International Journal of Pharmaceutics 363 (2008) 199–205

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Evaluation of generations 2, 3 and 4 arginine modified PAMAM dendrimers for gene delivery

Hye Yeong Nam[a], Hwa Jeong Hahn[a], Kihoon Nam[a], Woo-Hyung Choi[b], Yunseong Jeong[a], Dong-Eun Kim[b], Jong-Sang Park[a,*]

[a] *School of Chemistry & Molecular Engineering, Seoul National University, San 56-1, Shillim-dong, Kwanak-ku, Seoul 151-742, Republic of Korea*
[b] *Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea*

ARTICLE INFO

Article history:
Received 28 March 2008
Received in revised form 6 June 2008
Accepted 21 July 2008
Available online 30 July 2008

Keywords:
Biodegradation
PAMAM
Generation
Oligonucleotide
Transfection
Cytotoxicity

ABSTRACT

It is a matter of concern to develop and design synthetic non-viral gene carriers with high transfection efficiency and low cytotoxicity in gene therapy. Recently, various arginine conjugated dendrimers showed better performance in transfection and greater viability than polyethyleneimine (PEI). In this study, we synthesized and investigated e-PAM-R G2, 3 and 4 which are biodegradable polyamidoamine (PAMAM) dendrimers modified with arginine and compared that with PAMAM-R series containing amide bonds for gene carriers. For plasmid DNA delivery, the transfection efficiency of e-PAM-R G4 was higher than G3 and G2 and similar to PAMAM-R G4 with favorable cell viability. Moreover, they indicated significantly higher suppression of TEL/AML1 protein, maybe due to rapid olidonucleotide (ODNs) release through biodegradability of e-PAM-R. These results suggest that biodegradable and non-toxic e-PAM-R may be useful carriers for the gene including plasmid DNA, antisense ODNs and si-RNA.
© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Gene therapy is a new approach using genetic materials to test, cure, or prevent interactive disease like cancer, AIDS, Parkinson's and Alzheimer's disease and arthritis. For more than two decades, it has been attracting interest as one of the most promising clinical treatment in the post-genomic era (Shinya Kida et al., 2005; Choi et al., 2006).

For successful gene delivery, it is a matter of great importance to develop an effective and safe gene delivery vector. To this end various vectors have been developed by many groups (Itaka et al., 2003). In general, viral or non-viral vectors are used to deliver a therapeutic gene (DNA or si-RNA) to the body. Although viral vectors are efficient, the side effects, including cytotoxicity, mutagenesis, carcinogenesis and immune response, are a major obstacle to their application (Mulligan, 1993; Pedone et al., 2001). To avoid these problems of viral vectors, a large number of non-viral vectors, such as synthetic polymers, lipids and peptides, have been developed. Among them, synthetic polymers used as gene delivery vectors include linear, radiometric branched or block co-polymers and dendrimers (Brown et al., 2001). One of these groups of polymers, polyamidoamine (PAMAM) dendrimers, is safe, nonimmunogenic, and able to function as highly efficient cationic polymer vectors for gene delivery. They have been as efficient as (or more efficient than) both cationic liposomes and other cationic polymers for in vitro gene transfer. In addition, a significant quantity of information on PAMAM dendrimers has been collected during the past decade (Eichman et al., 2000). To develop the transfection efficiency of PAMAM dendrimers, various ligands were conjugated on the PAMAM surface. Many trials have been carried out to modify the surface periphery of PAMAM. Recently, some basic peptides known as protein transduction domains (PTD) or membrane translocalization signals (MTS) and magnetic nanoparticles (MNP) were identified, characterized, and introduced into polymers for the gene delivery (Tung and Weissleder, 2003; Choi et al., 2004; Brooks et al., 2005; Pan et al., 2007). Positively charged aminoacids in these peptides, of which the representative is arginine, are known to have excellent properties of cell penetration. A great deal of research has been done on introducing arginine into polymers such as PAMAM dendrimer or chitosan to improve their transfection efficiency (Choi et al., 2004; Gao et al., 2008).

In our previous study, PAMAM dendrimers conjugated with arginine (PAMAM-R) or lysine (PAMAM-K) through an amide bond were found to have excellent transfection efficiency and be less toxic than polyethyleneimine (PEI) which was the representative candidate for gene delivery at that time, but still had severe toxicity for

* Corresponding author. Tel.: +82 2 880 6660; fax: +82 2 877 5110.
E-mail address: pfjspark@plaza.snu.ac.kr (J.-S. Park).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.07.021

HUVEC or SMC. As is widely known, the cytotoxicity of dendrimers is influenced by the surface group. For example, the cationic dendrimers display more excellent transfection and more toxicity than anionic or neutral polymers such as PAMAM dendrimers with a carboxylate or hydroxyl surface group (Malik et al., 2000). Considering that the cytotoxicity of the polymers is due to accumulation of non-degradable, high molecular weight polymers with high charge density, the biodegradation of these polymers may solve the toxicity problems, at least for non-viral polymers with high charge densities and high molecular weights without loss of transfection efficiency (Jeong et al., 2001). Also, recently the importance of the efficient intracellular disassembly of polyplexes in siRNA delivery was reported, which described enhanced RNA interference activity through the rapid release of siRNA by polymer biodegradation (Jeong et al., 2007).

