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molecular pharmaceutics

Synergistic Effect of Low Cytotoxic Linear Polyethylenimine and Multiarm Polyethylene Glycol: Study of Physicochemical Properties and *In Vitro* Gene Transfection

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Received April 1, 2009; Revised Manuscript Received August 11, 2009; Accepted September 30, 2009

Abstract: Novel star-shaped copolymers consisting of multiarm polyethylene glycol and low molecular weight linear polyethylenimines (MAPEG-LPEIs) with a high transfection efficiency and low cytotoxicity were designed and synthesized as nonviral gene delivery carriers. The cationic polymers were prepared by conjugating low molecular weight linear PEI (2.5 kDa) to six-arm PEG-NHS (10 kDa) in two different compositions. Two copolymers, MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$ with molecular weights of 17.5 kDa and 25 kDa respectively, were synthesized. The MAPEG-LPEI$_3$/pDNA and MAPEG-LPEI$_6$/pDNA polyplexes are stably dispersed in aqueous media with a narrowly distributed size range of <200 nm as determined by dynamic light scattering. Furthermore, these polyplexes showed different surface charges depending upon the relative proportion of MAPEG and LPEI. Moreover, these polyplexes can protect pDNA from enzymatic degradation in serum containing media up to 24 h. These polyplexes were able to efficiently transfect luciferase-coded reporter gene into HeLa cancer cells and showed considerable gene transfection efficacy even in 50% serum-conditioned media *in vitro*. MAPEG-LPEI$_6$ exhibited higher transfection activity than that of MAPEG-LPEI$_3$ at the same weight ratios. Furthermore, MAPEG-LPEI/pDNA polyplexes were less toxic than LPEI/pDNA complexes as determined by MTT assay. These favorable results could be attributed to the combined effect of low molecular weight LPEI and multiarm PEG. The special structural features of the multiarm star-shaped central PEG core play an important role in achieving higher transfection efficiency as it imparts higher charge density to polyplexes and prevents the unwanted aggregation of the smaller polyplex particles. These two important factors contributed toward enhanced gene transfection. On the other hand, LPEI provides low cytotoxicity and effective complexation with pDNA in the designed architecture. Therefore it is possible to achieve enhanced gene transfection by using these two components, namely, pivotal multiarm PEG core and LPEI, in optimal ratio as observed in the case of MAPEG-LPEI$_6$.

Keywords: Star-shaped polymer; gene delivery; polyethylenimine; multiarm polyethylene glycol; transfection

Introduction

In gene therapy, viral gene carriers such as retroviruses, adenoviruses, and adeno-associated viruses have been known to have high transfection efficiency when compared to nonviral gene carriers. However these viral vectors have several drawbacks, such as targeting only dividing cells, random DNA insertion, low capacity for carrying large therapeutic genes, risk of replication, and possible host immune reaction.[1,2] Therefore, several nonviral gene delivery systems including cationic polymers or lipids have been

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10.1021/mp900096u CCC: $40.75
Published on Web 09/30/2009

investigated extensively as a means to overcome the disadvantages of viral vectors. One of the most promising nonviral gene carriers is polyethylenimine (PEI). These polymers, which have a great variety of molecular weights and degrees of branching, have been extensively studied *in vitro* as well as *in vivo* since their initial use as gene carrier. PEIs have an advantage over other polycationic polymers in that they possess remarkable DNA condensation ability, lysosomal buffering capacity, and potential ability to protect DNA from enzymatic degradation.[3−6] The molecular weights of commercially available PEIs vary from 423 Da to 800 kDa with both linear and branched geometries. The transfection efficiency of PEIs, along with their cytotoxicity, strongly depends on their molecular weight.[7,8] It is well-known that high molecular weight, branched PEI (HMW BPEI), with a molecular weight greater than 25 kDa, shows higher transfection efficiency than low molecular weight branched PEI (LMW BPEI). In spite of its excellent transfection ability, HMW BPEI is also known to induce substantial cytotoxicity.

LMW BPEI, which is less toxic, cannot effectively condense DNA and displays very poor transfection activity.[9,10] However, the relatively low cytotoxicity of LMW BPEI could be utilized to achieve relatively high gene expression by administration of higher doses of the polycation in gene delivery experiments.[8] But Nguyen et al. reported that the complexes derived from DNA and LMW PEI, which have a molecular weight of 2000 Da, were unstable and precipitated out of the buffer solution.[11] This made the determination of complex size and the gene expression activity in transfection experiments impossible.

In order to overcome the high toxicity of HMW PEI, several strategies have been adopted. Polyethylene glycol (PEG) has been conjugated to HMW PEI as a hydrophilic segment, and its efficacy has been investigated by several groups.[12−14] PEG-conjugated copolymers of HMW PEI show decreased cytotoxicity and improved serum stability.[15] However, the conjugation of PEG often results in diminished transfection efficiency due to decreased cellular association and internalization.[16−18] An alternative strategy is to synthesize biodegradable, modified, PEI derived from LMW PEI and PEG which exhibits both improved transfection efficiency and low cytotoxicity.[19] Cross-linked LMW PEI and PEG joined by a disulfide bond has also been shown to transfect DNA with higher efficacy and lower cytotoxicity.[20−22] The last two strategies are based on the fact that HMW PEI condenses the DNA more compactly than LMW PEI does. In the condensed form, the DNA is protected against digestion by enzymes and becomes a compact and small unit. This small size enables the DNA to move through diverse barriers toward the nucleus of the target cell where the gene can be expressed.

