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Low Molecular Weight PEI Conjugated Pluronic Copolymer: Useful Additive for Enhancing Gene Transfection Efficiency

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Abstract: For enhancing the gene delivery efficiency of polyplexes, a new formulation was developed using PEI conjugated Pluronic F127 copolymer as an effective additive. Low molecular weight, branched polyethylenimine Mw 600 (LMW BPEI 600) was conjugated to the terminal end of Pluronic F127. The PEI-modified Pluronic copolymers formed a micellar structure in aqueous solution, similar to that of unmodified Pluronic copolymer. PEI modification of Pluronic copolymer increased the size of micelles while concomitantly raising the critical micelle concentration (CMC). The PEI-modified Pluronic copolymer was used as a micellar additive to enhance the gene transfection efficiency of pre-formulated polyelectrolyte complex nanoparticles composed of luciferase plasmid DNA and branched PEI Mw 25k (BPEI 25k) or polylysine Mw 39k (PLL 39k). The luciferase gene expression levels were significantly enhanced by the addition of the BPEI-modified Pluronic copolymer for the two formulations of BPEI and PLL polyplexes. The results indicated that the BPEI-modified Pluronic copolymer micelles ionically interacted on the surface of DNA/ BPEI (PLL) polyplexes which might facilitate cellular uptake process.

Keywords: gene delivery, Pluronic, polyethylenimine, polyplexes, transfection.

Introduction

Nonviral gene delivery systems based on cationic lipids and polymers are recently gaining more attention for use in human gene therapy. Although the gene transfection efficiency is still lower than viral vector systems, there are some unique advantages for practical use such as safety, ease of scale-up production, and versatility in cell-specific targeting. Among the cationic polymers, polyethylenimine (PEI) has been widely used to form polyplexes with plasmid DNA.[1-3] The polyelectrolyte complex DNA/PEI nanoparticles exhibited superior gene transfection efficiency to various cells, probably due to stable complex structure appropriate for uptake by cells and the following "proton sponge" effect from PEI that facilitates escape of the complex from an endosomal compartment. High molecular weight branched PEI (HMW BPEI) demonstrates superior gene transfection, however shows severe cytotoxic effects with increasing nitrogen/phosphate (N/P) ratio between BPEI and DNA.[4-10] To solve this cytotoxic problem, chemically altered BPEI species such as poly(ethylene glycol) (PEG) grafted BPEI, PEG blocked BPEI, and self-degradable BPEI were synthesized. The less cytotoxic BPEI derivatives, however, showed reduced gene transfection efficiency compared to unmodified BPEI.[11-14]

Recently, amphiphilic copolymers that self-assemble to form micelles in aqueous solution were used as additives to promote gene transfection efficiency of naked DNA and DNA/PEI polyplexes.[15-19] Among the additives, Pluronic, poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-PPO-PEO triblock copolymer), is particularly effective in enhancing *in vivo* gene transfection efficiency of naked DNA in skeletal muscle.[20-22] We also recently reported that various polyplexes of a biodegradable tri-block copolymer composed of poly(D,L-lactic-*co*-glycolic acid)-*b*-poly(ethylene oxide)-*b*-poly(D,L-lactic-*co*-glycolic acid) (PLGA-PEO-PLGA) significantly increased gene transfection efficiency to different cell lines.[23] The biodegradable tri-block copolymers increased the gene expression level to a greater extent than the Pluronic copolymer. It was postulated that self-assembled polymeric micelles physically interacted with the cellular membrane, which facilitated cellular entry of nano-sized polyplexes by lowering the activation energy of endocytosis.

Previously, Pluronic P123 grafted BPEI (Mw 2,000), in combination with unmodified Pluronic copolymers, was used to form polyplexes with plasmid DNA.[21] The gene transfection levels were largely dependent on the formulation conditions, suggesting that the physical stability of polyplexes was a key determinant in cellular uptake and gene expression.