Based on these studies, we designed and synthesized a new biodegradable polymer named e-PAM-R modified with arginine by esterification, which was expected to have two advantages for gene delivery: (i) an enhanced transfection efficiency from introducing the arginine for DNA carriers, (ii) fast biodegradation able to lower cytotoxicity and deliver antisense ODNs and small interfering RNAs (si-RNAs) to modulate gene expression. For e-PAM-R G4, the synthesis, characterization and in vitro evaluation for DNA delivery is in the process of submission. Here we report the difference and characterization of e-PAM-R G*x* modified with different generation PAMAM-OH and evaluate the possibilities for their clinical application, respectively, for antisense ODNs and si-RNA delivery.

2. Materials and methods

2.1. Materials

PAMAM-OH G2, G3 and G4 (ethylenediamine core), PAMAM-NH_2 G2, G3 and G4 (Starburst), piperidine, *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Sephadex® LH-20 (lipophilic sephadex) were purchased from Sigma–Aldrich (St. Louis, MO). *N*-hydroxybenzotriazol (HOBt) and 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium (HBTU) were purchased from Anaspec Inc., (San Jose, CA). Fmoc-L-Arg(pbf)-OH was from Novabiochem (San Diego, CA). Luciferase Assay System and Reporter Lysis Buffer were from Promega (Madison, WI). Fetal bovine serum (FBS) and 100× antibiotic-antimycotic agent were purchased from GIBCO (Gaithersburg, MD). EBM-2 and EGM-2 medium were from Cambrex Bio Science (Walkersville, MD). Pico Green reagent was purchased from Molecular Probes (Eugene, OR). All chemicals were used without any further purification. The firefly luciferase expression plasmid, pCN-Luci was constructed by subcloning cDNA of Photinus pyralis luciferase with 21-amino acid nuclear localization signal from SV40 large T antigen to pCN.

2.2. Synthesis of e-PAM-R Gx (x = 2, 3, and 4)

As shown in Fig. 1, the synthesis of e-PAM-R is similar to PAMAM-R which is arginine-modified PAMAM through an amide bond except purification and deprotection of the pbf group. First, arginine coupling to PAMAM was performed with 4 eq. of Fmoc-L-Arg(pbf)-OH, HOBt, HBTU and 8 eq. of DIPEA per surface hydroxyl group of PAMAM-OH dendrimer in anhydrous DMF for 12 h at 37 °C. Then, the mixture was precipitated with cold diethyl ether. The crude product was eluted on a Sephadex® LH-20 (DMF only) to remove any remaining excess Fmoc-L-Arg(pbf)-OH, HOBT and HBTU. For deprotection, 90% of trifluoroacetic acid in DMF was used for the pbf protecting group and 30% of piperidine in DMF for the Fmoc group. Each step was performed for 30 min and followed with precipitation in cold diethyl ether 3times to purification. The polymers were verified by ^{1}H NMR spectra (Bruker DPX-300 NMR spectrometer, D_2O).

2.3. Hydrolytic degradation patterns by ^{1}H NMR spectroscopy

The degradation patterns of e-PAM-R G2, G3 and G4 in D_2O were determined by ^{1}H NMR spectrometry. Each polymer was dissolved in D_2O to a concentration of 5 mg/mL and incubated in an NMR tube at 37 °C. The NMR spectra of samples were obtained at the indicated time interval until products were almost degraded.

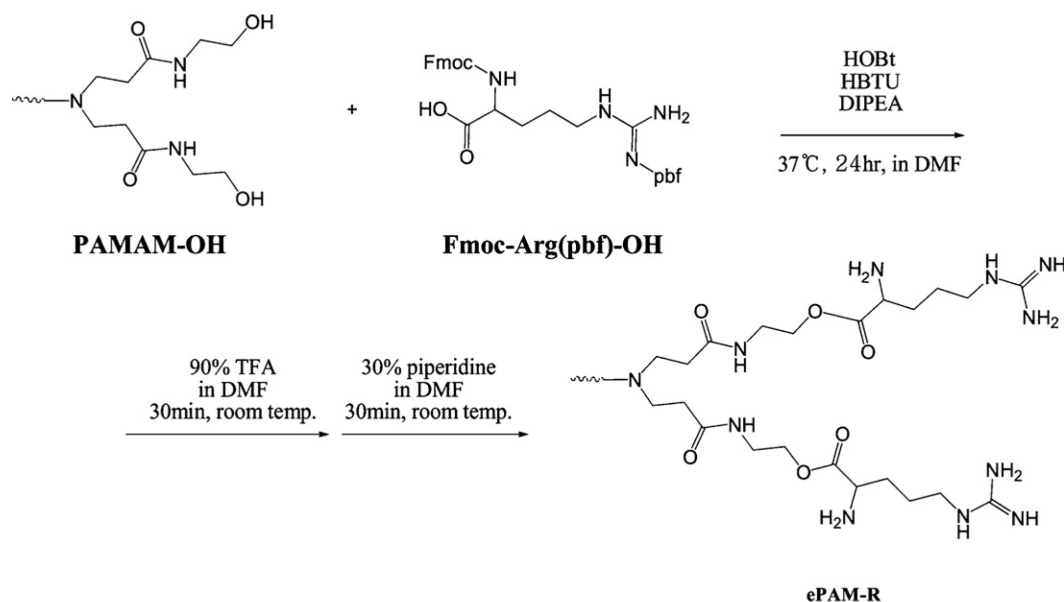


Fig. 1. The synthetic scheme of e-PAM-R G2, 3 and 4.