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More recently, several *in vitro* and *in vivo* studies have investigated the potential of linear PEI (LPEI)-derived vectors.[23,24] Though LPEI/DNA formulations display comparable transfection efficiency to BPEI/DNA complexes, they exhibit less cytotoxicity than that of BPEI/DNA formulations.[25]

In order to increase the transfection efficiency of LPEI, it is desirable to increase the cationic charge density of the polymer. To achieve this, a high charge density, star-shaped polymer has been designed. This polymer utilizes small molecular weight cationic polymers which are conjugated to a central multivalent polymer core.[26−28]

Recently, Uchida et al. reported on a novel star-shaped copolymer consisting of cationic chains as an inner domain and nonionic chains as an outer domain and evaluated its use as a polymeric transfectant.[26,27] Further, Reineke et al. reported that a peptide-functionalized multiarm PEG star copolymer is an efficient gene delivery vector. The multivalency of these star polymers may improve transfection by allowing simultaneous binding of the polymer to both the DNA and to the heparin sulfate domain on the surface of the cell.[28]

In contrast to conventional cationic polymers, which contain a long sequence of covalently bonded repeating units, the star-shaped gene carriers have been designed on the basis of a new strategy. Many cyclic cationic units are threaded onto a polymer chain to form a supramolecular entity, which functions as a macromolecular gene carrier. This kind of polymeric architecture has shown excellent DNA binding ability, low cytotoxicity, enhanced protection of the DNA, and high gene transfection efficiency.

Theoretically, conjugation of the LMW LPEI with multiarm PEG will provide a star-shaped copolymer which has a high charge density, improved gene transfection efficiency, and low cytotoxicity.

In this study, we synthesized novel cationic star-shaped polymers by conjugating LMW LPEI to multiarm PEG core. We found that these novel multiarm PEG-PEI star-shaped copolymers not only protected DNA from enzymatic degradation but also exhibited higher gene transfection efficiency in comparison to PEI having a similar molecular weight. We also investigated the physicochemical properties of DNA/polymer complexes in this report.

Experimental Section

Materials. The 6-arm polyethylene glycol succinimidyl succinate (6-arm PEG, MW 10 kDa) was purchased from SunBio, Inc. (Daejun, Korea). Linear polyethylenimine (LPEI, MW 2.5 kDa) and branched PEI (BPEI, MW 1.8 kDa) were obtained from Polyscience, Inc. (Warrington, PA). High molecular weight PEIs (BPEI25K, LPEI25K) were purchased from Sigma Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), and Dulbecco's phosphate buffered saline (DPBS) were purchased from Invitogen-Gibco (Carlsbad, CA). A luciferase assay system with reporter lysis buffer was purchased from Promega (Madison, WI), and a bicinchoninic acid (BCA) protein assay reagent kit was from Pierce Chemical Co. (Rockford, IL). Plasmid DNA (pDNA) was propagated in a chemically competent DH5α strain (GibcoBRL) and prepared from overnight bacterial cultures by alkaline lysis and column purification with a Qiagen plasmid Maxi kit (Qiagen, Valencia, CA). The concentration of pDNA solution was determined by measuring the absorbance at 260 nm, and its optical density at 260 to 280 nm was determined to be in the range of 1.8 to 1.9. Cell viability was estimated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO).

Synthesis of MAPEG-LPEI$_x$. LPEI was conjugated to the multiarm polyethylene glycol (MAPEG) at two different ratios to obtain MAPEG-PEI$_3$ and MAPEG-PEI$_6$. A solution of LPEI (450 mg, 0.18 mmol) dissolved in 5 mL of PBS and 2 M HCl (1.1 mL) was added dropwise to a solution of 6-arm PEG (300 mg, 0.03 mmol) in PBS buffer (1.5 mL). Six molar excess amount of LPEI was added to the PEG. The reaction mixture was stirred at room temperature for 24 h, followed by dialysis against distilled water (MWCO 3500 Da) for 1 day to remove the unreacted LPEI. The purified product was harvested by freeze-drying to generate MAPEG-PEI$_3$. MAPEG-PEI$_6$ was synthesized following the same procedure as above except LPEI (900 mg, 0.36 mmol) and 6-arm PEG (300 mg, 0.03 mmol) were used in different stoichiometric ratios. Twelve molar excess amount of LPEI was added to the PEG. The degree of conjugation for both the polymers was calculated using ^{1}H NMR spectroscopy performed on a Bruker DPX 300 MHz.

Gel Permeation Chromatography (GPC). The confirmation of reaction and molecular weight distribution (MWD) of these polymers was analyzed by GPC (Shimadzu, Kyoto,

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Japan) with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan) using a column (TSK gel GMPW, Tosoh Co. Ltd., Japan) at ambient temperature. Deionized water was used as the mobile phase with a flow rate of 1 mL/min.

Agarose Gel Retardation Assay. Polymer/pDNA complexes were prepared at various w/w ratios (the ratio of weight of polymer to DNA) ranging from 0 to 10 in PBS buffer and incubated for 30 min at room temperature. The polyplexes were then electrophoresed through a 1% (w/v) agarose gel containing ethidium bromide (EtBr) (0.5 μg/mL) in 0.5 X TAE (Tris-acetate-EDTA) buffer at 100 V for 20 min. Naked pDNA was used as a control. The gel was then analyzed on a UV illuminator (WiseUVWUV, DAIHAN Scientific, Seoul, Korea) to observe the position of the complexed pDNA relative to that of the naked pDNA.

Particle Size and Zeta Potential Measurements. Polyplexes were prepared at various w/w ratios from 0.5 to 30 by adding the polymer solution to the pDNA solution and diluted by PBS (pH 7.4, 140 mM NaCl). The final pDNA concentration was adjusted to 33 μg/mL. The mixtures were then incubated for 30 min at room temperature. The particle size of each sample was measured by particle size analyzer using a Zetasizer Nano S (Malvern Instruments, Malvern, U.K.). Surface charge was measured by determination of zeta potential using a Zetasizer Nano Z (Malvern Instruments, Malvern, U.K.) in PBS buffer.

Stability Test of pDNA in Serum. MAPEG-LPEI/pDNA complexes (w/w ratio = 5) or free pDNA (10 μg) was incubated at 37 °C in PBS solution containing 30% FBS. Ten microliter aliquots of each sample were taken at 0, 1, 3, 6, 12, and 24 h of incubation and pipetted into Eppendorf tubes. These tubes were stored at −80 °C until testing. The samples were then thawed and mixed with 2 μL of 10% sodium dodecyl sulfate (SDS) to dissociate the polymers from pDNA and then analyzed by electrophoresis using 1% agarose gel at 100 V for 20 min. Following electrophoresis, gels were then stained with EtBr and visualized on a UV transilluminator (WiseUVWUV, DAIHAN Scientific, Seoul, Korea).

