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Enhanced Gene Delivery by Palmitic Acid-Conjugated Low Molecular Weight Polyethylenimine

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Abstract: Palmitic acid-conjugated low molecular weight polyethylenimine (PEI-*g*-PEG-PA) was successfully synthesized to develop an efficient non-viral gene carrier. The judicious integration of hydrophobic palmitic acid and polyethylenimine *via* hydrophilic polyethylene glycol (PEG) facilitated the formation of nano-sized complex (nanoplex) with plasmid DNA (pDNA) which provided the protection of pDNA against the serumic enzymatic degradation. Furthermore, the delivery system demonstrated enhanced gene transfection efficiency in comparison to unmodified low molecular weight PEI without inducing any significant cytotoxicity.

Keywords: gene delivery, polyethylenimine, nanoplex, non-viral, palmitic acid.

Introduction

Viral gene carriers such as retroviruses, adenoviruses, and adeno-associated viruses have been known to have high transfection efficiency as compared to non-viral vectors. However, their applicability in clinical practice suffers a great setback due to several impediments associated with them. The demerits including their restricted targeting ability only to dividing cells, random DNA insertion, low capacity for carrying large therapeutic genes in size, risk of replication, and possible host immune reaction impaired their potential as gene delivery tool.[1,2] Therefore, non-viral gene delivery systems such as cationic polymers or lipids have been investigated intensively to overcome the disadvantages associated with viral vectors. Among the non-viral gene carriers, polyethylenimine (PEI) is one of the most potent carriers and has been extensively studied *in vitro* and *in vivo* since their initial use as gene carrier.[3] Transfection efficiency of PEI, along with its cytotoxicity, is heavily dependent on its molecular weights.[4-6] PEI with a molecular weight higher than 25 kDa exhibits high transfection efficiency and cytotoxicity, whereas low molecular weight PEI (<1.8 kDa) despite its low toxicity profile displays low transfection. The toxicity of PEI primarily arises from the strong interaction potential of positive charged PEI with negatively charged cell surfaces and associated membrane damage.[7] Several strategies have been adopted so far to address the toxicity concern. Polyethylene glycol (PEG) was conjugated to PEI as a hydrophilic segment and its efficacy has been investigated by several groups.[8,9] The non-viral gene delivery copolymers based on the high molecular weight PEI showed considerable transfection efficiency, however toxicity remained as a problem. In previous reports, biodegradable PEI derived from the low molecular weight PEI and PEG exhibited improved transfection efficiency and low cytotoxicity.[10-12] Also, water soluble lipopolymer (WSLP) obtained by integrating the cationic headgroup of branched PEI (BPEI, MW=1.8 kDa) with a hydrophobic lipid anchor, cholesterol chloroformate, showed low cytotoxicity, and enhanced transfection efficacy *in vitro* and *in vivo* was obtained.[13-15] These amphiphilic PEI has also been reported by other research groups.[16,17] The high molecular weight PEI (MW=25 kDa) conjugated with pendant palmitic acids chains improved the biocompatibility markedly when compared to the starting material, but also reduced transfection efficiency.[18] We hypothesized that a similar strategy with low molecular weight PEI, but different structure may generate the different complex formulation with DNA, thus improve transfection efficiency and biocompatibility.

In this study, we successfully synthesized palmitic acid-conjugated PEI (PEI-*g*-PEG-PA) and studied their physicochemical characteristics. Furthermore, the potential of PEI-*g*-PEG-PA as a novel gene carrier was evaluated through luciferase gene transfection assay.

Experimental

Materials. Glycerol ethoxylate (Glyceryl PEG, M_n=1,000

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Da), palmitoyl chloride, triethylamine, and *p*-nitrophenyl chloroformate were obtained from Aldrich Chemical Co. (Mikwaukee, WI). Branched PEI (BPEI) of molecular weight 1.2 kDa was purchased from Polysciences, Inc. (Warrington, PA). Glycerol ethoxylate was dried by azeotropic distillation. All other chemicals were of reagent grade and used without further purification.