2.4. Agarose gel electrophoresis studies

Polyplexes were prepared at various weight ratios between each of the polymers and pCN-Luciferase plasmid(pCN-Luci) was constructed by subcloning the cDNA of *Photinus pyralis* luciferase with 21-amino acid nuclear localization signal from SV40 large T antigen to pCN. The polyplexes were formed by incubating them in HEPES buffer (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$) at room temperature for 30 min. Then, each sample was subject to electrophoresis on 0.7% (w/v) of agarose gel and stained in a buffer containing ethidium bromide (EtBr, 0.5 g/mL) at 37 °C for 1 h. The stained gels were analyzed using an UV illuminator to show the location of the DNA band.

2.5. Pico Green assay for polyplex formation

200 μL of polyplex solutions of pCN-Luci DNA (1.0 μg) and each of the polymers were prepared in HEPES-buffered saline (HBS, 25 mM HEPES, 150 mM NaCl, pH 7.4) at various weight ratios and the mixtures were incubated for 30 min at room temperature. Then, 200 μL of the diluted Pico Green stock solution (1×) diluted 200-fold in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) before the experiment was mixed with the same volume of blank solution or polyplex solution. After 2 min incubation, the mixture was diluted with 1.6 mL of TE buffer to a total 2 mL. Fluorescence was measured with a spectrophotometer (JASCO, FP-750) at 480 and 520 nm for excitation and emission.

2.6. Size measurement

The size of the dendrimers/plasmid DNA complexes was measured using a Malvern Zetasizer 3000HAs (Malvern Instruments Ltd., Worcestershire, U.K.) using the PCS 1.61 software. About 2 mL of the polyplex solutions at various weight ratios were prepared to a final concentration of 5 μg/mL plasmid DNA in water for size measurement.

2.7. Cell culture

Human umbilical vein endothelial cells were grown in EGM-2 with 2% FBS. Human embryonic kidney 293 cells were grown in DMEM with 10% FBS. The cells were routinely maintained on plastic tissue culture dishes for 293 cells and cell binder (Corning) for HUVECs at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. The EGM-2 and DMEM contained 1× antibiotic–antimycitid agent.

2.8. Transfection of plasmid DNA

HUVEC (2×10^4 cells/well) were seeded on 24-well cell binder plates in 600 μL (aliquots) of EGM-2 containing 2% FBS and maintained for a day. Before transfection, the medium was exchanged with growth factor-free medium (EBM-2) containing 2% FBS. HUVEC were treated with polyplex solution prepared with 2 μg of plasmid DNA and different weight ratios of e-PAM-R G*x* each in 150 μL of serum-free medium, with growth factor to avoid polymer-serum interactions. The polyplex solutions were incubated for 30 min at room temperature before treatment on cells. After 4 h treatment of the polyplex, the medium was replaced by fresh 600 μL medium with EGM-2 and the cells were incubated for a further 20 h. For assay the growth medium was removed and the cells were rinsed with PBS. Then, the cells were lysed for 30 min at room temperature with 150 μL of Reporter lysis buffer (Promega, Madison, WI). Luciferase activity in the transected cells was measured using a LB 9507 luminometer (Berthold, Germany) with ten microliters of the lysate dispensed into a luminometer tube and automatic injection of 50 μL of Luciferase Assay Reagent.

2.9. Cell cytotoxicity

For the cytotoxicity assay, the color metric MTT assay was performed. Briefly, HUVEC were seeded to a density of 6×10^3 cells/well in a 96-well cell binder plate and grown in 90 μL of EGM-2 containing 2% FBS media for 1 day prior to incubation with polymer. After 24 h, each polymer was treated with various concentrations. In particular, PEI 25 kDa was treated as the control to undertake a comparison of cell cytotoxicity.

After 24 h growth, 26 μL of MTT stock solution (2 mg/mL) was added to each well and incubated for a further 4 h. The media was then removed and 150 μL of DMSO was added and the absorbance was measured at 570 nm using a micro-plate reader.

2.10. Transfection of DNAzyme and assessment of TEL/AML1 expression

A slightly modified transfection procedure with plasmid DNA was used for the transfection of mammalian expression plasmid (pcDNA3.1 (Invitrogen) + TEL/AML1 fusion gene) on 293 cells. Before polyplex addition, the medium was changed to serum free medium. The cells were treated with a polyplex containing solution for 4 h at 37 °C. After the exchange of medium with serum, the cells were further incubated for 24 h at 37 °C. Then, the cells were rinsed with DPBS to remove the serum and polymer and serum-free medium was added to each well. DNAzyme (Dz) (oligonucleotides, 58 nts)/e-PAM-R G4 complex incubated for 30 min at room temperature was treated and the cells were further incubated for 24 h. The cells were harvested and transferred to a new tube. Lysis buffer (20 mM tris–HCl, 20 mM EDTA, 10% glycerol, 100 mM KCl, 0.1% nonidet P-40) was added to each tube. After vortexing and storing on ice for 1 h, the cells were centrifuged at 1300 rpm for 15 min at 4 °C and the cell lysate was obtained. The total cellular proteins from the transfected cells were mixed with 5× SDS ample buffer, denaturized at 95 °C and subjected to electrophoresis in an 8% SDS-polyacrylamide gel. For Western blot analysis, the proteins were transferred to an immobilion-P membrane and the membrane was incubated in primary antibodies, mouse monoclonal His-tag antibody and antibody β-actin monoclonal antibody solution for 1 h. The membrane was washed twice with PBST (0.05% Tween 20 in PBS) and incubated in secondary antibody solution (anti-mouse IgG peroxidase conjugate, Sigma) for 1 h. The bands were visualized using chemiluminescence (WEST-ZOL, Intron Biotechnology).