Cell Culture. Human cervix epithelial carcinoma (HeLa), murine fibroblast (NIH3T3) cell lines were cultured in DMEM medium, and human prostate adenocarcinoma (PC-3) cells were cultured in RPMI medium, both containing 10% FBS and 1% antibiotics. Every other day, the medium was replaced until cells became 80% confluent and then subcultured using 0.25% trypsin/EDTA.

Luciferase Reporter Gene Expression. Cells were seeded on 24-well culture plates at an initial density of 4×10^4 cells/well and were incubated for 24 h in 500 μL of DMEM containing 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2. In order to form polyplexes, a solution of 1 μg of pDNA in 10 μL of PBS buffer was added to 10 μL of polymer solution at predetermined w/w ratios and incubated for 30 min at room temperature. The cells were then incubated with the polyplexes in 250 μL of serum-free (or 50% FBS) DMEM media for 4 h, followed by an additional incubation for 20 h in 500 μL of DMEM media containing

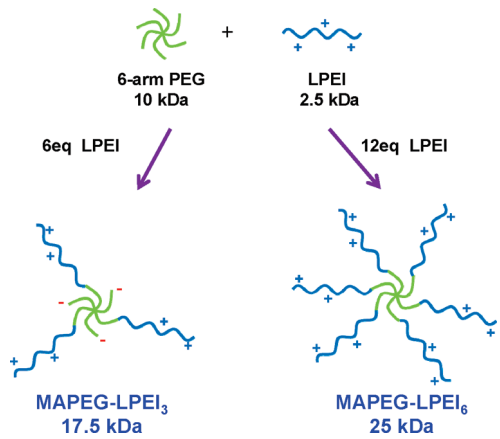


Figure 1. Schematic illustration of MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$.

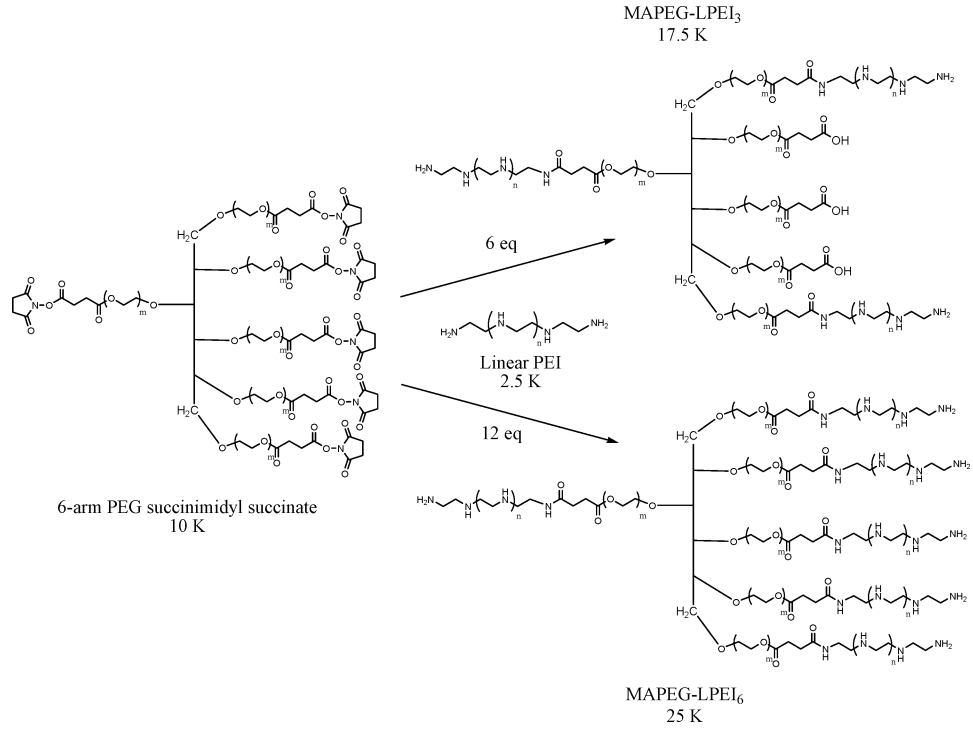
10% FBS. The cells were then washed twice with 500 μL of PBS and lysed by the addition of 200 μL/well of lysis buffer. Luciferase gene expression was evaluated using a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer, Wellesley, MA). The results are presented as mean and standard deviation of the mean obtained from three samples. In the case of PC-3 cell and NIH3T3 cells, the reporter gene expression tests also followed the same procedures as HeLa cells except media.

Cell Viability Assay. The cytotoxicity of polymer/pDNA complexes was evaluated using the standard MTT assay protocol. Briefly, HeLa cells were seeded onto 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. pDNA (0.2 μg/μL) was complexed with the polymer at predetermined w/w ratios in PBS buffer and incubated for 30 min before use. Polyplexes were incubated with the cells for 4 h in 100 μL of serum-free DMEM media followed by 20 h in 200 μL of DMEM media containing 10% FBS. These mixtures were replaced with 200 μL of fresh media and 20 μL of 5 mg/mL MTT solution and incubated for another 4 h. The media was then removed, and 150 μL of DMSO was added into each well to dissolve the internalized purple formazan crystals. An aliquot of 100 μL was taken from each well and transferred into a fresh 96-well plate. The absorption was measured at 570 nm using a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer-Wellesley, MA). The relative percentage of the control (untreated) cells, which were not exposed to the transfection system, was used to represent 100% cell viability.