Synthesis of PEI-*g*-PEG-PA Conjugate. PEI-*g*-PEG-PA was synthesized following the scheme depicted in the Figure 1. Dry glycerol ethoxylate (1 molar equiv.) was treated with palmitoyl chloride (2 molar equiv.) in the presence of triethylamine (2.5 molar equiv.) in anhydrous methylene chloride. Resulting glyceryl PEG-di-palmitate was purified by precipitation in petroleum ether. The purified glyceryl PEG-di-palmitate (1 molar equiv.) was reacted with *p*-nitrophenyl chloroformate (*p*-NPC) (1.5 molar equiv.) in the presence of triethylamine (2 molar equiv.) in anhydrous methylene chloride. The activated glyceryl PEG-di-palmitate (Gly-PEG-NPC) was purified by recrystallization from ethyl acetate. Gly-PEG-NPC was further reacted with BPEI of molecular weight 1.2 kDa at a molar ratio of 1:1.5 in a 50 mM sodium bicarbonate solution for 24 h. Resulting PEI-*g*-PEG-PA was purified by dialysis in double distilled water. The polymer solution was lyophilized overnight to have brown oily PEI-*g*-PEG-PA.

Preparation of PEI-*g*-PEG-PA/pDNA Nanoplexes. Cationic polymer/plasmid DNA (pDNA) complexes were prepared by mixing equal volumes of aqueous solution of cationic polymer and pDNA to give a net molar excess of nitrogen (polymer) to phosphate (nucleic acid) over the range of 0 to 20. The electrostatic interactions between PEI-*g*-PEG-PA and pDNA resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to as nanoplexes here onwards. Nanoplexes were prepared by first preparing an aqueous solution of cationic polymer containing PEI-*g*-PEG-PA or unmodified BPEI in 5% glucose solution. In a separate tube, pDNA were dissolved in the same buffer in the same total volume as the cationic polymer solution. The two solutions were then mixed and vortexed for 30 s, and incubated for 30 min to make nanoplexes at room temperature.

Agarose Gel Retardation Assay. PEI-*g*-PEG-PA plasmid DNA expression luciferase under a cytomegalovirus promoter (pCMV-Luc pDNA) complexes were prepared at various N/P ratios ranging from 0 to 20 in 5% glucose solution. After the samples were electrophoresed through a 1.0% agarose