3. Result and discussion

3.1. Synthesis and characterization of e-PAM-R G*x* (*x* = 2, 3 and 4)

Each generation of PAMAM-OH dendrimer was modified with L-arginine for non-viral gene carriers by a similar method for the synthesis of e-PAM-R G4. The synthesis of the e-PAM-R G*x* was confirmed by proton NMR spectrum. Little difference was seen among the spectra of e-PAM-R G2, G3 and G4. The rate of arginine modification was calculated between the methyl protons in the interior of PAMAM, δ 2.63 ($-NCH_2CH_2CO-$ of PAMAM-OH unit) and the methyl peck of PAMAM terminal, δ 4.25 ($-CONHCH_2CH_2OCO-$ of PAMAM-OH unit) and it was confirmed that above 90% of each PAMAM-OH were conjugated with arginine (about 98% for G2, about 95% for G3 and about 94% for G4). As the generation of e-PAM-R increases, the number of the arginine is nearly doubled, but the charge dendity

increases more rapidly due to the guanidine of the arginie (besides primary amine).

3.2. Hydrolytic degradation patterns by ^1H NMR spectroscopy

The e-PAM-R G*x* was degraded to PAMAM-OH G*x* and free L-arginine in hydrolytic environments. We determined the nature of the ester bond degradation over time in a hydrolytic environment. The degradation profiles of free polymer in D_2O at 37 °C were determined by NMR spectroscopy. In the process of the degradation of the ester bond, a peak shift of e-PAM-R was noticeable, in which the δ 4.25 (–CONHCH$_2$C*H$_2$*OCO- of PAMAM-OH unit) peak shifted to δ 3.66 (–CONHCH$_2$C*H$_2$*OH of PAMAM-OH unit) (Fig. 2A). From this NMR peak shift, the relative percentage of the number of conjugated residues at zero time was calculated. e-PAM-R G4 was degraded about 50% within 4.2 h, e-PAM-R G3 within 4.7 h and G2 within 4.9 h, that is without a significant difference in the degradation rates of e-PAM-R G4, G2 and G3 after 4 h (Fig. 2B). Therefore, it was expected that e-PAM-R had a higher positive charge density as the generation increased.

3.3. Characterization of e-PAM-R G2, G3 and G4 polyplex

To assess the formation of polymer-DNA polyplex, agarose gel electrophoresis was preformed at various weight ratios. The e-PAM-R, arginine-rich cationic polymer has abundant primary amine groups and guanidine groups at the surface. Therefore, it is possible that e-PAM-R interacts and condenses completely DNA with negative charge As shown in Fig. 3, the polyplexs of e-PAM-R G*x* did not have negative charge at weight ratio 2 and above. That is, each polymer made a compact polyplex with plasmid DNA at 2 or more wt ratio in HBS buffer solution.

For a more accurate analysis, the Pico Green reagent assay was performed. The Pico Green reagent is more sensitive then EtBr.