Results and Discussion

Synthesis of MAPEG-LPEI$_x$. MAPEG-LPEI$_x$ were synthesized following the scheme as depicted in Figure 1. A 6-arm PEG was treated with LPEI at two different molar ratios separately. To synthesize MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$, we used MAPEG and LPEI in molar ratio of 1:6 and 1:12 respectively. As the reaction progressed, NHS was parted from the end group of each arm of the PEG molecule resulting in the formation of amide bonds between PEG and PEI (Scheme 1). At the same time, the unreacted ester functional groups of each PEG were hydrolyzed. The degrees

Scheme 1. Synthetic Scheme of MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$



of conjugation were calculated from the ^{1}H NMR spectrum. The proton signals of the 6-arm PEG and LPEI were observed at 3.69 (Figure 2A) and 2.71 ppm (Figure 2B) respectively. Both of the MAPEG-LPEI conjugated products showed two peaks, one at 3.70 and one at 3.01 ppm (Figure 2C,D). The integration ratios of these peaks at δ 3.70 and 3.01 ppm of MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$ were found to be 6:3 and 6:6 respectively. Thus in the former case, each 6-arm PEG is conjugated to three LPEI chains, and in the latter case, six LPEI chains were attached to each molecule of the 6-arm PEG. Therefore it is confirmed that MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$ were synthesized with the desired compositions. There is no ambiguity in establishing the structural composition of MAPEG-LPEI$_6$ as the unreacted

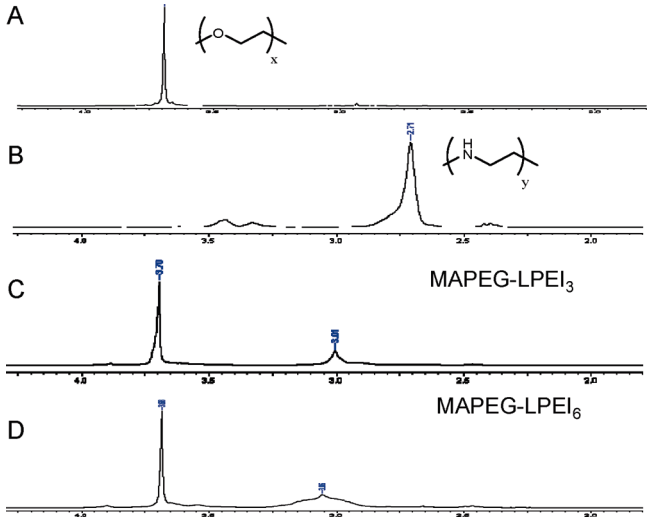


Figure 2. ^{1}H NMR spectra of PEG (A), LPEI (B), MAPEG-LPEI$_3$ (C) and MAPEG-LPEI$_6$ (D).

LPEI was removed by dialysis through membrane (MWCO 3500 Da). In the case of MAPEG-LPEI$_3$ the conjugation ratio is obvious from the ^{1}H NMR data, however the structure could not be established so emphatically as done with MAPEG-LPEI$_6$. Though the conjugated ratio of LPEI and PEG-core was indicated by the ^{1}H NMR as 1:3 for MAPEG-LPEI$_3$, there is a possibility that other polymers having different conjugation ratio may also be present in the sample. However the proportions of these polymers are expected to be insignificantly small with respect to the transfection efficiency and other physiochemical properties. Other concerns regarding the synthesis of MAPEG-LPEI$_3$ were the potential intramolecular conjugation and intermolecular cross-linking. To minimize the intramolecular conjugation which could arise due to the possible intramolecular conjugation of the two ends of the same LPEI chain to the two arms of the same PEG core, we have carried out the reaction by dropwise addition of LPEI to MAPEG solution. We further argued that the linearity of the attached LPEI chain might prevent it from approaching the adjacent PEG arm of same multiarm core which required bending of the LPEI chain. The potential steric hindrance may also inhibit such intramolecular conjugation. The most comprehensive evidence for such a structural disposition could be deduced from the zeta potential value. The low positive zeta potential value is indicative of the free carboxylic moieties which neutralize the positive charge of LPEI moieties (Figure 6). The intramolecular cross-linking is expected to minimize the number of the free carboxylic moieties, and no such lowering of the positive zeta potential value could have taken place. Hence the observed lowering of the zeta potential rules out any intramolecular cross-linking.

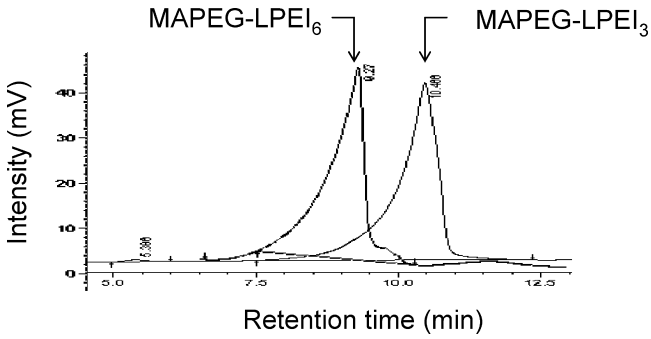


Figure 3. GPC chart of MAPEG-LPEI$_6$ (25 kDa, 9.27 min) and MAPEG-LPEI$_3$ (17.5 kDa, 10.47 min).

Again to prevent or minimize the possible intermolecular cross-linking we have carried out the reaction in relatively dilute conditions. Indirect evidence can be obtained if we consider the solubility of the product. The intermolecular cross-linking is expected to impart low solubility to the polymer. Therefore considering the good solubility of the polymers in aqueous media (PBS) it can be argued that the extent of intermolecular cross-linking is less, if it even takes place at all.

These NMR results were confirmed by gel permeation chromatography (Figure 3). Retention times of MAPEG-LPEI$_6$ and MAPEG-LPEI$_3$ were 9.27 and 10.47 min respectively. This indicates that MAPEG-LPEI$_6$ is larger than MAPEG-LPEI$_3$, and this result is in accord with NMR results.

Polyelectrolyte Complexation Profile of Polymer with pDNA. Polyplexes were prepared by mixing each polymer solution with the pDNA solution at various w/w ratios. To study the formation of polyplexes, gel retardation assay was carried out using 1% agarose gel (Figure 4). Naked pDNA was used as a control. The polyplexes successfully retarded pDNA migration at w/w ratio 1, 2, and 1 for LPEI, MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$, respectively, as shown in Figure 4. These results indicate that each polymer and pDNA forms a polyplex through effective and complete complexation at certain w/w ratios. In spite of a hydrophilic PEG core, MAPEG-LPEI$_6$ was found to possess almost the same magnitude of attractive force toward the negatively charged phosphate backbone of pDNA as possessed by LPEI. However, the differential attractive force in MAPEG-LPEI$_3$ and LPEI might arise due to the structural and compositional diversity of LPEI and MAPEG-LPEI$_3$. LPEI, which has a linear configuration, can compact pDNA efficiently due to its highly exposed positive surface charge. In the case of MAPEG-LPEI$_3$, the complexation between pDNA and MAPEG-LPEI$_3$ was greatly reduced due to the steric hindrance offered by the partially conjugated central multiarm core, and therefore we assumed that a lesser amount of LPEI surface is available for complexation. Another important factor which may dominate the complexation with pDNA is the possibly generated negatively charged carboxylic groups at the end of free arms of the star-shaped PEG core. These negatively charged end groups should repel the negatively charged pDNA. This phenomenon is substantiated by experimental