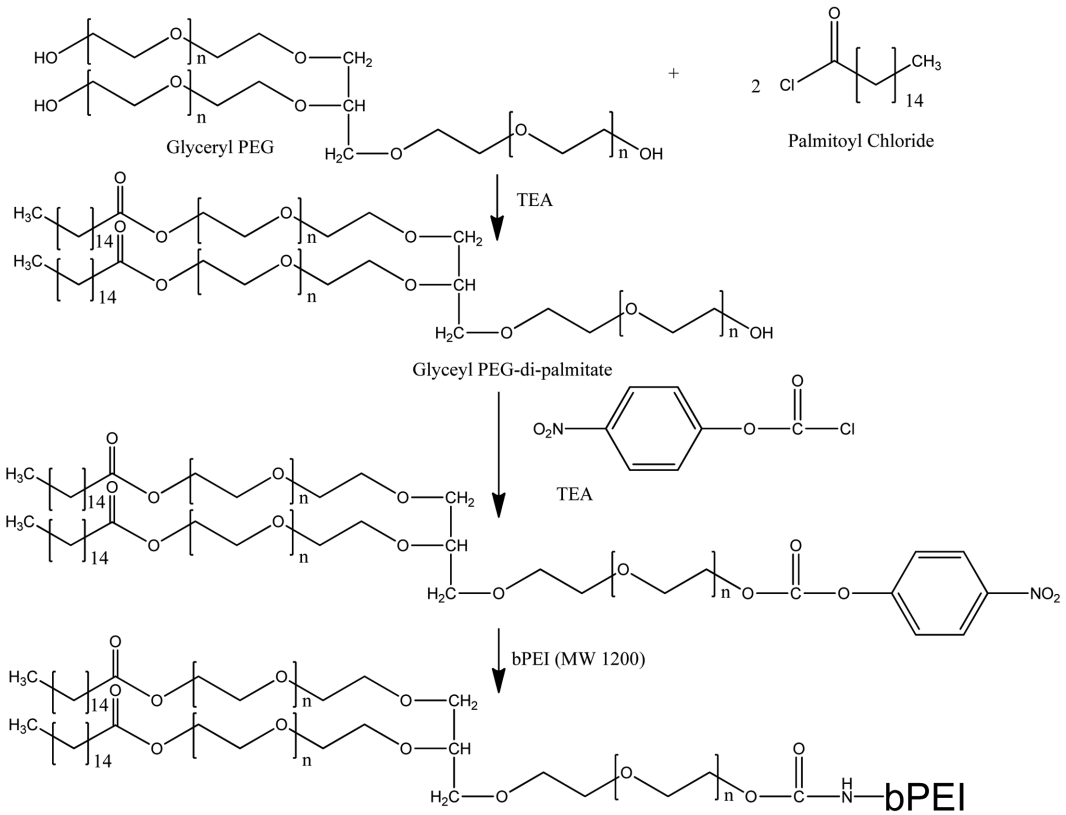

Figure 1. Synthesis scheme of PEI-*g*-PEG-PA conjugate. Dry glycerol ethoxylate was treated with palmitoyl chloride in the presence of triethylamine, and then the resulting glyceryl PEG-di-palmitate was purified by precipitation in petroleum ether. The purified glyceryl PEG-di-palmitate was reacted with *p*-nitrophenyl chloroformate (*p*-NPC), and this activated glyceryl PEG-di-palmitate (Gly-PEG-NPC) was further reacted with BPEI.

gel in 0.5 TAE (Tris-acetate-EDTA) buffer at 100 V for 30 min, and stained with ethidium bromide (EtBr, 0.5 μg/mL) for 20 min. Finally pDNA was visualized on a UV transilluminator (wiseUV® WUV, DAIHAN Scientific, Seoul, Korea).

Stability of Polymer/pDNA Nanoplexes in Serum-Containing Media. PEI-*g*-PEG-PA/pCMV-Luc nanoplexes or free pCMV-Luc pDNA were incubated at 37 °C in the 5% glucose solution containing 20% final concentration of fetal bovine serum (FBS). At 0, 0.5, 1, 2, 3, 6, 20, and 30 h postincubation, 10 μL of samples were taken into Eppendorf tubes and were frozen, and stored at -70 °C. The samples were thawed and mixed with 2 μL of 2% sodium dodecyl sulfate (SDS) to dissociate pDNA from cationic polymer, and analyzed using a 1.0% agarose gel electrophoresis. Following electrophoresis, gels were stained with EtBr and visualized on a UV-transilluminator.

Measurement of Zeta Potential and Particle Size of Nanoplexes. PEI-*g*-PEG-PA nanoplexes were prepared at various N/P ratios ranging from 0 to 40 for particle size measurements (N/P ratios were from 1 to 20 for zeta potential measurements) by adding various amounts of PEI-*g*-PEG-PA into the same volume of pCMV-Luc pDNA solution (5 μg in 5% glucose solution). The mixtures were then incubated for 30 min at room temperature for complex formation. Zeta potential and size distribution of each sample were determined in three serial measurements with Zetasizer 3000HSA (MALVERN Instruments, Worcestershire, UK).

Cell Culture. PC-3 human prostate adenocarcinoma cell lines were grown and maintained in RPMI 1640 medium which was supplemented with 10% fetal bovine serum (FBS) at 37 °C and humidified 5% CO_2.

Cell Viability. For the evaluation of cytotoxicity, the colorimetric MTT assay was employed. Briefly, PC-3 cells were seeded at a density of 8×10^4 cells/well in 24-well plate and grown for 24 h prior to transfection. After transfection with cationic polymers and pCMV-Luc pDNA nanoplexes at various N/P ratios ranging from 0 to 20, the mixtures were replaced by 0.5 mL of fresh medium and 20 μL of 10 mg/mL MTT solution, and incubated further for 4 h. The media was removed and 200 μL of dimethyl sulfoxide (DMSO) was added, and then the absorbance was measured at 570 nm using a microplate reader.