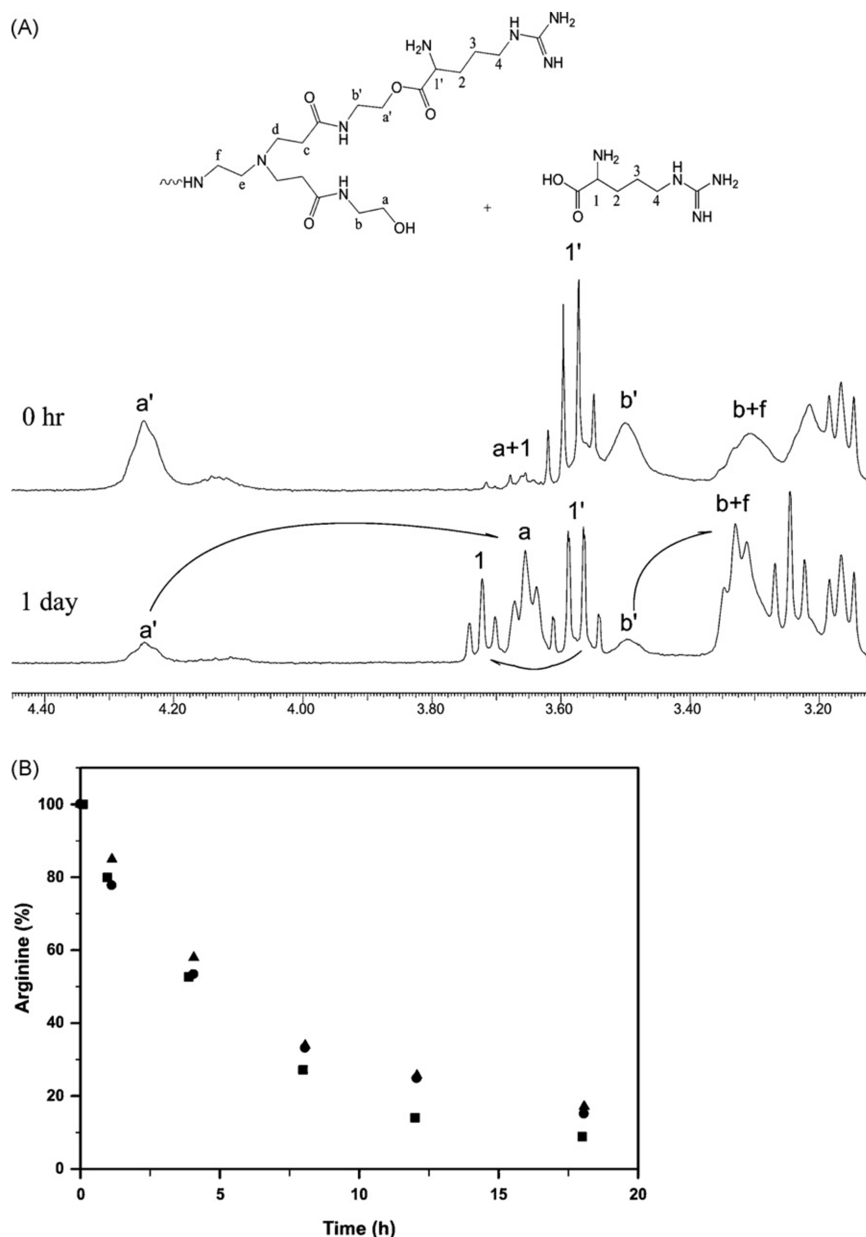


Fig. 2. Degradation profiles of free polymer in D_2O at 37 °C. (A) NMR spectrum of e-PAM-R G3 at 0 h and 24 h. (B) Percent of arginine conjugation at designated times. e-PAM-R G2 (●), e-PAM-R G3 (▲) and e-PAM-R G4 (■).

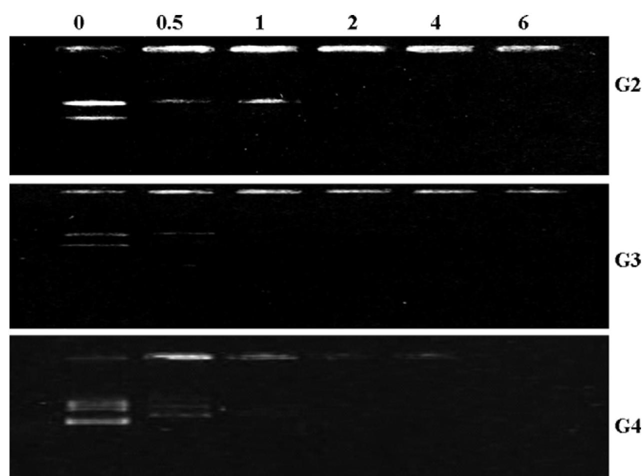


Fig. 3. Gel retardation at various weight ratios. Polymer/DNA = 0.5, 1, 2, 4 and 6 (lanes 2, 3, 4, 5 and 6, respectively).

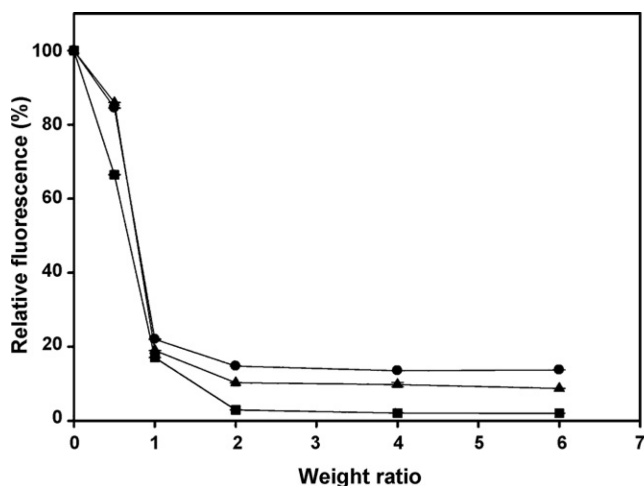


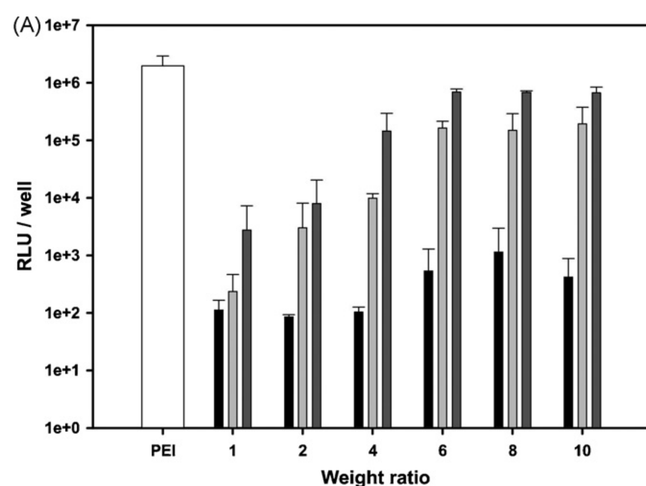
Fig. 4. Pico Green reagent assay, e-PAM-R G2 (●), e-PAM-R G3 (▲) and e-PAM-R G4 (■).

As shown in Fig. 4, the polyplexes were formed at weight ratio 2, which corresponded to the gel electrophoresis result. No significant difference was noticed between each generation, showing a strong complex in order of generations G4, G3, G2. It may be due to the increase in primary amine groups on the surface of polymer according to the generation of e-PAM-R G*x*.

The polyplex sizes were determined by DLS at specific weight ratios, which were desirable ratios for polyplex formation for each generation (Table 1). The e-PAM-R G*x* efficiently condensed DNA at sizes ranging from 200 to 230 nm and e-PAM-R G4, respectively, condensed DNA at sizes in the range of 200 nm that had a narrow size distribution at their optimal weight ratios. It seems that e-PAM-R G4 has the most abundant positive charge to interact with pDNA. Thus the e-PAM-R G*x*, especially G4, is small enough to be taken up into the cell when it forms a polyplex (Vroman et al., 2007).

