Synthesis and Characterization of a Hydroxylated Dendrimeric Gene Delivery Carrier

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Arginine conjugated PAMAM dendrimer, PAMAM-R was modified with propylene oxide *via* hydroxylation of primary amines of arginine residues. About 49 amines were detected to be converted to amino alcohols by ^{1}H NMR. The newly synthesized polymer, PAMAM-R-PO was able to completely retard pDNA from a charge ratio of 2. The average diameter of PAMAM-R-PO polyplex was found to be 242 nm at a charge ratio of 30. The Zeta-potential value of PAMAM-R-PO polyplex was able to reach 20-30 mV over a charge ratio of 10. PAMAM-R-PO indicated higher cell viability than unmodified PAMAM-R on HeLa and 293 cells because of its hydroxylated amines. Transfection experiments on 293 cells showed that the transfection efficiency of PAMAM-R-PO was found to be 1.5-1.9 times higher than that of PEI25kDa at a charge ratio of 30. The polymer eventually displayed about 2 times greater transfection efficiency than PAMAM-R at the same charge ratio in the absence of serum. Therefore, we concluded that the modification of primary amines of PAMAM-R to amino alcohols gives positive effects such as reduced cytotoxicity and enhanced transfection efficiency on 293 cells for gene delivery potency of PAMAM-R.

Key Words : Hydroxylation, Propylene oxide, Dendrimer, Gene delivery

Introduction

Nonviral gene delivery carriers including cationic polymers and lipids have been studied as alternatives to viral carriers because of their lower cytotoxicity, non-immunogenicity, more convenient handling and larger capacity of gene to deliver.[1-4] Among them, dendrimers have been utilized and examined for drug and gene delivery systems because they have unique and interesting characteristics such as well-defined structures, inner cavities able to encapsulate guest molecules, and controllable multi-valent functionalities in their inner or outer part.[5-9]

Poly(amido amine) (PAMAM) dendrimer is a representative dendrimer and it has been utilized and examined extensively for drug and gene delivery systems *in vitro* and *in vivo*.[10] In addition, it is relatively easy to synthesize this dendrimer in comparison with other dendrimers. PAMAM dendrimers have also been modified with PEG, amino acids, or ligands in order to enhance the gene delivery potency.[11-14]

Our group also reported the synthesis of a novel gene delivery carrier, PAMAM-R by conjugating arginine residues to the periphery of PAMAM dendrimer G4.[15] It showed a much higher transfection efficiency than unmodified dendrimer but also revealed some cytotoxicity in various mammalian cell lines. Meanwhile, Tziveleka *et al.* reported that the hydroxylation of primary amines reduced the cytotoxicity of guanidylated poly(propylene imine) dendrimers.[16] However, they found that their transfection efficiency decreased in accordance to the increase of the degree of hydroxylation. So we intend to examine the hydroxylation effect on the transfection efficiency and the cytotoxicity of PAMAM-R by reacting propylene oxide with the primary amines of PAMAM-R.

Here, we report the synthesis of propylene oxide-reacted PAMAM-R, PAMAM-R-PO and its characterization including cytotoxicity and transfection efficiency for gene delivery systems.

Experimental Section

Materials. Poly(ethylenimine) (25 kDa), PAMAM dendrimer G4, N,N-diisopropylethylamine (DIPEA), piperidine, propylene oxide, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). N-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Anaspec, Inc. (San Jose, CA). Fmoc-L-Arg(pbf)-OH was purchased from Novabiochem (San Diego, CA). Luciferase 1000 Assay System and Reporter Lysis Buffer were purchased from Promega (Madison, WI). The luciferase expression plasmid, pCN-Luci was constructed by subcloning cDNA of Photinus pyralis luciferase with a 21-amino acid nuclear localization signal from an SV40 large T antigen to pCN.[17] Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD). Micro BCA^{TM} protein assay kit was purchased from PIERCE (Rockford, Il). All chemicals were used without any further purification.