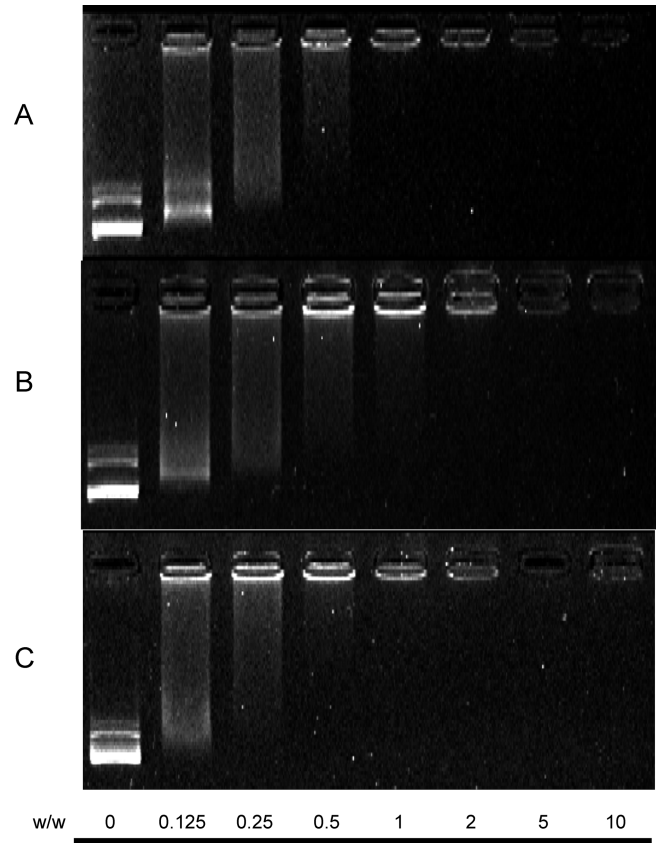


Figure 4. Electrophoretic patterns of pDNA complexes with LPEI25 K/pDNA (A), MAPEG-LPEI$_3$/pDNA (B), and MAPEG-LPEI$_6$/pDNA polyplex (C) at various w/w ratios.

data such as gel retardation assay and zeta potential measurements. Therefore MAPEG-LPEI$_3$ generates a successful polyplex only at a relatively high w/w ratio despite its star-shaped structure. On the contrary, MAPEG-LPEI$_6$ does not have any free carboxylic acid group as all PEG arms are conjugated with LPEI and so pDNA does not encounter any sort of electrostatic repulsion in approaching the LPEI residues. Moreover, MAPEG-LPEI$_6$ contains a higher proportion of LPEI hinged to its central core through amide linkage. This is evident from the zeta potential data. MAPEG-LPEI$_6$ possesses a higher positive surface charge compared to that of MAPEG-LPEI$_3$. Another interesting and promising observation is that MAPEG-LPEI$_6$ possesses less positive surface charge than that of LPEI and still displayed comparable transfection efficiency to that displayed by LPEI. This unique characteristic feature of MAPEG-LPEI$_6$ may arise due to its branched star-shaped structural architecture.

Size and Zeta Potential of Polymer/pDNA Complexes. In order to successfully internalize into the cell, polyplexes must have proper size and surface charge. The particle sizes of these polyplexes were measured by dynamic light scattering (DLS) at various w/w ratios (Figure 5). The investigation revealed that though LPEI can make a large polyplex particle (>1000 nm) with pDNA, both MAPEG-LPEI$_6$ and MAPEG-LPEI$_3$ can condense pDNA effectively into smaller polyplex particles (<200 nm). The large particle size of the LPEI polyplex may be due to the aggregation of

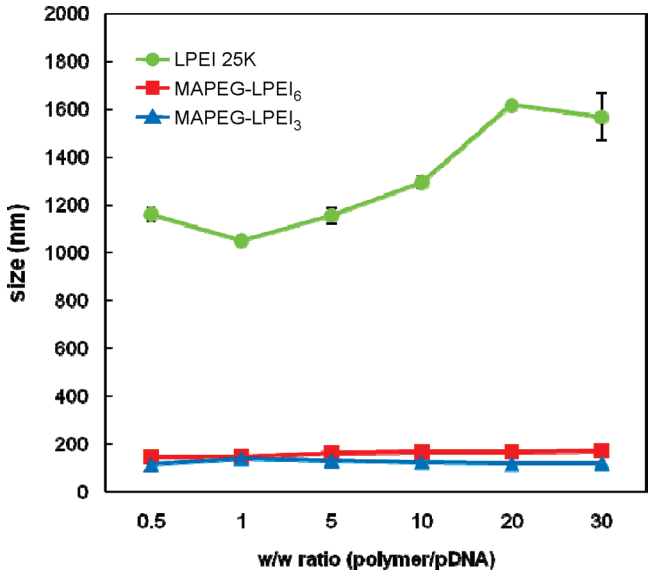


Figure 5. Size of the LPEI 25 K/pDNA (green), MAPEG-LPEI$_3$/pDNA (blue), and MAPEG-LPEI$_6$/pDNA polyplex (red) at various w/w ratios.