***In vitro* Transfection.** PC-3 cells were seeded in six-well flat-bottomed microassay plates at a density of 3×10^5 cells/well and incubated at 37 °C in a 5% carbon dioxide incubator. Prepared polymer/pCMV-Luc nanoplexes were incubated for 30 min prior to transfection. As cells attained 80% confluency, media was replaced with serum-free RPMI 1640 fresh media containing the polymer/pCMV-Luc nanoplexes (2 μg of DNA/well). Following 4 h incubation at 37 °C, transfection media was then removed and replaced with fresh media with 10% FBS. After an additional 24 h incubation, cells were lysed and luciferase gene expression was quantified using a luciferase system and 96-well plate luminometer (Dynex Technologies Inc., Chantilly, VA). Luciferase enzyme activity was expressed in terms of relative light units integrated over 60 s, per mg of cell protein determined by BCA assay (RLU/mg).

Results and Discussion

Synthesis of PEI-*g*-PEG-PA Conjugate. In order to synthesize PEI-*g*-PEG-PA conjugate, the glyceryl PEG was initially treated with palmitoyl chloride under basic condition using triethylamine to afford glyceryl PEG-di-palmitate (*g*-PEG-PA). Then the free OH groups in *g*-PEG-PA was activated with *p*-nitrophenyl chloroformate to yield activated Gly-PEG-NPC which was subsequently treated with BPEI to afford PEI-*g*-PEG-PA conjugate containing stable urethane bonds (Figure 1).

The structure of PEI-*g*-PEG-PA conjugate was confirmed by ^{1}H NMR spectroscopy (Figure 2). The typical peaks of PEG showed at 3.5 ppm (-CH_2CH_2O-) and those of BPEI (-CH_2CH_2NH-) appeared around 2.6 ppm. In addition to these peaks, the characteristic peaks of PA clearly were discernible at 2.3, 1.5, 1.2, and 0.8 ppm. The ratio between PEG and PEI in the synthesized PEI-*g*-PEG-PA conjugate was found to be 1:1 as per the relative integral values of ethylene signals in the proton spectrum of the conjugate. The degree of PA modification was estimated as 0.8 per glyceryl PEG molecule. The low conjugation ratio in spite of using two molar equivalence of PA might be attributed to the *in situ* hydrolysis of PA molecule under the reaction condition. However, the final conjugation reaction between BPEI and *g*-PEG-PA demonstrated almost quantitative yield

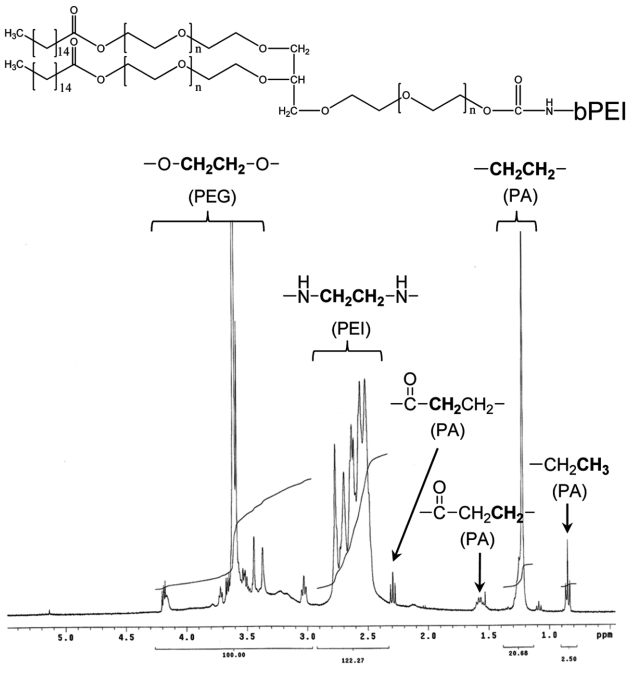

Figure 2. ^{1}H NMR spectra of PEI-*g*-PEG-PA conjugate.

having PEG and PEI in 1:1 ratio as evident from the ^{1}H NMR integral values.