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However, overall gene transfection efficiency of polyplexes formulated with Pluronic grafted BPEI was still far lower than those formulated with branched HMW BPEI. Molecular weight of BPEI plays a crucial role in stabilizing the polyelectrolyte complex structure and subsequently enhancing the extent of cellular uptake. Although increasing molecular weight of BPEI enhances gene transfection efficiency, it adversely elicits a cytotoxic problem.

In this study, Pluronic F127 was end-capped with LMW BPEI 600 to prepare a molecular structure of PEI-PEO-PPO-PEO-PEI. The LMW BPEI end-capped Pluronic F127 has a different molecular architecture from the above mentioned BPEI-*g*-Pluronic P123 that has multiple Pluronic chains grafted onto the medium MW branched PEI (Mw 2,000). It was expected that the LMW BPEI modified Pluronic copolymers, while maintaining a cationic tri-block copolymer structure, self-assembled to form less cytotoxic cationic micelles that could serve as an effective additive for intracellular uptake of DNA/HMW BPEI complexes. The micellar characteristics of PEI conjugated Pluronic F127, such as size and critical micelle concentration, were evaluated compared to those of unmodified Pluronic F127, and their additive effects on gene transfection of BPEI and polylysine (PLL) polyplexes were examined in a dose-dependent manner.

Experimental

Materials. Pluronic F127 was purchased from BASF Corporation (Mount Olive, NJ). Branched poly(ethylenimine) (Mw 25,000, HMW BPEI; Mw 600, LMW BPEI) were obtained from Aldrich (Milwaukee, WI). *p*-nitrophenyl chloroformate and poly(L-lysine) (Mw 39,000) were purchased from Sigma (St. Louis, MO). Phosphate-buffered saline (PBS), RPMI 1640 medium, fetal bovine serum (FBS), Lipofectamine reagent, penicillin G sodium, streptomycin sulfate, amphotericin B, and trypsin were the products of Gibco-BRL (Grand Island, NY). Dialysis membrane (MWCO 1,000) was purchased from Spectrum (Houston, TX). Microbicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). 293T cells were supplied from the Korean Cell Line Bank (Seoul, South Korea). Plasmid DNA (pCMV-Luciferase) was extracted from transformed *E. coli* by a standard alkaline lysis technique[24] and purified by a DNA-purification column (QIAGEN, CA). The purity and concentration of plasmid DNA was determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer (UV-1601, Shimadzu, Japan).

Activation of Pluronic F127 with *p*-Nitrophenyl Chloroformate. Pluronic F127 was activated with *p*-nitrophenyl chloroformate as described previously.[25,26] Two grams of Pluronic F127 dissolved in 6 mL of benzene was slowly added to a stirred solution of 192 mg of *p*-nitrophenyl chloroformate (stoichiometric ratio of *p*-nitrophenyl chloroformate and -OH was 3:1) in 6 mL of benzene. The reaction was progressed at room temperature for 24 h under nitrogen atmosphere, and the product was precipitated with diethyl ether, recovered by filtration, and re-dissolved in benzene. The above procedure was repeated three times and the activated Pluronic F127 was completely dried under vacuum. The degree of activation was determined spectrophotometrically by measuring the concentration of *p*-nitrophenolate ion released in an alkaline solution. For this purpose, an accurately weighed sample of the *p*-nitrophenyl chloroformate-activated Pluronic F127 was dissolved in 0.2 M NaOH. After 2 h of mixing under rotation, its absorbance was measured at 410 nm and converted to a concentration, using a molar extinction coefficient of 17,400 cm^{-1} M^{-1}. The activated Pluronic F127 was also confirmed by ^{1}H-NMR analysis in *d6*-DMSO (Bruker, DRX 400).

Synthesis of PEI-Pluronic-PEI Conjugate. A three fold molar excess of BPEI 600 (173.92 mg) to the activated Pluronic F127 was dissolved in 5 mL deionized water at pH 8.2. To the solution, 5 mL of deionized water containing 5 g of the activated Pluronic F127 was slowly added. The reaction was stirred for 15 h at 25 °C. The resulting solution was purified by dialysis and then dried by lyophilization. The synthesis of PEI-Pluronic-PEI was confirmed by ^{1}H-NMR analysis in *d6*-DMSO (Bruker, DRX 400).