Synthesis of PAMAM-R-PO. The backbone polymer, PAMAM-R, was synthesized according to a previously reported method.[15] Briefly, PAMAM-R was synthesized by conjugating Fmoc-Arg(pbf)-OH to the periphery of PAMAM G4 dendrimer with HOBt/HBTU coupling method. After the completion of the reaction, the reaction mixture was precipitated twice with an excess of diethyl ether to remove

unreacted Fmoc-Arg(pbf)-OH and other coupling reagents. The residual precipitate was dissolved in DMF and mixed with an equal volume of piperidine (30% in DMF) at room temperature for 20 min to remove the Fmoc groups of coupled Fmoc-Arg(pbf)-OH and precipitated again with diethyl ether. Then, 1.1 equiv. of propylene oxide in comparison to primary amines was added to the Fmoc-removed PAMAM-R/DMF solution in order to modify primary amines of arginine residues to amino alcohols. The reaction was maintained at 50 °C overnight. The reaction mixture was precipitated with ethyl ether. Finally, the reagent (95:2.5:2.5, trifluoroacetic acid/triisopropylsilane/water, v/v) was used to deprotect the pbf groups of coupled arginine residues at room temperature for 1 h. After the reaction, the final product, PAMAM-R-PO was dialyzed by a dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA, MWCO = 1000) against ultra-pure water overnight and lyophilized before use for analysis and assay.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra of the polymers were obtained using a Bruker DPX-300 NMR spectrometer (300 MHz). For analysis, the polymer samples were dissolved in D_2O containing 3-(trimethylsilyl)propionic-2,2,3,3-*d*4 acid sodium salt as an internal reference (0 ppm).

Gel Retardation Assay. PAMAM-R-PO/plasmid complexes at various charge ratios ranging from 0.5 to 8, were prepared in Hepes buffered saline (10 mM Hepes, 1 mM NaCl, pH 7.4). After 30 min incubation at room temperature for formation of the complex, the samples were electrophoresed on a 0.7% (w/v) agarose gel and stained in an ethidium bromide solution (0.5 μg/mL), and analyzed on a UV illuminator to show the location of the DNA.

Polyplex Size Measurements. The hydrodynamic diameters of the PAMAM-R-PO/plasmid DNA complexes were determined by dynamic light scattering. 2 mL of polyplex solutions containing 5 μg of DNA were prepared at charge ratios of 6, 10, and 30 in Hepes buffered saline (10 mM Hepes, 1 mM NaCl, pH 7.4). After 30 min incubation, polyplex sizes were measured using a Zetasizer 3000HS (Malvern Instruments, UK). The laser used was a nominal 5 mW HeNe laser having a 633 nm wavelength. Scattered light was detected at a 90° angle. The refractive index (1.33) and the viscosity (0.89) of ultrapure water were used at 25 °C for measurements. Zetasizer 3000 (Advanced) Size mode v1.61 software was used for data acquisition. Data analysis was performed in automatic mode. Measured sizes were represented as the average values of 5 runs.

Zeta-potential Measurements. The Zeta-potentials of the PAMAM-R-PO/plasmid DNA complexes were determined. Each polyplex solution was prepared using the same method as above size measurement experiments. After 30 min incubation, each polyplex solution was diluted to a 12 ml final volume prior to measurements. Zeta-potential measurements were carried out using a Zetasizer 3000HS (Malvern Instruments, UK) at 25 °C. Zetasizer 3000 (Advanced) Zeta mode v1.61 software was used for data acquisition. The sampling time was set to automatic. Potential values were presented as the average values of 5 runs.

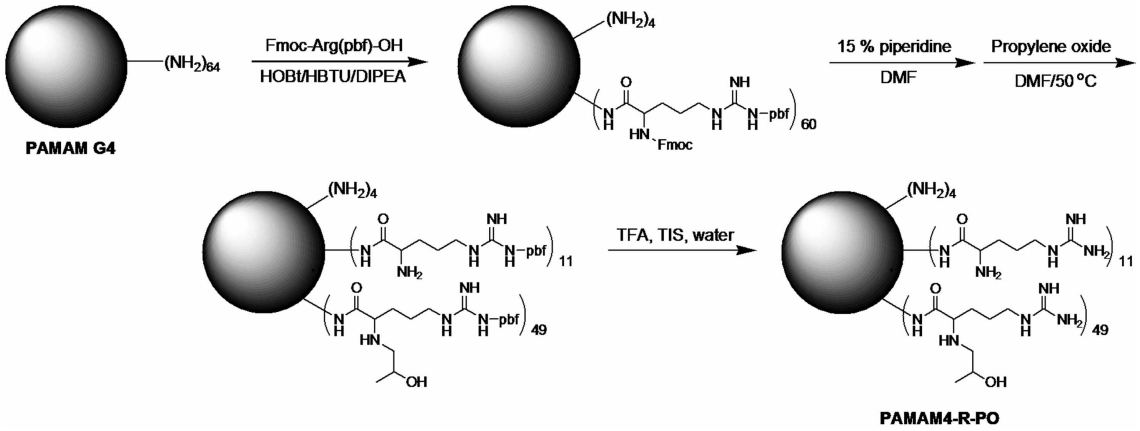
Cytotoxicity Assay. The cytotoxicity of the polymers was measured by MTT assay. Human cervical carcinoma (HeLa) cells and 293 human kidney transformed cells were seeded in a 96-well tissue culture plate at 10^4 cells per well in 90 μL DMEM medium containing 10% FBS. Cells achieving 70-80% confluence after 24 h were exposed to 10 μL of the polymer solutions having various concentrations for 48 h. Then, 26 μL of stock solution of MTT (2 mg/mL in PBS) was added to each well. After 4 h of incubation at 37 °C, each medium was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystals formed by proliferating cells. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA) and recorded as a percentage relative to untreated control cells.

