscope with appropriate filters.

Cytotoxicity assay. Evaluation of cytotoxicity was performed by the 3-(4,5-dimethyl)thiazol-2,5-diphenylterzo-

limum bromide (MTT) assay. The cells were seeded at a density of 2×10^4 cells/well in 96-well microassay plates (Falcon Co., Becton Dickenson, Franklin Lakes, NJ), and incubated for 24 hrs before transfection. PEI25K/pCMV-Luc complex was prepared at a 5/1 N/P ratio and each PEI-LP/pCMV-Luc complex was prepared at various ratios. The media were replaced with fresh DMEM without FBS before the transfection. The polymer/pCMV-Luc complexes were added to the cells. The amount of pCMV-Luc was fixed at $0.2 \mu\text{g}/\text{well}$. After the incubation at 37°C for 4 hrs, the transfection mixture was replaced with $100 \mu\text{L}$ of fresh DMEM medium supplemented with 10% FBS. The cells were incubated for an additional 20 hrs at 37°C . After the incubation, $24 \mu\text{L}$ of $2 \text{ mg}/\text{mL}$ MTT solution in PBS was added to the wells. The cells were incubated for an additional 4 hrs at 37°C and the MTT-containing medium was aspirated off and $150 \mu\text{L}$ of DMSO was added to dissolve the formazan crystal formed by live cells. Absorbance was measured at 570 nm . The cell viability (%) was calculated according to the following equation:

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

Where the $\text{OD}_{570}(\text{sample})$ represents the measurement from the wells treated with polymer/pCMV-Luc complex and the $\text{OD}_{570}(\text{control})$ represents the measurements from the wells treated with PBS buffer only.

Results

Synthesis of PEI2K-LP and gel retardation assay of complexes with DNA. PEI-LP was synthesized by the conjugation of mitochondrial LP to PEI (Mw 25,000 or 2,000) using SPDP as a cross-linking reagent as described previously.²⁶ The synthetic scheme was presented in Figure 1. A gel retardation assay was performed to confirm that the PEI-LPs form complexes with DNA (Fig. 2). Various amounts of PEI-LPs were added to the fixed amount of DNA. While PEI25K-LP retarded DNA completely at a 0.6/1 weight ratio (polymer/DNA), PEI2K-LP retarded DNA at a 1.0/1 weight ratio (Fig. 2). It may be due to low charge density of PEI2K-LP, suggesting that larger amount of polymers are required to construct complete complexes with plasmid DNA in the case of PEI2K-LP.

DNase I protection experiments. DNA is easily degraded by various nucleases in the process of gene delivery. Therefore, DNA protection from nuclease enzymes is one of the requirements as a gene delivery carrier.^{27,28} To investigate whether PEI2K-LP could protect DNA, DNase I protection assay was performed. PEI2K-LP/DNA complexes were prepared at various weight ratios ranging from 0.6/1 to 1.8/1. After complex formation, DNase I was added to each solution. The degradation of DNA was evaluated by agarose gel electrophoresis. After 20 min of incubation with DNase I, the plasmid DNA without any polymers was completely degraded (Fig. 3, lane 1). Similarly, DNA in PEI2K-LP/DNA complexes (0.6/1 weight ratio) was completely

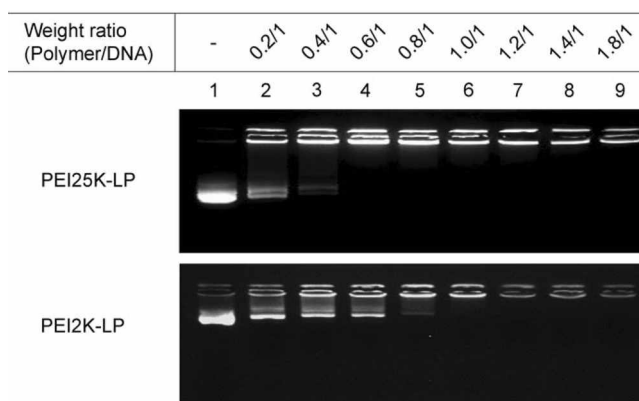


Figure 2. Gel retardation assay. PEI25K-LP/pCMV-Luc or PEI2K-LP/pCMV-Luc complexes were prepared at various weight ratios and left for 20 min at room temperature for complex formation. The complexes were analyzed by 1% (w/v) agarose gel electrophoresis.

degraded within 20 min (Fig. 3, lane 2). This result indicated that the complex is not formed tightly and PEI2K-LP could not protect DNA at this weight ratio. However, DNA in the complexes at 1.2/1 and 1.8/1 weight ratios was protected effectively up to 60 min (Fig. 3, lanes 3 and 4). Therefore, PEI2K-LP had DNA protection ability when the complex was formed at or above a 1.2/1 weight ratio.