CMC Measurement. The critical micelle concentration (CMC) of PEI modified Pluronic micelles was determined using pyrene as an extrinsic fluorescence probe, as described previously.[27,28] The concentration of PEI-Pluronic-PEI was varied from 0.0001 to 5 mg/mL and incubated with 6×10^{-7} M of pyrene. The fluorescence intensity was measured at 25 °C using a spectrofluorophotometer (Shimadzu, RF-5301 PC, Japan). Fluorescence spectra were obtained at an excitation and an emission wavelength of 334 and 339 nm, respectively.

Particle Size Measurement. Above the CMC values of Pluronic micelles, the sizes of Pluronic and PEI- Pluronic-PEI micelles were measured by a dynamic light scattering (DLS) instrument (Zeta plus, Brookhaven, NY) equipped with a He-Ne laser at a wavelength of 632.2 nm. The sizes of HMW BPEI/DNA polyplexes in the presence of varying amounts of PEI-Pluronic-PEI were also determined at room temperature. All experiments were performed in triplicate.

Cell Culture. 293T cells (kidney epithelial cell line) were grown in DMEM supplemented with 10% FBS, penicillin G sodium at 10 U/mL, streptomycin sulfate at 10 μg/mL, and 25 ng/mL amphotericin B. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. Fifty thousand cells per well were plated in a 24 well plate in 0.5 mL DMEM with 10% FBS. After 24 h, the cell culture medium was replaced with serum-free medium prior to addition of BPEI/DNA polyplexes formulated with varying amounts of PEI-Pluronic-PEI.

Preparation of BPEI/DNA/PEI-Pluronic-PEI Complexes

and Transfection. Cell transfection experiments were performed in the absence of serum proteins. Fifty μL of the DMEM solution containing BPEI/DNA complex particles with varying amounts of PEI-Pluronic-PEI was added in each well. BPEI (40 μg/mL) and DNA (40 μg/mL) stock solution were prepared in HBS (10 mM HEPES, 30 mM NaCl, 1.5 mM sodium phosphate dibasic, pH 7.4). The BPEI stock solution (1 mL) and DNA solution (1 mL) were mixed at a nitrogen/phosphate (N/P) ratio of 8, where the maximum transfection efficiency was attained.[29] After 4 h incubation, the medium was removed and supplemented with fresh medium containing 10% FBS. Transfected cells were incubated for 24 h before measuring luciferase activity in each well. The extent of luciferase gene expression was determined by using a luciferase assay kit (Promega, Madison, WI). Transfected cells were harvested with subsequent lysis and the cell lysate was centrifuged at 14,000 rpm for 30 min. The supernatant (20 μL) was used for measuring luciferase activity and the protein concentration was measured by using a Micro-BCA protein assay kit. The luciferase activity was monitored for 15 s in a Lumat LB 9501 luminometer (Berthold, Wilbach, Germany). The transfection efficiency was expressed as relative light unit per mg of cell protein. All transfection experiments were performed in triplicate.

Results and Discussion

Synthesis of PEI-Pluronic F127-PEI. A schematic diagram of synthesis of PEI-Pluronic-PEI is shown in Figure 1. The two terminal hydroxyl groups of Pluronic F127 were activated by *p*-nitrophenyl chloroformate. Because the activated Pluronic F127 instantly turned yellow upon incubating at an alkaline condition, the activation degree could be easily determined. The degree of activation was 99.4%. Figure 2 (A) shows ^{1}H-NMR spectra of *p*-nitrophenyl chloroformate activated Pluronic, where doublet proton peaks appear at 7.5