Table 1
Polyplex Sizes by DLS

Polymers	Weight ratio	Size (nm)	Standard deviation
e-PAM-R G2	3	213.1	±1.7
e-PAM-R G3	3	232.9	±4.7
e-PAM-R G4	6	202.5	±2.5



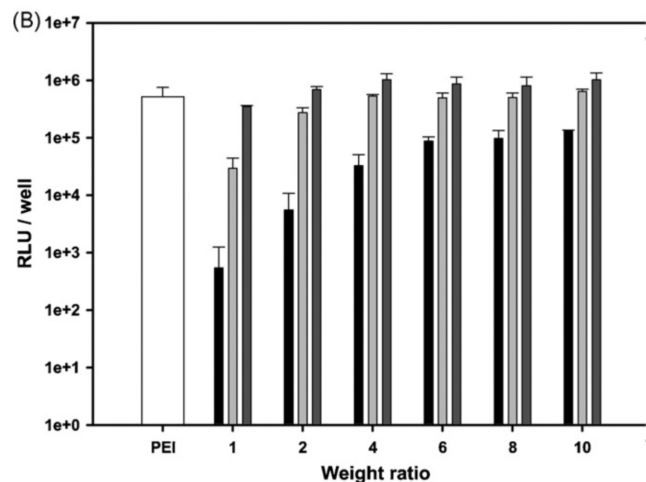


Fig. 5. Transfection efficiency in HUVECs. Results were expressed as RLU/well and each data point represents the mean ± standard deviation ($n = 3$). PEI (□), e-PAM-R G2 (A,■), e-PAM-R G3 (A, light grey), e-PAM-R G4 (A, dark grey), PAM-R G2 (B,■), PAM-R G3 (B, light grey) and PAM-R G4 (B, dark grey).

3.4. Transfection efficiency and cytotoxicity on the HUVECs

The PAMAM-OH has low cell toxicity and transfection efficiency because the hydroxyl groups on the surface of the dendrimer are neutral enough not to interact with DNA and cell membrane for transfection. To evaluate the transfection efficiency of e-PAM-R in each generation, in vitro transfection assay was performed on the sensitive primary cells, HUVECs with a Luciferase gene assay system. e-PAM-R/pCN-Luci complexes were prepared with 2 μg of plasmid DNA at various weight ratios. In Fig. 5, two remarkable characteristics are shown. First, both PAMAM-R and e-PAM-R showed increased transfection activity in higher PAMAM generation. It may be due to more primary amines of high generation, resulting in them interacting with plasmid DNA more strongly. Second, the e-PAM-R showed similar or slightly lower transfection efficiency than PAMAM-R. It is explained that this is due to a defect, –OH groups of e-PAM-R from incomplete modification or hydrolysis during incubation for transfection experiment. However the e-PAM-R G4 showed transfection efficiency as much as peptide-derivate PAMAM-R G4 or PEI 25 kDa at weight ratio of 6 or more.

The cytotoxicity of free polymers, e-PAM-R G*x* was compared with PEI and PAMAM-R G*x* at high concentrations (up to100 μg/mL). As shown in Fig. 6, significant toxicity was observed for PEI 25 kDa (less then 1%), even for the lowest concentration (20 μg/mL). PAMAM-R G4 with the most efficient transfection abil-

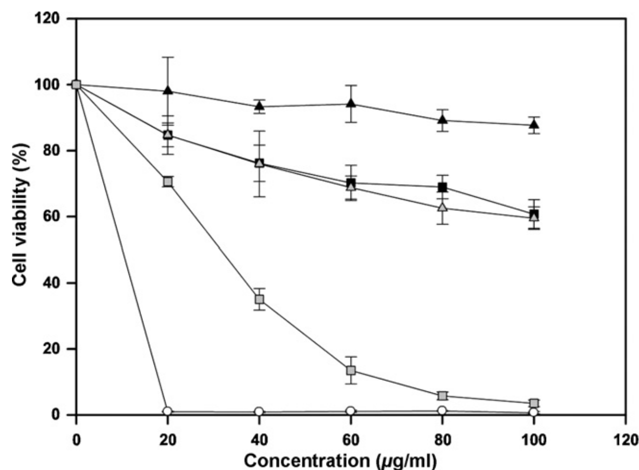


Fig. 6. Cytotoxicity assay on HUVECs at various concentration of polymers (n = 5, error bars represent standard deviation). PEI (○), e-PAM-R G3 (▲), e-PAM-R G4 (■), PAM-R G3 (△) and PAM-R G4 (□).

ity exhibited under 40% cell viability at the 40 μg/mL, whereas other polymers (respectively, e-PAM-Rs) showed negligible levels of toxicity under all conditions. The e-PAM-R G3 showed approximately 87% cell viability and e-PAM-R G4 showed approximately 60% cell viability at the highest concentration 100 μg/mL, which represented nearly non-toxic status for in vivo application. We analyzed e-PAM-R G2 and PAMAM-R G2 but there was little deference between them (about 90% of cell viability). It showed the e-PAM-R had low cytotoxicity, even for sensitive cells, like HUVECs.