Cytotoxicity results of PEI2K-LP/DNA complexes. To evaluate the cytotoxicity of PEI2K-LP, MTT assay was carried out. PEI2K-LP/DNA complexes were prepared at 0.6/1, 1.2/1, and 1.8/1 weight ratios and treated to 293 cells. After 24 hrs, the cell viability was evaluated by MTT assay. As controls, PEI25K/DNA and PEI25K-LP/DNA complexes (0.8/1 weight ratio) were treated to the cells. The results showed that PEI2K-LP was not toxic to cells in the range of 0.6/1 to 1.8/1 weight ratios (Fig. 4). However, PEI25K or

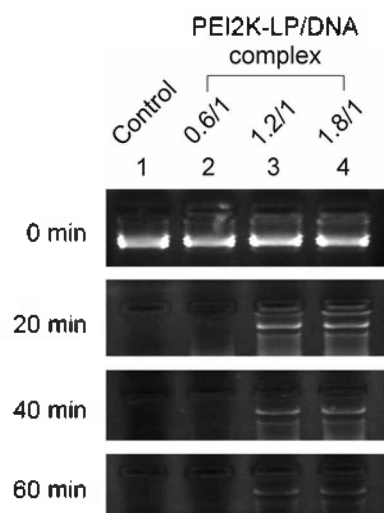


Figure 3. DNase I protection assay. PEI2K-LP/pCMV-Luc complex was prepared as described in experimental sections. The complex solution was incubated with DNase I for 20, 40 or 60 min. After incubation, DNA was analyzed by 1% agarose gel electrophoresis.

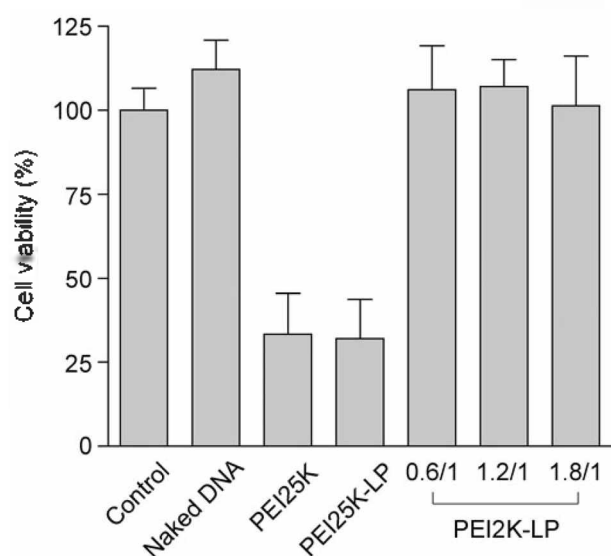


Figure 4. Cytotoxicity of PEI25K, PEI25K-LP and PEI2K-LP. 293 cells were seeded in 96-well microassay plates. PEI25K/DNA (0.8/1), PEI25K-LP/DNA (0.8/1) and PEI2K-LP/DNA complexes were prepared as described in experimental sections. Naked DNA or polymer/DNA complexes were added to the cells and incubated for 4 hrs at 37°C. After the incubation, the transfection mixture was replaced with 100 ml of fresh DMEM medium supplemented with 10% FBS. The cells were incubated for an additional 20 hrs at 37°C. After the incubation, cell viability was measured by MTT assay. The data is expressed as mean values (\pm standard deviation) of five experiments.

PEI25K-LP showed higher level of toxicity compared to PEI2K-LP.