Characterization of Nanoplex. In order to establish the formation of PEI-*g*-PEG-PA/pDNA nanoplexes, agarose gel electrophoresis was performed at different N/P ratios (Figure 3(a)). The movement of the pDNA in the gel was retarded as the amount of the PEI-*g*-PEG-PA conjugate was increased, demonstrating that the PEI-*g*-PEG-PA conjugate binds to the pDNA, neutralizing its negative charge. At N/P ratios exceeding the neutralization composition, the complexes migrated slightly toward the anode, suggesting that they have a small positive charge. Complete complex formations were achieved at N/P ratios from approximately 0.8 and 1.2 (Lane 3 and 4).

Protection of pDNA in Nanoplex from Degradation in Serum. The protection efficiency of PEI-*g*-PEG-PA was determined by incubating freshly prepared complexes at a N/P ratio of 10 with FBS. The gel retardation of free pDNA or complexed pDNA with PEI-*g*-PEG-PA after incubation with FBS is shown in Figure 3(b). After 30 min incubation time, all of the non-complexed pDNA control were degraded by the enzyme in serum. In contrast, when complexed with PEI-*g*-PEG-PA, intact pDNA was observed even after 3 h incubation time. These results demonstrated that the full compaction of pDNA within a polycation core restricted the access of nucleases resulting in protection of pDNA.

Particle Size and Zeta Potential Measurements. Physicochemical characterizations of PEI-*g*-PEG-PA/pDNA nanoplexes, such as surface charge and effective particle size were investigated. The study of particle size measurement by dynamic light scattering (DLS) showed that PEI-*g*-PEG-PA conjugate effectively condenses pDNA into small

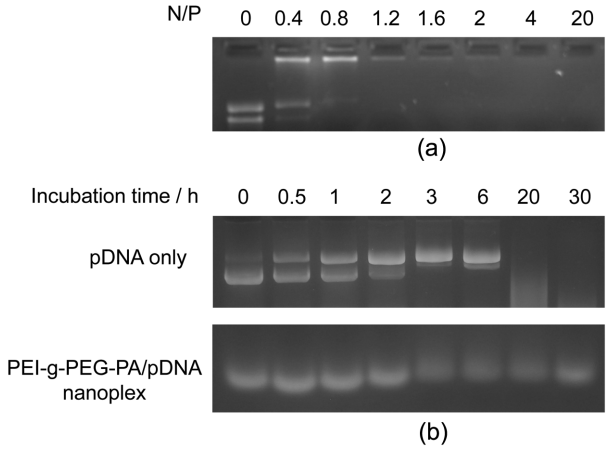

Figure 3. (a) Electrophoretic patterns of PEI-*g*-PEG-PA/pDNA nanoplexes at various N/P ratios. (b) pDNA protection assay in serum-containing media. Degradation of pDNA exposed to serum was observed for free pDNA and PEI-*g*-PEG-PA/pDNA nanoplexes. The nanoplexes were incubated in 20% FBS media from 0 to 30 h at N/P ratio 10. Undegraded intact pDNA was detected following electrophoresis and EtBr staining.

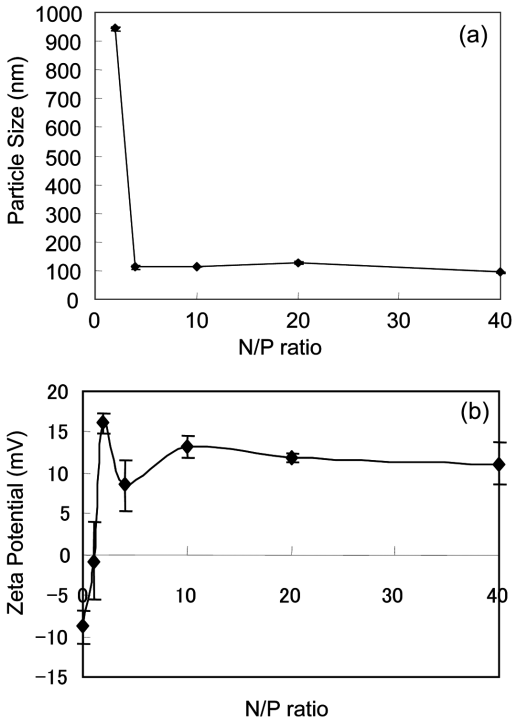

Figure 4. Particle size of PEI-*g*-PEG-PA/pDNA nanoplexes (a) and Zeta potential measurements (b) at various N/P ratios. PEI-*g*-PEG-PA nanoplexes were prepared at various N/P ratios ranging from 0 to 40 for particle size measurements and from 1 to 20 for zeta potential measurements by adding various amounts of PEI-*g*-PEG-PA into the same volume of pCMV-Luc pDNA solution. The mixtures were then incubated for 30 min at room temperature for complex formation.