***In vitro* Transfection.** 293 cells were seeded at a density of 5×10^4 cells/well in a 24-well plate in 600 μL of DMEM medium containing 10% FBS and grown to reach 70-80% confluence prior to transfection. In the case of serum-absent condition, the medium was exchanged with fresh serum-free medium before transfection. The cells were treated with polyplex solution containing 2 μg of plasmid DNA at different charge ratios for 4 h at 37 °C. After exchange with fresh serum-containing medium, cells were further incubated for 2 days after transfection. In the case of serum-present condition, polyplex solutions was added to cells without exchange of medium and cells were incubated for 2 days after transfection. Then, the growth medium was removed, and the cells were rinsed with PBS and shaken for 30 min at room temperature in 120 μL of Reporter Lysis Buffer. Luciferase activity was measured by a luminescence assay. 10 microliters of the lysate was dispensed into a luminometer tube and luciferase activity was integrated over 10 s with a 2 s measurement delay in a Lumat LB 9507 luminometer (Berthold, Germany) with an automatic injection of 50 μL of Luciferase Assay Reagent. The final results were reported in terms of RLU/mg protein.

Results and Discussion

Synthesis of PAMAM-R-PO. First, Fmoc-Arg(pbf)-OHs were conjugated to PAMAM dendrimer G4 by HOBt/HBTU coupling method. A small portion of PAMAM-R was collected and analyzed by ^{1}H NMR after removal of all protecting groups in order to confirm the conjugation of arginine residues. About 60 arginine residues were found to be conjugated to PAMAM dendrimers from the NMR result. After confirmation of PAMAM-R synthesis, Fmoc groups of residual polymer were removed by using 30% piperidine/DMF solution. Then, propylene oxide was added to form amino alcohol *via* ring opening reaction and the reaction was maintained at 50 °C overnight. Lastly, pbf groups of arginine residues were removed to expose guanidine groups. The final product, PAMAM-R-PO, was dialyzed and lyophilized. All synthetic procedures are represented in Scheme 1.

The synthesis of purified PAMAM-R-PO was confirmed by ^{1}H NMR as follows.



Scheme 1. The synthetic scheme of PAMAM-R-PO.

^{1}H NMR(D_2O): δ propylene oxide (-CH_2CH(OH)CH_3) = 1.35; δ arginine (-HCCH$_2$C**H$_2$**CH$_2$NH-) = 1.71; δ arginine (-HCC**H$_2$**CH$_2$CH$_2$NH-)=1.91; δ PAMAM(-**CH$_2$**CONH-) = 2.54; δ propylene oxide (-C**H$_2$**CH(OH)CH_3) and PAMAM (protons next to amines) = 2.81–2.97; δ arginine (-HCCH$_2$-CH$_2$C**H$_2$**NH-) = 3.25; δ PAMAM (-CONHC**H$_2$**- and -CONHCH$_2$C**H$_2$**NHCO-) = 3.38; δ propylene oxide (-CH_2-C**H**(OH)CH_3) and arginine (-**H**CCH$_2$CH$_2$CH$_2$NH-) = 3.95.

The degree of amine modification was calculated by comparing the integration of proton peaks at 2.54 ppm (PAMAM) and peaks at 1.35 ppm (propylene oxide). About 49 molecules of propylene oxide were observed to react with primary amines of the dendrimer. The molecular weight of the dendrimer was calculated as 26432.09 Da from the NMR result. The charge density of the polymer was estimated as 213.16 Da/(+) by considering all the primary amines of the polymer, and the guanidine groups and secondary amines of arginine residues.