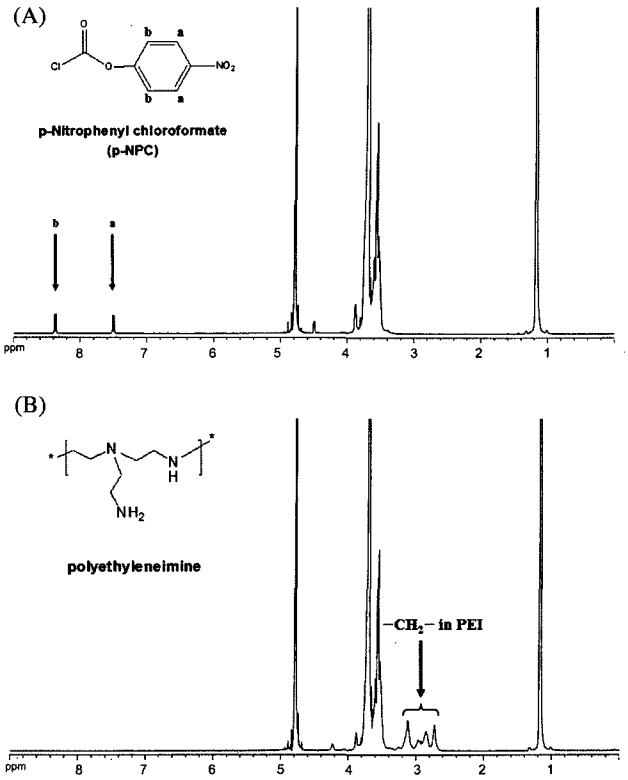

Figure 2. ^{1}H-NMR spectra of (A) *p*-NPC activated Pluronic F127 and (B) PEI-Pluronic-PEI.

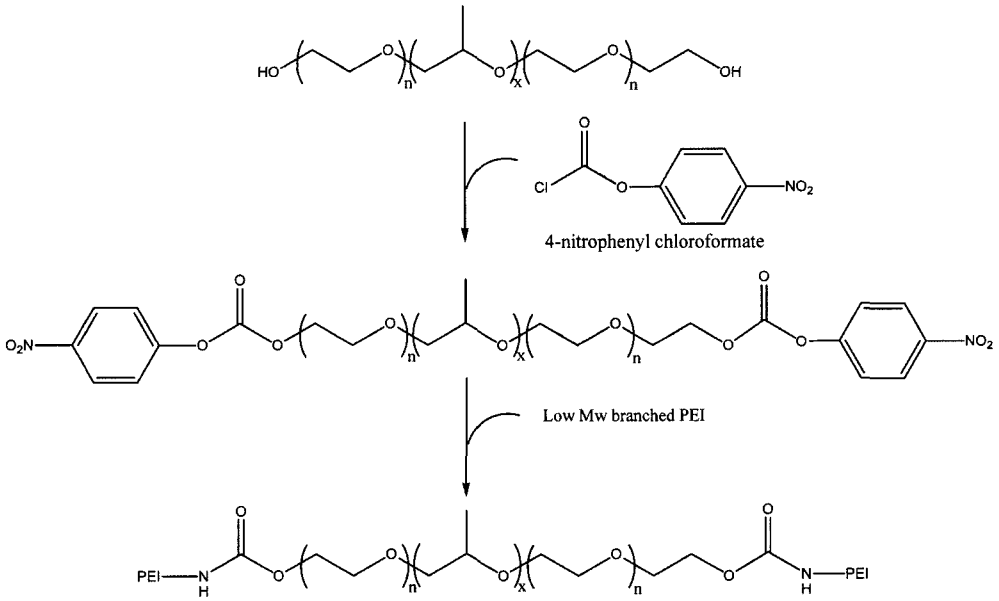

Figure 1. A synthetic scheme of PEI-Pluronic-PEI.

and 8.3 ppm from the aromatic ring of *p*-nitrophenyl group conjugated to the ends of Pluronic. The activation percent as determined from ^{1}H-NMR was 97.0%, close to the value determined spectrophotometrically. Figure 2(B) shows ^{1}H-NMR spectra of PEI-Pluronic-PEI where new multiple proton peaks of -CH_2- from LMW BPEI appear at 2.8~ 3.1 ppm. It can be seen that after reacting BPEI to the activated Pluronic F127, *p*-nitrophenyl doublet proton peaks appearing at 7.5 and 8.3 ppm disappear completely, indicating that LMW BPEI was conjugated to the terminal ends of Pluronic F127. From the proton peak ratio of -CH_2- between BPEI and PEO (4.2 ppm), the conjugation percent of LMW BPEI to Pluronic F127 was 76.4%.