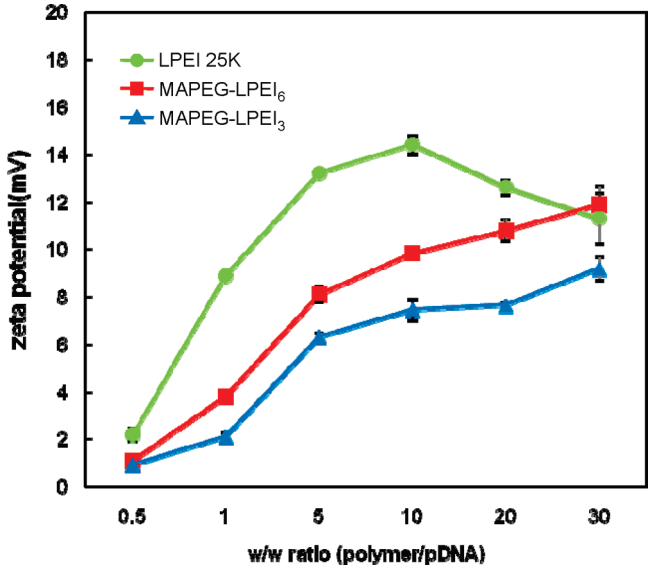


Figure 6. Zeta potential of the LPEI 25K/pDNA (green), MAPEG-LPEI$_3$/pDNA (blue), and MAPEG-LPEI$_6$/pDNA polyplex (red) at various w/w ratios.

small polyplexes, which lack a hydrophilic spacer such as PEG. In addition to this, the long length and linearity of LPEI restricts its flexibility, resulting in an overall larger size. On the other hand, star-shaped MAPEG-LPEI does not aggregate due to the presence of hydrophilic PEG chains. These features could make MAPEG-LPEI polyplexes potential candidates for gene delivery vectors.

The surface potentials of these polyplexes were also measured at various w/w ratios (Figure 6). All polyplexes have positive surface potential over the w/w ratio 0.5. LPEI showed the highest surface charge over the entire range, because it does not have a hydrophilic spacer which may shield the positive surface charge. These results are in accordance with the observation of the gel retardation assay. MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$ showed lower surface charge than unmodified LPEI due to the presence of the neutral central PEG core which to some extent shields the positive surface charge of LPEI. However MAPEG-LPEI$_3$ possesses an even smaller positive charge than that of MAPEG-LPEI$_6$. The presence of free carboxyl groups, which were generated by the hydrolysis of unreacted end groups of PEG-NHS, accounted for the further decrease in zeta potential. These anionic carboxylic groups are presumed to neutralize the positive charge of the LPEI moieties. Generally, particles with smaller size and higher positive charge display higher transfection efficiency. Therefore it can be inferred that MAPEG-LPEI$_6$ could be a more efficient gene carrier than LPEI or MAPEG-LPEI$_3$ considering its complexing ability and particle size.

Protection of pDNA in Serum Containing Media. Polyplexes protect DNA by physically shielding it from nucleolytic enzymes in serum. Unprotected pDNA is degraded by DNAase within several hours. We evaluated the effectiveness of MAPEG-LPEI polymers in protecting pDNA by the following test. MAPEG-LPEI$_3$/pDNA polyplex and MAPEG-LPEI$_6$/pDNA polyplex were incubated at 37 °C in DMEM media containing 30% FBS, and free pDNA was used as a control. The extent of degradation of pDNA was observed by agarose gel electrophoresis at the indicated time intervals. For free pDNA, the intact DNA band disappeared within 6 h of incubation (Figure 7). In the case of the MAPEG-LPEI$_3$/pDNA polyplex and the MAPEG-LPEI$_6$/pDNA polyplex, however, the band of pDNA was preserved up to 24 h. This suggests that MAPEG-LPEI protects pDNA from degradation by serum enzymes. This protective polymer envelope reduces the chance of enzymes coming in contact with the pDNA. MAPEG-LPEI can protect pDNA from serum enzymes, and this phenomenon could be a merit to *in vitro* and *in vivo* experiments.

***In Vitro* Gene Transfection.** To estimate the transfection efficiency of MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$, their complexes with pCMV-Luc reporter gene encoding luciferase were incubated with several cell lines including HeLa, NIH3T3, and PC-3 cells at various w/w ratios ranging from 5 to 30. Unmodified LPEI (25 kDa) polyplex was used as a control. The MAPEG-LPEI$_6$ showed the highest transfection efficiency through all w/w ratios and attained a maximum value at w/w ratio of 5 in HeLa cancer cell lines, as shown in Figure 8A. Indeed the MAPEG-LPEI$_6$ expressed at least 10-fold higher gene transfection efficiency than MAPEG-LPEI$_3$ and LPEI polyplex at all w/w ratios. MAPEG-LPEI$_3$ showed similar transfection efficiency to LPEI except at a w/w ratio 30.

Despite having a lower proportion of PEI than LPEI25 K, MAPEG-LPEI$_6$ shows higher transfection efficiency than that of LPEI. The functional differences could be attributed to structural differences of the polymers. MAPEG-LPEI$_6$ has a star-shaped structure, which exposes a large density of LPEI on its exterior. As a result of this special structure, MAPEG-LPEI$_6$ can make a more highly charged and compact polyplex particle than LPEI does. The highly

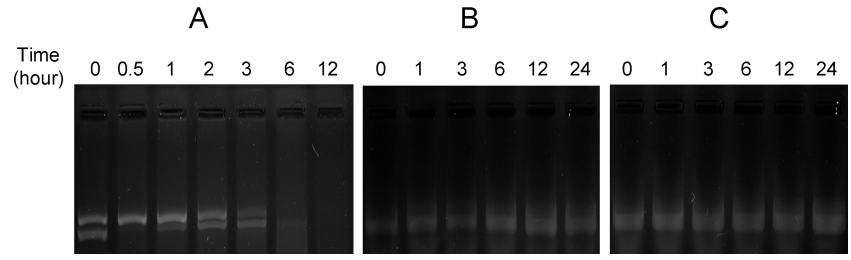


Figure 7. pDNA protection assay in serum. Degradation of pDNA exposed to serum was observed for free pDNA (A), MAPEG-LPEI$_3$/pDNA polyplex (B), and MAPEG-LPEI$_6$/pDNA polyplex (C). The polyplexes were incubated in 30% FBS media from 0 to 24 h at w/w ratio 5. Undegraded, intact pDNA was detected following electrophoresis and EtBr staining.