Delivery of polymer/DNA complexes to the isolated mitochondria. For a cell-free assay, mitochondria were isolated from 293 cells. DNA was labeled with fluorescent RITC reagent to identify the localization of polymer/DNA complexes by confocal microscopy. PEI2K-LP/DNA complexes were prepared at a 1.2/1 or 1.8/1 weight ratio and PEI25K-LP/DNA complex was prepared at 0.8/1 weight ratio (polymer/DNA). The isolated mitochondria were incubated with naked DNA, PEI25K-LP/DNA complex or PEI2K-LP/DNA complex. After washing, the mitochondria and RITC-labeled DNA/polymer complexes were observed by a confocal microscopy. Mitochondria were stained with Mitotrack green. Naked DNA or PEI/DNA complex showed a few positive signals (Fig. 5A and 5B). However, PEI25K-LP/DNA complex showed much higher level of fluorescent signals of the RITC-labeled DNA, suggesting that PEI25K-LP/DNA complexes were associated with mitochondria (Fig. 5C). PEI2K-LP/DNA complexes showed different results depending on the weight ratios of PEI2K-LP/DNA (Fig. 5D and 5E). PEI2K-LP/DNA complex at a 1.8/1 weight ratio had higher efficiency than that at a 1.2/1 weight ratio. Although the efficiency of PEI2K-LP was slightly lower than that of PEI25K-LP, at a 1.8/1 weight ratio of PEI2K-LP/DNA complexes, more amount of fluorescent particles were observed at mitochondria than that of RITC-DNA only or PEI25K/RITC-DNA complexes.