CMC Measurement and Particle Size of PEI-Pluronic-PEI Micelles. The fluorescence intensity ratio at the two excitation lengths (I_{339}/I_{334}) was measured as a function of polymer concentration at room temperature. Critical micelle concentration values of Pluronic F127 and PEI-Pluronic-PEI were 5.075×10^{-6} and 1.978×10^{-5} M, respectively. The CMC value of PEI-Pluronic-PEI was higher than that of Pluronic F127, because the incorporation of ionic and hydrophilic BPEI units to the terminal ends of PEO side-blocks in Pluronic F127 increased the hydrophilic-lipophilic balance (HLB) value for the PEI-Pluronic-PEI copolymer structure. The sizes of PEI-Pluronic-PEI micelles were determined at a 0.1 w/v% concentration by DLS. Effective diameter of Pluronic F127 was 24.8 ± 3.7 nm, while that of PEI-Pluronic-PEI was 39.1 ± 8.7 nm. This suggests that the conjugation of LMW BPEI to the two PEO ends increased the size of micelles while concomitantly destabilizing their self-assembly structure. The increment in effective diameter can be attributed to the increased CMC value.

Size Change of BPEI/DNA Polyplexes in the Presence of PEI-Pluronic-PEI Micelles. Figure 3 shows changes in effective diameters of HMW BPEI/DNA polyplexes (N/P ratio=8) in the presence of different amounts of Pluronic and PEI-Pluronic-PEI. It can be seen that the sizes of HMW BPEI/DNA polyplexes increase with addition of Pluronic and PEI-Pluronic-PEI in the formulation. The effective diameters gradually increased and leveled off at 0.1% (w/v) concentration for both PEI conjugated and unconjugated Pluronics. Since CMC values of Pluronic F-127 and PEI-Pluronic-PEI were 0.05 and 0.1% (w/v), respectively, it is reasonable to say that the increment in effective diameters was attributed to nonspecific interactions of Pluronic and PEI-Pluronic-PEI micelles onto the surface of BPEI/DNA polyplex nanoparticles. The effective diameter of BPEI/DNA polyplexes was 162.1 ± 20.6 nm. By adding Pluronic and PEI-Pluronic-PEI at a concentration larger than 0.1% (w/v) in the formulation, the effective diameters of polyplexes increased to ca. 180 nm for Pluronic and to ca. 250 nm for PEI-Pluronic-PEI. From the results, it is conceivable that the increased size of BPEI/DNA polyplexes in the presence of PEI-Pluronic-PEI micelles, which was about twice the size of PEI-Pluronic-PEI micelles, was most likely due to their interactions on the surface of BPEI/DNA polyplexes. The sizes of the BPEI/DNA polyplexes coated with PEI-Pluronic-PEI were larger than those with Pluronic with increasing the polymer concentration, suggesting that the surface interaction might occur via ionic interactions between surface exposed LMW BPEI residues of PEI-Pluronic-PEI micelles and DNA in HMW BPEI/DNA polyplexes. Surface zeta potential value of HMW BPEI/DNA polyplexes coated with PEI-Pluronic-PEI micelles at 0.5% (w/v) concentration was + 29.0 ± 3.6 mV, while that of uncoated polyplexes was + 20.5 ± 4.5 mV. The increased surface zeta potential value also suggests that PEI-Pluronic-PEI micelles could bind onto the surface of HMW BPEI/DNA polyplexes.