3.5. In vitro transfection of DNAzyme using e-PAM-R G4

TEL/AML1 contains the first 336 amino acids of TEL that are linked to residues 21–480 of AML and the fusion protein acted as a transcription repressor to various target genes (Bernardin et al., 2002). RNA-cleaving antisense oligonucleotide, DNAzyme, which targets against TEL/AML1 junction and represses the expression of TEL/AML1 gene, was used to evaluate the transfection efficiency of polymers. The transfection of DNAzyme using fourth generation modified PAMAM-R and e-PAM-R as delivery agents was investigated on 293 cells applying Western blotting analysis. Dendrimer-DNAzyme of weight ratios 2 and 4 and concentrations of 0.1 μM and 1 μM DNAzyme (Dz) were used in transfection tests. The e-PAM-R/DNAzyme complex (a weight ratio 2) exhibited much higher transfection efficiency than complexes prepared with PAMAM-R and Lipofectamin (optimal weight ratio 4), followed by significantly higher suppression of TEL/AML 1 protein (Fig. 7). The result of quantitative analysis showed that the 2x e-PAM-R/DNAzyme polyplex displayed the lowest protein expression, even

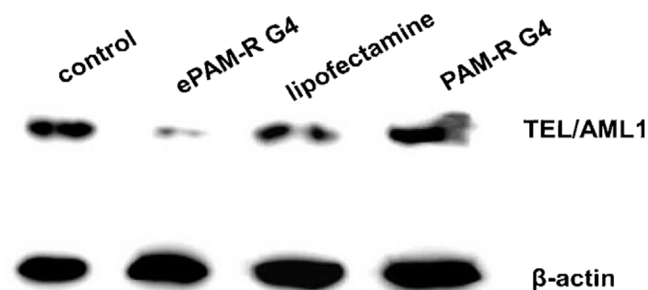


Fig. 7. Western blot analysis of DNAzyme transfection at optimal weight ratio of polymer/0.1 μM DNAzyme.

at 0.1 μM. The comparison of PAMAM-R G4 and e-PAM-R G4 for DNAzyme indicates that the introduction of biodegradability is significantly important for si-RNA and antisense oligonucleotides. The potential of the e-PAM-R G4 for genetic materials was also confirmed by the shRNA-mediated suppression of HMGB1 (high-mobility group box 1, a nonhistone DNA-binding protein) expression test, leading more suppression of HMGB1 than PAMAM-R G4. Anti-inflammatory effect of shRNA-mediated HMGB1 suppression using PAMAM-R G4 was previously published (Kim et al., 2006). Before the application to animal model, the preliminary ex vivo test was carried about with e-PAM-R G3 and G4 to evaluate for the effect according to the generation. Preliminary results showed that the e-PAM-R G3 was preferred to e-PAM-R G4 (data not shown). That is, while PAMAM-R G4 shows better efficiency or similar than e-PAM-Rs, e-PAM-R G3 is more effective than PAMAM-R G4 and e-PAM-R G4 for si-RNA and antisense oligonucleotides.

4. Discussion

Low transfection efficiency and high cell toxicity has limited in vivo applications of non-viral carriers. Until now, PEI has been considered the most excellent non-viral gene carriers. Our previously reported PAMAM-R has shown equal or better ability than PEI for in vitro transfection of plasmid DNA on various cell lines and primary cells with beneficial result for cell viability. However, PAMAM-R still has a problem of toxicity for primary cells, SMC and HUVEC for clinical applications, even though preferable to PEI. Newly synthesized e-PAM-R displays similar transfection efficiency and cell viability as PAMAM-R. Cell transfection procedures are well known, such as: cellular membrane interaction, cellular uptake and endosomal escape and followed by gene expression. To enter the cytoplasm and nucleus, it is very important to escape from the endosome of a polyplex, which is generally accomplished with a “proton sponge” effect. PAMAM dendrimers including PAMAM-OH have tertiary amines in their interior capable of being protonated at acidic endosomal pH; therefore, e-PAM-R having PAMAM backbone is considered to be able to escape easily from the endosome and show favorable transfection efficiency (Godbey et al., 1999; Eichman et al., 2000).

Generally cytotoxicity of PAMAM dendrimers increases as generations are increased because the size has influence on the dendrimers' cytotoxicity. The nature and density of charged groups are another factor that determines dendrimers' toxicity. Cationic charges at the surface are usually toxic, but it depends on the specific groups like primary amines (Dufes et al., 2005).

Once the cationic polymers form complexes, they usually have neutral or positive charge at a charge ratio of 1 or more. These complexes usually showed less cytotoxicity than cationic free-polymers. The charge density of cationic polymers has been felt to account for crucial interactions with the cell membrane. However, because that interaction may cause damage to cells, the development of non-toxic cationic polymers is very important. The e-PAM-R was hydrolyzed and produced the PAMAM-OH and free arginines. The PAMAM-OH has neutral hydroxyl groups on its surface instead of primary amine groups, resulting in nearly cellular non-cytotoxicity and non-aggregation. Moreover, arginine is the kind of amino acid in the cellular system. Therefore, e-PAM-R was very biocompatible and non-toxic due to biodegradability.

While PAMAM-R demonstrated more efficient ability for plasmid DNA delivery, e-PAM-R was better for oligonucleotide and si-RNA delivery. This result shows that the biodegradability of these dendrimers may affect the delivery of small size nucleic acids such as antisense ODNs and si-RNAs as well as cytotoxicity. A similar result exhibiting the importance of adequate intracellular disas-

sembly of polyplexes for si-RNA delivery was recently reported by another group (Jeong et al., 2007). The excellent result for the delivery of these small nucleic acids may be due to the easy release of oligonucleotides through fast hydrolysis of e-PAM-R, and more e-PAM-R G3 showed proper interaction (slightly weak interaction) than G4. Considering their ability to regulate gene expression in a highly sequence specific manner, antisense oligonucleotide (like DNAzyme) and small interfering RNAs have gathered a lot of attention in diverse fields. Therefore, the excellent transfection capability of e-PAM-R for these therapeutic small nucleic acids is a very important result.