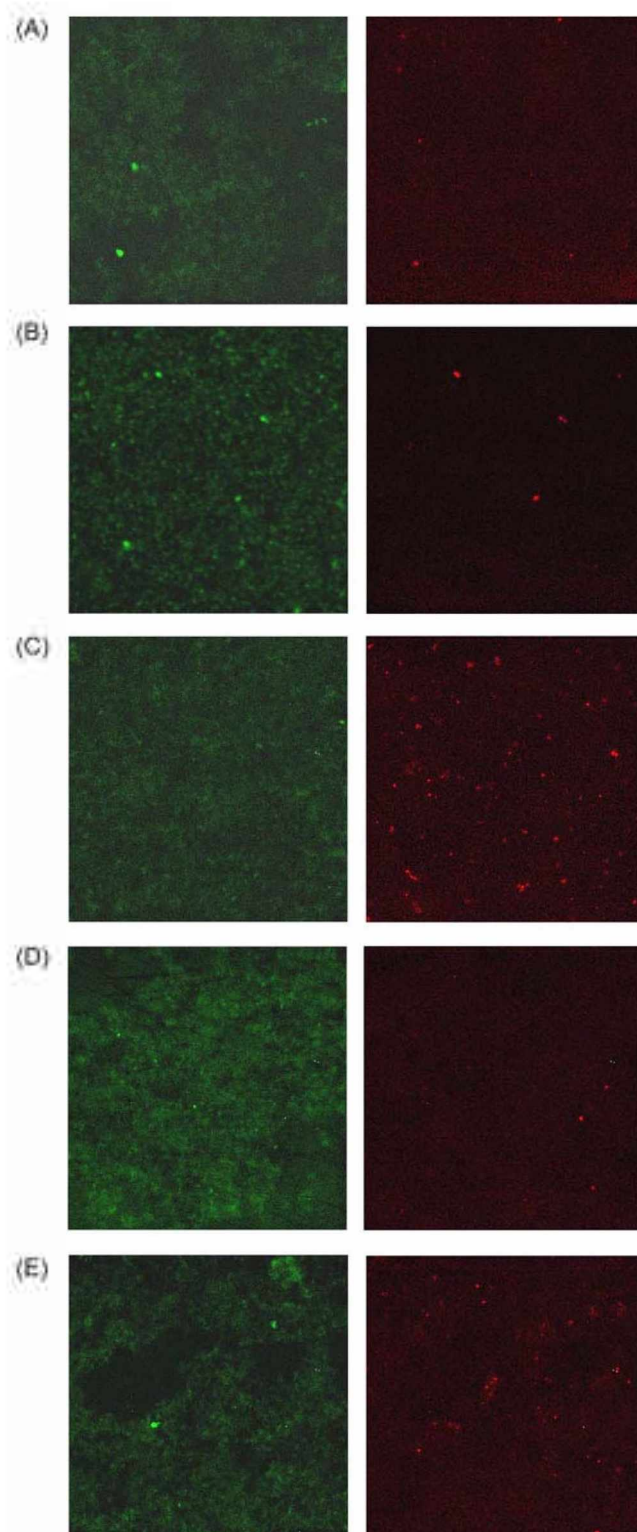


Figure 5. Cell free assay with isolated mitochondria. Mitochondria were isolated from 293 cells. The RITC-labeled DNA was complexed with PEI25k, PEI25K-LP, or PEI2K-LP. DNA only, PEI25K/DNA, PEI25K-LP/DNA or PEI2K-LP/DNA complex was incubated with isolated mitochondria. Mitochondria were stained with Mitotrack green. After washing, the mitochondria were observed on a confocal microscopy. (A) DNA only, (B) PEI25K/DNA (0.8/1), (C) PEI25K-LP/DNA complex at a 0.8/1 weight ratio, (D) PEI2K-LP/DNA complex at a 1.2/1 weight ratio, (E) PEI2K-LP/DNA complex at a 1.8/1 weight ratio.

Discussion

Mitochondrial genetic defects are involved in many diseases such as diabetes, blindness and weakness.^{8,9,12,13} Direct gene delivery to mitochondria has been suggested as a therapeutic option for these diseases. However, currently any effective gene delivery method to mitochondria is not available. Previously, we synthesized PEI25K-LP as a gene carrier to mitochondria.²⁶ Cell free assay with isolated mitochondria or *in vitro* transfection into a living cell showed that PEI25K-LP could deliver DNA to mitochondrial sites. However, PEI25K has significant toxicity to cells and clinical application of PEI25K has been limited. To address the cytotoxicity of the carrier, PEI2K-LP was synthesized and evaluated as a gene carrier to mitochondria.

PEI has been investigated as a gene carrier to various cells because of its high transfection efficiency.^{21,29} In addition, various derivatives or molecular conjugates of PEI have been developed to reduce the cytotoxicity of PEI or to target polymer/DNA complex to a specific organ.^{21,22} It was previously reported that transfection efficiency and cytotoxicity are closely related to the size of PEI.³⁰ It was shown that PEI with higher molecular weight had higher transfection efficiency and cytotoxicity.²⁵ To reduce the cytotoxicity, polyethylene glycol (PEG) has been conjugated to PEI.²² The conjugation of PEG effectively reduced the cytotoxicity of PEI and furthermore increased the solubility of polymer/DNA complex in aqueous solution. However, the transfection efficiency of PEG conjugated PEI was decreased compared to PEI without PEG. Another approach is to use low molecular weight PEI to reduce the cytotoxicity of PEI. PEI2K has much lower cytotoxicity than PEI25K.²⁵ PEI binds on the surface of the plasma membrane by charge interaction and precipitates in huge clusters adhering to the cell surface.³¹ Therefore, charge density of the polymeric carrier is one of the important factors that affect their cytotoxicity. PEI2K has low charge density than PEI25K when complexed with DNA. In Figure 2, PEI2K-LP retarded DNA at a 1.0/1 weight ratio, while PEI25K-LP did at a 0.6/1 weight ratio. It may be due to the lower charge density of PEI2K. In addition, the cytotoxicity of PEI2K-LP was much lower than that of PEI25K-LP. In Figure 4, PEI2K-LP did not show any detectable cytotoxicity to cells in the tested range of weight ratio. Instead, PEI2K-LP has lower DNA delivery efficiency than PEI25K-LP. However, PEI2K-LP had substantial delivery efficiency compared to naked DNA or PEI/DNA complex. Also, at a 1.8/1 weight ratio, PEI2K-LP had DNA delivery efficiency comparable to PEI25K-LP.

One of the requirements for gene therapy is the stability of DNA in the process of delivery. DNA is easily degraded by enzymatic attack and loses its activity.²⁸ Therefore, gene delivery carrier should be able to protect DNA from nucleases. As shown in Figure 3, PEI2K-LP protected DNA up to 60 min when the complex was prepared at a 1.8/1 weight ratio.

Gene delivery to mitochondria has been thought as

speculative, since a definite gene delivery method has not been developed. Many barriers should be overcome to realize the direct mitochondrial gene therapy. The previous studies showed that LP-linked DNA could be internalized into mitochondria.¹⁵ They showed that substantially large DNA-LP could be imported to mitochondrial matrix. However, DNA without a gene carrier displays very low efficiency in cellular uptake,^{20,21,27,32-34} suggesting that a gene carrier is required to facilitate cellular uptake of DNA. Current study showed that LP-conjugated PEI2K could deliver DNA to mitochondrial sites without significant cytotoxicity to cells. Therefore, PEI2K-LP may be useful for the development of mitochondrial gene delivery system.

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