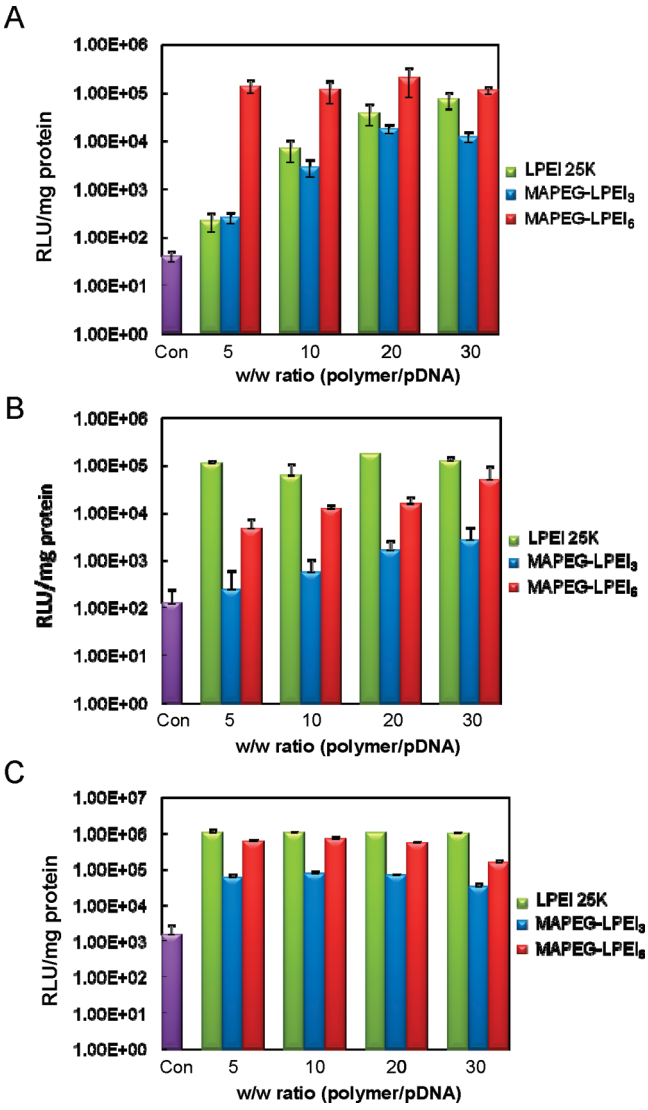


Figure 8. Transfection efficiency of control (purple), LPEI 25 K/pDNA (green), MAPEG-LPEI$_3$/pDNA (blue), and MAPEG-LPEI$_6$/pDNA polyplex (red) in HeLa cells (A), pc-3 cells (B), and NIH3T3 cells (C) lines at various w/w ratios.

positively charged MAPEG-LPEI$_6$ polyplex surface enables it to attach to a negatively charged cell membrane more easily. Moreover, the small size of the MAPEG-LPEI$_6$ polyplex assists in effective endocytosis. Therefore, the MAPEG-LPEI$_6$ can deliver pDNA to cells more efficiently than LPEI. In comparison to the MAPEG-LPEI$_6$, MAPEG-LPEI$_3$ has half the PEI content with respect to each MAPEG core. Unlike MAPEG-LPEI$_6$, which has excess LPEI that generates a positive surface charge upon complexation with pDNA, MAPEG-LPEI$_3$ does not have enough LPEI on the polyplex. The less positive charge of MAPEG-LPEI$_3$ polyplex obstructs its attempt to pass through the cell membrane. This factor can explain the lower transfection efficiency of MAPEG-LPEI$_3$ than MAPEG-LPEI$_6$. These observations show that a higher density of PEI induces higher transfection efficiency. In other cell lines such as NIH3T3 and PC-3 cell lines, MAPEG-LPEI$_6$ showed a little lower or similar transfection efficiency as compared to LPEI, as shown in Figure 8B (PC-3) and Figure 8C (NIH3T3). These results suggested that MAPEG-LPEIs have cell line dependency, however MAPEG-LPEI$_6$ showed higher gene expression than MAPEG-LPEI$_3$ in all three cell lines. Further cell line screening and discussion are required for intensive evaluation as a gene carrier.

We then investigated the transfection efficiency of polyplexes in serum-conditioned media. The transfection efficiency of BPEI was reduced in 50% serum-conditioned media as compared to serum free media as shown in Figure 9A. *In vitro*, a reduced transfection efficiency is often been observed for BPEI in the presence of serum. In fact, serum may strongly interfere with polyplex size, by preventing particle clustering, and affect particle stability. On the contrary, LPEI25K showed slightly enhanced gene expression even in serum-conditioned media. These results imply that the conformation of BPEI in serum is quite different compared to LPEI. Further, LPEI-based star-shaped polymers showed considerable gene expression in serum and MAPEG-LPEI$_6$ showed higher transfection efficiency than that exhibited by LPEI25K in 50% serum medium at w/w ratio of 10 (Figure 9B). MAPEG-LPEI$_6$ showed almost similar transfection efficiency as LPEI25K displayed in serum medium at other w/w ratio. These results imply that although the conformation and the orientation of the LPEI chains were changed in branched type star-shaped PEG core, LPEI preserved its intrinsic property associated with transfection.

We also investigated the effect of shape and MW of polymers upon the transfection efficiency with or without serum. Interestingly, in 50% serum-conditioned media BPEI25K, BPEI1.8K and LPEI2.5K mediated transfections