Gel Retardation Assay. The complex formation of PAMAM-R-PO/pDNA was examined by agarose gel retardation assay. The positively charged dendrimer would bind with negatively charged pDNA *via* electrostatic interaction, leading to the formation of positively charged polyplexes that are retarded in gel electrophoresis. As shown in Figure 1, PAMAM-R-PO could completely retard pDNA from a charge ratio of 2, although PAMAM-R showed almost complete retardation of pDNA from a charge ratio of 1.[15] This may be attributed to the low pKa value and steric hindrance of secondary amines formed by reaction with propylene oxide. CH_3(OH)$CHCH_2$- groups would shield secondary amines of the dendrimer from electrostatic interaction with pDNA. This tendency was also identified in a previous report by Tziveleka *et al.*[16]

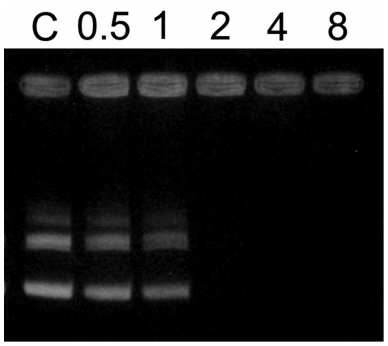


Figure 1. Gel retardation assay of PAMAM-R-PO polyplex. Each number represents charge ratio. C: DNA only.

Size and Zeta-potential Measurements. The average diameter and Zeta-potential of PAMAM-R-PO/pDNA complex was measured to examine primary amine modification effects by Zetasizer. The size of the polyplex is generally known to be an important factor for efficient gene delivery.[18] Positive Zeta-potential of the polyplex is also considered to be essential to adsorption onto the negatively charged cellular membrane of the polyplex. As shown in Table 1, the average diameters of PAMAM-R-PO polyplexes were gradually decreased as the charge ratios of the polyplexes were increased. PAMAM-R-PO polyplex was found to have a size of 242 nm at a charge ratio of 30 although unmodified PAMAM-R polyplex showed a size of 200 nm even at a charge ratio of 6. This result indicates that the pDNA condensing ability of PAMAM-R-PO is weaker than that of PAMAM-R due to its hydroxylated amines.

In the case of Zeta-potential, the potential values of PAMAM-R-PO polyplexes were increased according to the increase of charge ratio. At a charge ratio of 6, PAMAM-R-PO polyplex showed a potential of about 4 mV although PAMAM-R polyplex displayed a plateau value ranging from 20 to 25 mV. The potential value of PAMAM-R-PO polyplex could reach a similar value only at charge ratios greater than 10. This result indicates that Zeta-potential of PAMAM-R-PO polyplex is reduced because of the low pKa value and steric hindrance of hydroxylated amines in the same manner as the former gel retardation result.

Cytotoxicity Test. The cytotoxicity of polymers is a very

Table 1. The average sizes and Zeta-potential values of PAMAM-R-PO polyplexes

Charge ratio	6	10	30
Zave (nm)	428.1 ± 22.0	388.6 ± 50.8	242.2 ± 8.2
Zeta-potential (mV)	3.9 ± 0.1	19.7 ± 9.5	29.7 ± 5.0

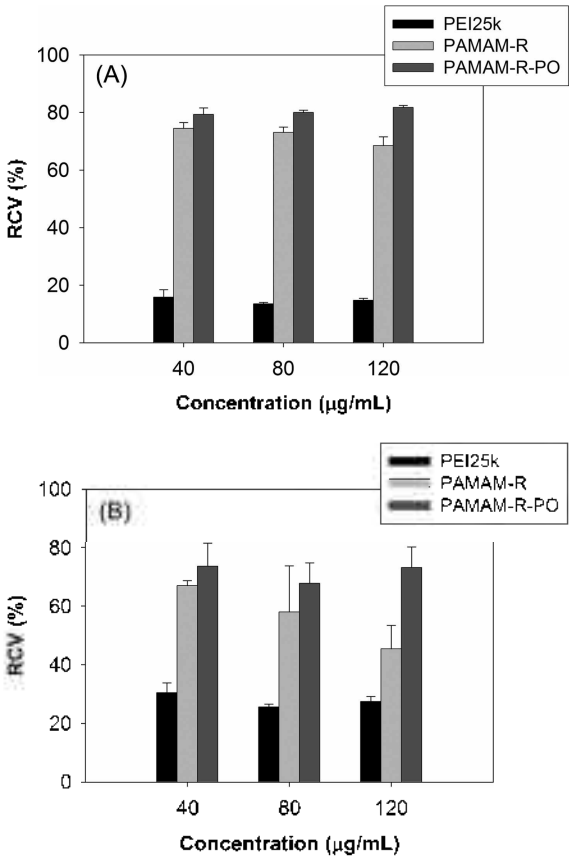


Figure 2. MTT assay results of PAMAM-R-PO on (A) HeLa cells and (B) 293 cells. RCV: relative cell viability.