nano-sized particles (~100 nm) at N/P ratio range from 5 to 40 (Figure 4(a)). The zeta potential profile showed that PEI-*g*-PEG-PA/pDNA nanoplexes have moderate zeta potentials of approximately +5 ~ +15 at N/P ratio range between 5 to 40 (Figure 4(b)). The obtained zeta potential value reached a plateau around +12 mV, which is significantly lower than those observed for high molecular weight BPEI (MW=25 kDa, ~30 mV, data not shown) as the N/P ratio increased. The shielding of the positively charged surface of the nanoplexes by the non-ionic PEG chains is responsible for the reduced zeta potential value as also reported in other articles.[8,19,20]

***In vitro* Gene Transfection.** *In vitro* gene transfecion potential of PEI-*g*-PEG-PA copolymer was assessed in PC-3 cell lines treated with PEI-*g*-PEG-PA/pCMV-Luc reporter gene complex. As shown in Figure 5(a), the transfection efficiency of PEI-*g*-PEG-PA/pCMV-Luc nanoplex first increased and then decreased with increasing N/P ratios, in which the highest transfection level was observed at the N/P ratio of 10. Above the N/P ratio 10, transfection efficiency could be inhibited due to the non-specific interaction of extra polymers with cells. Extra amine group of BPEI which is not able

to form complex with pDNA interacts with cell membrane then it inhibits the interaction of polyplexes with cells, resulting in reduced transfection efficiency. The transfection efficacy of PEI-*g*-PEG-PA copolymer was compared with either high molecular weight of BPEI (MW=25 kDa) or low molecular weight of BPEI (MW=1.2 kDa). At the N/P ratio of 10, the transfection level of PEI-*g*-PEG-PA was 55-fold higher than that of low molecular weight BPEI, whereas slightly lower than high molecular weight BPEI.

Cytotoxicity. A successful gene delivery carrier should be able to deliver transcriptionally active gene to the cell nucleus without adversely affecting the normal functions of the host cell. Therefore, the *in vitro* cytotoxicity of nanoplexes based on PEI-*g*-PEG-PA conjugates was studied as a function of N/P ratio using MTT assay. High molecular weight BPEI 25 kDa and low molecular weight BPEI 1.2 kDa were used as references. As shown in Figure 5(b), BPEI 25 kDa/pDNA complexes have higher toxicity than either BPEI 1.2 kDa or PEI-*g*-PEG-PA conjugate at the N/P ratio of 10 in agreement with the previous literatures. Moreover, the PEI-*g*-PEG-PA/pDNA nanoplexes are less toxic than BPEI 25 kDa (N/P=10) even at the high N/P ratio of 20 (Figure 5(b)), while BPEI 25 kDa shows high toxicity at the N/P ratio of 20 (data not shown). The toxicity of BPEI 25 kDa is a well-known phenomenon and has been reported by a number of investigators in both *in vitro* and *in vivo* experiments.[21,22]