These consequences suggested that the attachment of arginine to a PAMAM dendrimer through a biodegradable ester bond has two advantages. First, the introduced arginine affects polyplex formation and penetration into the cell membrane and nucleus. Second, the biodegradability of polymers affects the efficiency of intracellular disassembly for oligonucleotides and si-RNAs and the appropriate cell viability.

Therefore, we have concluded that arginine modified biodegradable e-PAM-R has advantages for the delivery of therapeutic genetic materials, including DNA, antisense ODNs and si-RNAs according to the generation of the dendrimers.

References

Bernardin, F., Yang, Y., Cleaves, R., Zahurak, M., Cheng, L., Civin, C.I., Friedman, A.D., 2002. TEL-AML1, expressed from t(12;21) in human acute lymphocytic leukemia, induces acute leukemia in mice. Cancer Res. 62, 3904–3908.

Brooks, H., Lebleu, B., Vives, E., 2005. Tat peptide-mediated cellular delivery: back to basics. Adv. Drug Deliv. Rev. 57, 559–577.

Brown, M.D., Schatzlein, A.G., Uchegbu, I.F., 2001. Gene delivery with synthetic (non viral) carriers. Int. J. Pharm. 229, 1–21.

Choi, J.S., Ko, K.S., Park, J.S., Kim, Y.-H., Kim, S.W., Lee, M., 2006. Dexamethasone conjugated poly(amidoamine) dendrimer as a gene carrier for efficient nuclear translocation. Int. J. Pharm. 320, 171–178.

Choi, J.S., Nam, K., Park, J.-Y., Kim, J.-B., Lee, J.-K., Park, J.-S., 2004. Enhanced transfection efficiency of PAMAM dendrimer by surface modification with L-arginine. J. Control. Release 99, 445–456.

Dufes, C., Uchegbu, I.F., Schatzlein, A.G., 2005. Dendrimers in gene delivery. Adv. Drug Deliv. Rev. 57, 2177–2202.

Eichman, J.D., Bielinska, A.U., Kukowska-Latallo, J.F., Baker, J.R., 2000. The use of PAMAM dendrimers in the efficient transfer of genetic material into cells. Pharm. Sci. Technol. Today 3, 232–245.

Gao, Y., Xu, Z., Zhang, H., Ding, S., Gu, W., Cheng, L., Li, Y., 2008. Arginine-chitosan/DNA self-assemble nanoparticles for gene delivery: in vitro characteristics and transfection efficiency. Int. J. Pharm 359, 241–246.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999. Poly(ethylenimine) and its role in gene delivery. J. Control. Release 60, 149.

Itaka, K., Yamauchi, K., Harada, A., Nakamura, K., Kawaguchi, H., Kataoka, K., 2003. Polyion complex micelles from plasmid DNA and poly(ethylene glycol)-poly(-lysine) block copolymer as serum-tolerable polyplex system: physicochemical properties of micelles relevant to gene transfection efficiency. Biomaterials 24, 4495.

Jeong, J.H., Lane, C.V., James, Y.W., Zhong, Z., Johan, E.F.J., Kim, J.W., Feijen, J., Kim, S.W., 2007. Reducible poly(amido ethylenimine) directed to enhance RNA interference. Biomaterials 28, 1912.

Jeong, J.H., Song, S.H., Lim, D.W., Lee, H., Park, T.G., 2001. DNA transfection using linear poly(ethylenimine) prepared by controlled acid hydrolysis of poly(2-ethyl-2-oxazoline). J. Control. Release 73, 391.

Kim, J.-B., Choi, J.S., Nam, K., Lee, M., Park, J.-S., Lee, J.-K., 2006. Enhanced transfection of primary cortical cultures using arginine-grafted PAMAM dendrimer, PAMAM-Arg. J. Control. Release 114, 110–117.

Malik, N., Wiwattanapatapee, R., Klopsch, R., Lorenz, K., Frey, H., Weener, J.W., Meijer, E.W., Paulus, W., Duncan, R., 2000. Dendrimers: relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. J. Control. Release 65, 133–148.

Shinya Kida, Mitsuko Maeda, Keiko Hojo, Yusuke Eto, Jian-Qing Gaob, Shinnosuke Kurachi, Fumiko Sekiguchi, Hiroyuki Mizuguchi, Takao Hayakawa, Tadanori Mayumi, Shinsaku Nakagawa, Koichi Kawasaki, 2005. Design and synthesis of a peptide-PEG transporter tool for carrying adenovirus vector into cells. Bioorg. Med. Chem. Lett. 15, pp. 621–624.

Mulligan, R.C., 1993. The basic science of gene therapy. Science 260, 926–932.

Pan, B., Cui, D., Sheng, Y., Ozkan, C., Gao, F., He, R., Li, Q., Xu, P., Huang, T., 2007. Dendrimer-modified magnetic nanoparticles enhance efficiency of gene delivery system. Cancer Res. 67, 8156–8163.

Pedone, E., Cavallaro, G., Richardson, S.C.W., Duncan, R., Giammona, G., 2001. [alpha], [beta]-poly(asparthylhydrazide)-glycidyltrimethylammonium chloride copolymers (PAHy-GTA): novel polymers with potential for DNA delivery. J. Control. Release 77, 139–153.

Tung, C.-H., Weissleder, R., 2003. Arginine containing peptides as delivery vectors. Adv. Drug Deliv. Rev. 55, 281–294.

Vroman, B., Ferreira, I., Jerome, C., Jerome, R., Preat, V., 2007. PEGylated quaternized copolymer/DNA complexes for gene delivery. Int. J. Pharm. 344, 88.