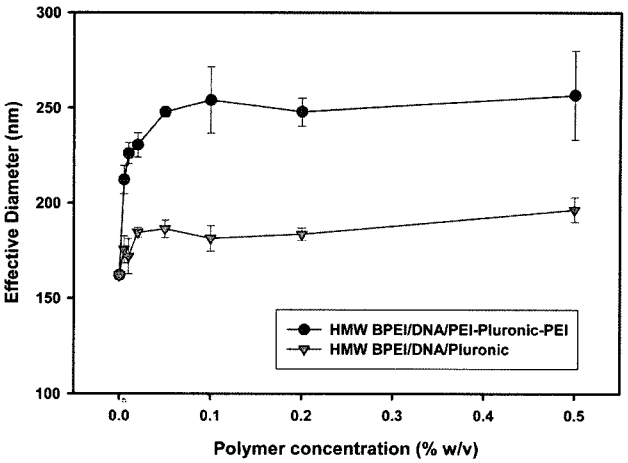

Figure 3. Change in effective diameters of BPEI/DNA polyplexes (N/P ratio=8) with increasing concentrations of Pluronic F127 and PEI-Pluronic-PEI.

***In vitro* Transfection.** Luciferase gene transfection efficiency of BPEI/DNA polyplexes (N/P ratio=8) was examined with increasing the concentration of Pluronic or PEI-Pluronic-PEI in the formulation (Figure 4). The gene transfection efficiency gradually increased with addition of PEI-Pluronic-PEI. The BPEI/DNA polyplexes exhibited its maximum gene transfection efficiency of 2.05×10^7 RLU/mg protein at an N/P ratio of 8. By co-incubating PEI-DNA-PEI micelles with the polyplexes, the extent of gene transfection steadily increased to 6.45×10^7 RLU/mg protein with increasing the PEI-Pluronic-PEI concentration. In contrast, for unmodified Pluronic, the gene transfection efficiency was modestly increased to 2.98×10^7 RLU/mg protein over the same concentration range. Figure 5 shows the effect of PEI-Pluronic-PEI micelles on the gene transfection efficiency of PLL/DNA polyplexes (N/P ratio = 8). A similar trend of enhancing gene transfection by adding PEI-Pluronic-PEI micelles can be seen. It is of interest to note that the extents of luciferase gene transfection for both of BPEI/DNA and PLL/DNA polyplexes tend to show their saturated levels

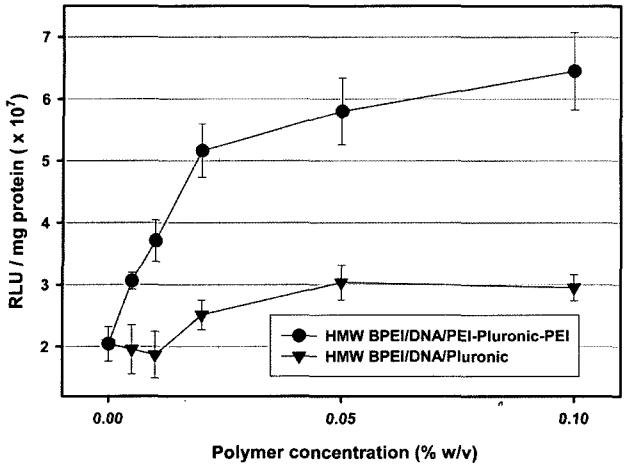

Figure 4. Luciferase gene transfection efficiencies of BPEI/DNA polyplexes (N/P ratio=8) with increasing concentrations of Pluronic F127 and PEI-Pluronic-PEI.

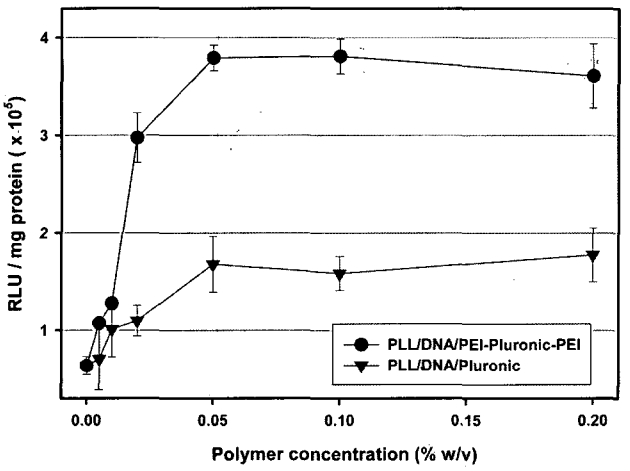

Figure 5. Luciferase gene transfection efficiencies of PLL/DNA polyplexes (N/P ratio=8) with increasing concentrations of Pluronic F127 and PEI-Pluronic-PEI.