important factor for gene delivery carriers. Cytotoxicity of cationic polymers is thought to be induced from interaction between amines of polymers and cellular compartments, or accumulation of non-degraded polymer in cells. We performed MTT assay in order to examine the hydroxylation effect of primary amines on cytotoxicity. PEI25 kDa and unmodified PAMAM G4-R were used as controls. PEI25 kDa showed significant cytotoxicity on both HeLa and 293 cells as shown in Figure 2. The cytotoxicity of PEI25 kDa is well known. In Figure 2A, PAMAM-R-PO indicated 80% of cell viability even at a concentration of 120 μg/mL although PAMAM-R displayed 68% of viability at the same concentration on HeLa cells. In the case of 293 cells (Figure 2B), PAMAM-R-PO still showed a high cell viability of over 70% at most concentrations but the cell viability of PAMAM-R decreased to 45% at a concentration of 120 μg/mL. The decreased cytotoxicity of PAMAM-R-PO is thought to be the result that hydroxylated amines reduce the interaction between cationic amines and anionic cellular components, because these two polymers have the same chemical structures except for amine moieties. This result showed that the modification of primary amines of PAMAM-R to hydroxylated amino alcohols reduced their cytotoxicity, giving good biocompatibility to PAMAM-R-PO for gene delivery systems.

***In vitro* Gene Delivery on 293 Cells.** We performed transfection experiments of synthesized PAMAM-R-PO in order to examine the hydroxylation effect of primary amines

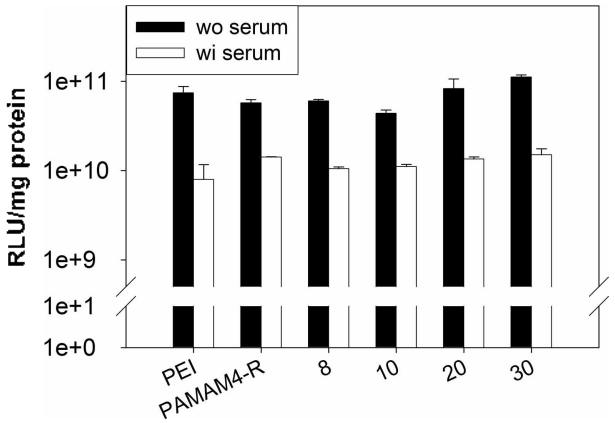


Figure 3. Transfection efficiency on 293 cells. Each number represents charge ratio of PAMAM-R-PO polyplex.

on 293 cells. As shown in Figure 3, transfection efficiency of PAMAM-R-PO was found to be 1.5-1.9 times higher than that of PEI25kDa at a charge ratio of 30. In the presence of serum, PAMAM-R-PO showed a similar transfection efficiency value to unmodified PAMAM-R. However, the polymer eventually displayed about 2 times greater transfection efficiency than PAMAM-R at the same charge ratio in the absence of serum. Although PAMAM-R-PO showed weaker DNA condensing ability than PAMAM-R at low charge ratios, it is thought that this factor does not significantly affect its transfection efficiency at high charge ratios in which physicochemical properties of each polyplex are similar. In addition, lower cytotoxicity of PAMAM-R-PO may lead to more cell proliferation that brings more protein expression on 293 cells than that of PAMAM-R. So, this result shows that the hydroxylation of PAMAM-R-PO does not give a negative effect but rather a positive effect on the transfection efficiency of PAMAM-R on 293 cells and it may be due to reduced cytotoxicity of the polymer.

Conclusion

We hydroxylated primary amines of arginine-conjugated cationic gene delivery carrier, PAMAM-R *via* reaction with propylene oxide and evaluated its gene delivery potency. The newly synthesized polymer, PAMAM-R-PO showed somewhat weaker pDNA condensing ability than unmodified PAMAM-R in view of the gel retardation assay, its average size, and Zeta-potential measurements of the polyplex. However, its cytotoxicity was found to be reduced on mammalian cells and the polymer displayed enhanced transfection efficiency on 293 cells in comparison with unmodified PAMAM-R. Therefore, this strategy based on primary amine modification *via* hydroxylation, would be a potential method for tuning up the existing gene delivery carrier, PAMAM-R.

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