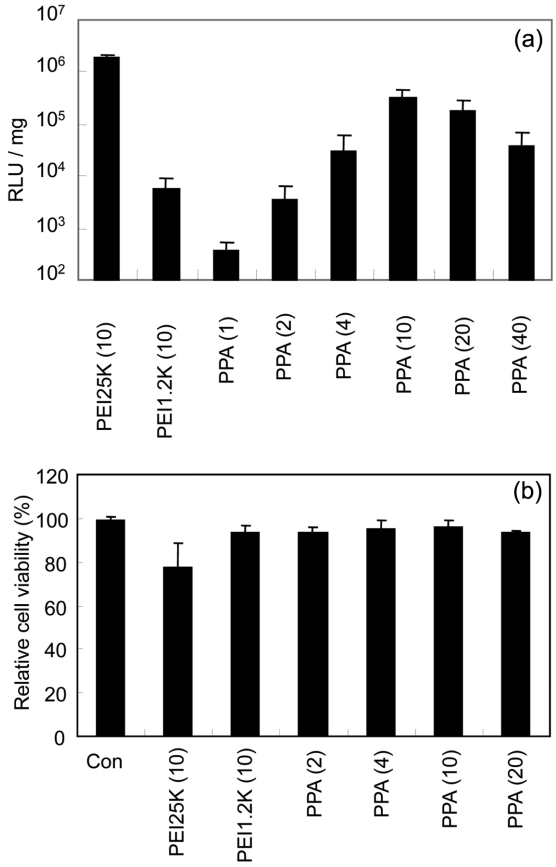

Figure 5. Luciferase gene transfection efficiency (a) and cell toxicity (b) of PEI-*g*-PEG-PA/pDNA nanoplexes assay at various N/P ratios. (a) PC-3 cells were seeded in six-well flat-bottomed microassay plates at a density of 3×10^5 cells/well. After transfection with the polymer/pCMV-Luc nanoplexes, transfection efficiency was investigated by luciferase assay. (b) For the evaluation of cytotoxicity, PC-3 cells were seeded at a density of 8×10^4 cells/well in 24-well plate and grown for 24 h prior to transfection. After transfection with nanoplexes, the mixtures were replaced by 0.5 mL of fresh medium and 20 μL of 10 mg/mL MTT solution.

The development of safe and efficient gene delivery vector is of paramount importance in realizing the gene therapy in prevalent clinical applications. Vectors based on polyelectrolyte complexes of nucleic acids and synthetic cationic polymers (polyplexes or nanoplexes) represent the potential alternatives to viral vectors as polymeric vectors usually do not elicit the safety issues as encountered with virus. However, unfortunately due to their low transfection efficiency, despite enormous progress in recent years, non-viral polymeric vector still lags miles behind in terms of sophistication and efficiency in comparison to their viral counter parts. The objective of this study is to design more efficient and less toxic polymeric gene carrier for delivering nucleic acids using modified low molecular weight PEI. In order to enhance the transfection efficacy, we conjugated palmitic acid to low molecular weight PEI *via* hydrophilic PEG. The data in Figure 5(a) clearly demonstrates the benefits of palmitic modification of PEI for transfection activity. PEI-*g*-PEG-PA/pDNA nanoplexes display approximately 55-fold enhancement in transfection activity in comparison to ummodified PEI, and the observation corroborated the earlier reports which suggested the role of lipid modification in enhancing the cellular uptake of nanoplexes.[23] Several groups have reported that the modification of cationic polymers with PEG not only reduced the toxicity considerably but also adversely affected the transfection activity arising due to the inefficient cellular uptake.[24,25] However enhanced transfection activity can be achieved by lipid-induced enhanced cellular uptake and low cytotoxicity. These PEG-modification effect can be shown in size distribution and surface charge measurements. It has been considered that complex formation between cationic polymer and pDNA through ionic interactions is affected by N/P ratios of polymer/DNA; relatively large particles are formed at low N/P ratios due to the aggregation of particles by neutralizing their surface charges, whereas small particles are detected at the higher N/P ratio owing to net electrostatic repulsive forces between particles. However, as shown in Figure 4(a), PEI-*g*-PEG-PA/pDNA nanoplexes forms very small nanosized complexes even in neutralizing charge ratios, suggesting that PEG chain in the nanoplexes prevents aggregation of particles in physiological condition and is in agreement with previously reported literatures.[26-28]

Conclusions

The work demonstrates the successful synthesis of efficient non-viral polymeric gene carrier, PEI-*g*-PEG-PA containing hydrophilic spacer. The developed gene delivery vector, PEI-*g*-PEG-PA, are endowed with the several favorable attributes namely formation of nano-sized complex, protection of pDNA, low cytotoxicity, enhanced transfection efficiency that are indispensible for efficient gene delivery. Furthermore, incorporation of appropriate cell-specific target ligand to the developed vector may potentially offers a tunable vector system to achieve target-specific gene therapy.

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