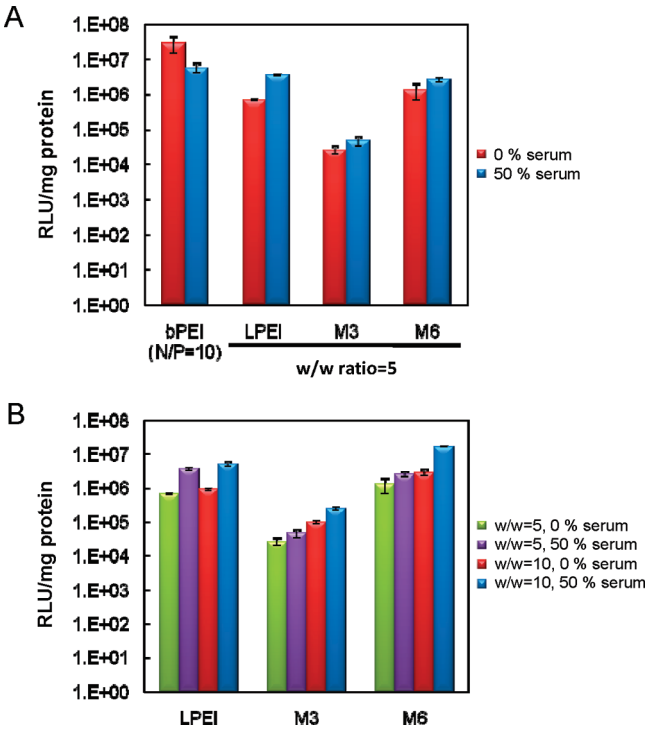


Figure 9. Effect of serum in luciferase gene expression in HeLa cell line. Transfection efficiency of various carriers in serum free (red) or 50% serum (blue). BPEI25K/pDNA (N/P = 10), LPEI25K/pDNA (w/w = 5), MAPEG-LPEI$_3$/pDNA (M3, w/w = 5), and MAPEG-LPEI$_6$/pDNA (M6, w/w = 5) (A). Transfection efficiency of various carriers at w/w ratio 5 or 10 in serum free or 50% serum. LPEI25K/pDNA, MAPEG-LPEI$_3$/pDNA (M3), and MAPEG-LPEI$_6$/pDNA (M6) (B).

were inhibited, whereas LPEI25K exhibited enhanced transfection efficiency in 50% serum-conditioned media as shown in Figure S1 in the Supporting Information. It is also noteworthy that, despite the decreased transfection profile of LMW LPEI2.5K in serum-conditioned media, the star-shaped MAPEG-LPEI showed increased transfection efficiency even in serum-conditioned media. Therefore the star-shaped core and its conjugation with LMW LPEI have imparted these favorable traits into these unique polyplexes.

Cytotoxicity of Polymer/pDNA Complexes. The cytotoxicity of polyplexes formed from MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$ was examined by MTT assay using LPEI25K as a control. MAPEG-LPEI$_3$ polyplexes have exhibited the lowest cytotoxicity (Figure 10). The MAPEG-LPEI$_6$ polyplexes had similar cytotoxicity to LPEI25K. The results of the cytotoxicity study on MAPEG-LPEI$_3$ and MAPEG-LPEI$_6$ are also in agreement with previous outcomes. Owing to the presence of less LPEI, the cytotoxicity is reduced in MAPEG-LPEI$_3$. MAPEG-LPEI$_6$ shows a little higher toxicity than LPEI due to the large density of PEI in MAPEG-LPEI$_6$. The highly positive charge of PEI not only induces higher transfection efficiency but also elicits an increased cytotoxicity. Despite this, the cell viability was not substantially reduced when compared to control cells. Therefore, MAPEG-

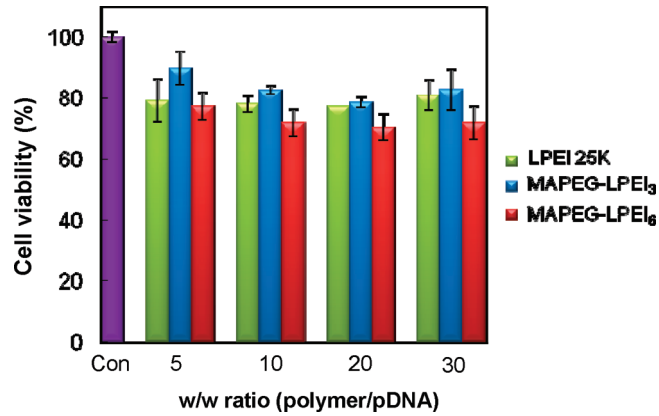


Figure 10. Relative cell viability of control (purple), LPEI 25 K/pDNA (green), MAPEG-LPEI$_3$/pDNA (blue), and MAPEG-LPEI$_6$/pDNA polyplex (red) in HeLa cell lines at various w/w ratios.

LPEI$_3$ and MAPEG-LPEI$_6$ both could be considered as potential gene carriers.

We also studied cell viability of various polymers. As expected enhanced cell viability was observed when transfection was carried out in serum-conditioned media (Figure S2in the Supporting Information). Moreover, HMW BPEI25K showed the highest cell toxicity among these polymers as reported in other literatures.

Conclusions

This work demonstrated the synthesis of novel star-shaped copolymers of 6-arm PEG at two different proportions of LPEI and evaluation of the physicochemical properties of the polymer/pDNA complexes. This study dealt with the transfection efficiency of these polyplexes. The different properties of polyplexes such as size, charge and complexation ability were optimized for these structurally unique copolymers. Individual attributes of the separate components were infused efficiently and judiciously in these newly developed star-shaped polymers. The core multiarm PEG provides the unique structural template which facilitates the spreading of the cationic charges arising from LPEI moieties. This structural architecture also allows LPEI to undergo effective complexation with pDNA which is otherwise difficult to achieve using LPEI alone due to its low charge density.

The transfection profile of MAPEG-LPEI$_3$/pDNA and MAPEG-LPEI$_6$/pDNA polyplexes provides an insight into the correlation between the charge density, relative amount of LPEI, complexation with pDNA and transfection efficiency.

This unique combination of cationic and hydrophilic polymeric moieties along with the special structural architect exerted remarkable effect in enhancing *in vitro* gene transfection efficiency by providing appropriate combination of charge density, complexation ability, nanosized polyplexes and minimum cytotoxicity and even better serum stability. This observation opens up the possibilities to develop more efficient vectors. To our knowledge, this is the first case where such a unique strategy has been employed to

conglomerate all these favorable traits within the same vector utilizing the synergistic effect of star-shaped multiarm PEG and LMW LPEI. We believe that this kind of highly efficient novel star-shaped gene carrier has an immense potential to direct future research toward the development of this type of transfecting reagent for *in vitro* as well as target specific *in vivo* applications.

Acknowledgment. This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A080919). We thank Prof. Kimoon Kim for assistance in the particle size and zeta potential measurement studies.

Supporting Information Available: Bar graphs depicting transfection efficiency (Figure S1) and cell viability (Figure S2) of various carriers. This material is available free of charge via the Internet at http://pubs.acs.org.

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