starting from 0.05% (w/v) of polymer concentration, which is close to a CMC value of PEI-Pluronic-PEI micelles. This indicates that self-assembled PEI-Pluronic-PEI micelles, not in the form of unimers, played a critical role in enhancing the gene transfection efficiency. As mentioned earlier, PEI-Pluronic-PEI micelles were likely to be adsorbed onto the surface of BPEI/DNA or PLL/DNA polyplexes via electrostatic interactions, which might perturb the structural integrity of cell membrane upon contact with cells. Surface anchored PEI-Pluronic-PEI micelles around the polyplexes might promote the intracellular uptake process of the polyplexes. It was previously shown that amphiphilic Pluronic copolymers modified with cationic polymers such as BPEI (MW 2 K) and poly(*N*,*N*'-dimethylaminoethyl methacrylate), when

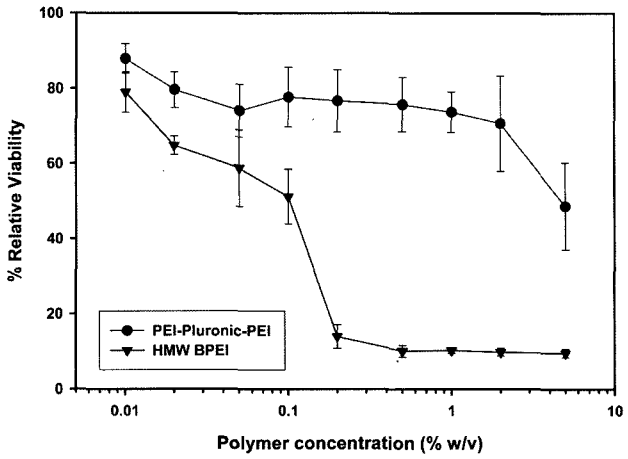

Figure 6. Cell viabilities with increasing concentrations of BPEI and PEI-Pluronic-PEI.

complexed with DNA, exhibited reduced gene transfection efficiencies, compared to that of BPEI/DNA complexes, presumably because the resultant polyplexes had an unstable nano-particulate structure in aqueous solution.[21,22] In the current study, the structural integrity of polyplexes was first ensured by using an optimized amount of BPEI (branched, Mw 25 K) for complexation with DNA. By adding PEI-Pluronic-PEI micelles to the BPEI/DNA formulation, the gene transfection efficiency was significantly enhanced without compromising cell viability. One serious concern about using an excess amount of BPEI for DNA complexation was its cytotoxic problem.[1,2] Although HMW BPEI demonstrated superior transfection efficiencies with increasing its MW and N/P ratio, the cells were severely damaged. Figure 6 shows cell viabilities for BPEI and PEI-Pluronic-PEI at different concentrations. It can be seen that PEI-Pluronic-PEI maintains the cell viability about 80% up to 2% (w/v) concentration, whereas BPEI sharply gradually decreases the cell viability to the concentration of 0.1% (w/v), and elicits sudden cell death thereafter. The well tolerated cell viability for PEI-Pluronic-PEI was due to the fact that LMW PEI (Mw 0.6 K) was conjugated to the Pluronic terminal ends. This indicates that PEI-Pluronic-PEI micelles can be used as a safe and efficient formulation excipient for enhancing gene transfection.

Conclusions

In this study, PEI-Pluronic-PEI was successfully synthesized, and their role as an additive for polyplexes was investigated. PEI-Pluronic-PEI formed micelles with slightly increasing CMC value and effective diameter, compared to those of Pluronic F127. PEI-Pluronic-PEI exhibited much better cell viability compared to BPEI. PEI-Pluronic-PEI coated BPEI/DNA polyplexes showed enhanced gene transfection efficiency. The additive effect was also clearly

observed for PLL/DNA polyplexes. Cationic Pluronic micelles can be used as a useful additive in non-viral gene delivery.

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