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Bioorganic & Medicinal Chemistry 15 (2007) 1708–1715
Bioorganic & Medicinal Chemistry

Synthesis and characterization of poly (amino ester) for slow biodegradable gene delivery vector

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Received 30 October 2006; revised 4 December 2006; accepted 4 December 2006
Available online 15 December 2006

Abstract—Many therapeutic carrier materials were exploited for human gene therapy from viral to polymeric vectors. This research describes the evaluation of two biodegradable ester-bonded polymers synthesized by double-monomer polycondensation for a non-viral cationic polymer-based gene delivery system. The backbone was constructed to include inner tertiary amines and outer primary amines. Self-assembly with DNA resulted in the production of regularly nano-sized spherical polyplexes with good transfection efficiency, especially in the presence of serum. The polymers showed a relatively slow degradability for an amine-containing ester polymer, as they maintained DNA/polymer complex for 7 days in physiological buffer conditions. Finally, the low toxicity and slow degradation concluded these polymers reliable for long-term therapeutic applications.

1. Introduction

The application of gene therapy has led to improvements in the treatment of several diseases arising from genetic deficiencies, such as cancers and cystic fibrosis.[1–4] The delivery systems are viral and non-viral vectors, but the possibility of insertional oncogenesis and immune response limits the repeated administration of most viral systems.[5] Therefore, low-toxic and safe non-viral systems are potentially attractive compared with viral systems, and are also superior in their ease of manufacturing, R&D/production costs, and the possibility of large-scale production, including DNA loading capacity. In non-viral systems, cationic polymers have played a big part in the delivery system, since DNA is negatively charged. They have the advantage of self-condensing ability when mixed with DNA producing regular sized polyplexes and they also cover proton sponge effects for endosomal escape by some polymers.[6] Among those cationic vectors, some biodegradable polymers show relatively lower toxicity due to their degradable scaffolds compared with conventional non-degradable polymers.[7,8] The degradable polymers vary from linear to hyperbranched types and their degradation profiles differ according to their structure. Linear degradable polymers degrade relatively fast and lack long-term stability under physiological conditions.[9–11] For in vivo conditions, stable and long enduring vector is necessary to form a safe DNA/polymer complex to withstand the harsh physiological conditions.[12] Some hyperbranched polymers have been prepared by a double-monomer methodology and reported for gene delivery as well as various other applications such as coatings, additives, drug delivery, and for use as macromolecular building blocks. Simple direct polycondensation of two monomers-containing multiple reacting groups generally ends up with gelation which results in insoluble polymers, but several researchers have succeeded in preparing soluble polymers of high molar mass.[3–17]

Here, we report the development of three-dimensional, complex-structured slow biodegradable amino ester polymer with excellent solubility, constructed by double-monomer polycondensation method. The polymers contain repeating internal tertiary amine-linkage and abundant surface hydroxyl groups that enable good

Keywords: Gene delivery; Biodegradable polymer; Ester polymer; DNA condensation; DNA delivery.
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doi:10.1016/j.bmc.2006.12.004

solubility and further modification. The polymers were also designed to introduce surplus primary amines by modification of the surface hydroxyl groups of the product for effective DNA condensation.

2. Results and discussion

2.1. Polymer synthesis and characterization

Bulk polycondensation of monomer 1 and monomer 2 by slow temperature increase resulted in a soluble, biodegradable complex-structured poly (amino ester). Numerous reactive arms, such as four methyl esters from monomer 1 and five hydroxyls from monomer 2, reacted at an appropriate speed without vacuum and a relatively high molecular mass polymer was obtained without gelation, exhibiting honey color and sticky semi-solid properties. The polymer had excellent solubility not only in aqueous condition, but also in organic solvents such as DMF, DMSO. Two polymers were obtained with different reaction times, such as 24 h and 72 h at step (i) in Figure 1. The surface hydroxyl groups were coupled with Fmoc–ε-Ahx–OH (ii) and deprotection of Fmoc groups (iii) yielded two polymer-NH_2s, named as PAE 24 and PAE 72, by the reaction time. Primary amine quantification by fluorescamine assay revealed that PAE 24 and PAE 72 had 2.4 and 3.6 μmol primary amines/mg, respectively. The average M_w data with polydispersity ratios were collected at three angles, extrapolated in a Zimm plot, at a laser wavelength of 690 nm with sample concentration of 20 mg/mL (Table 1). The exponent α values were 0.53 and 0.59 for PAE 24 and PAE 72, respectively, obtained by the scaling law $< s^2 >^{1/2} = QM^{\alpha}$. The value of α between 0.5 and 0.6 is an indication of a random coiled structure as the values are 0.33 for spherical and 1 for rod-like structures, which indicated that both PAE 24 and PAE 72 had a random coiled structure. The polymer was mixed with DNA and left for 30 min before adding the buffer containing PicoGreen reagent and data were obtained assessing the formation of polymer/DNA complexes at increasing weight ratios, as shown in Figure 2. PicoGreen is known as a very sensitive, highly reproducible probe for double-stranded DNA quantification and the fluorescence increases as the degree of the exposed DNA increases from complexes.[18] Both PAE 24 and PAE 72 showed polymer/DNA complex formation at a weight ratio of 5, reaching minimum values.

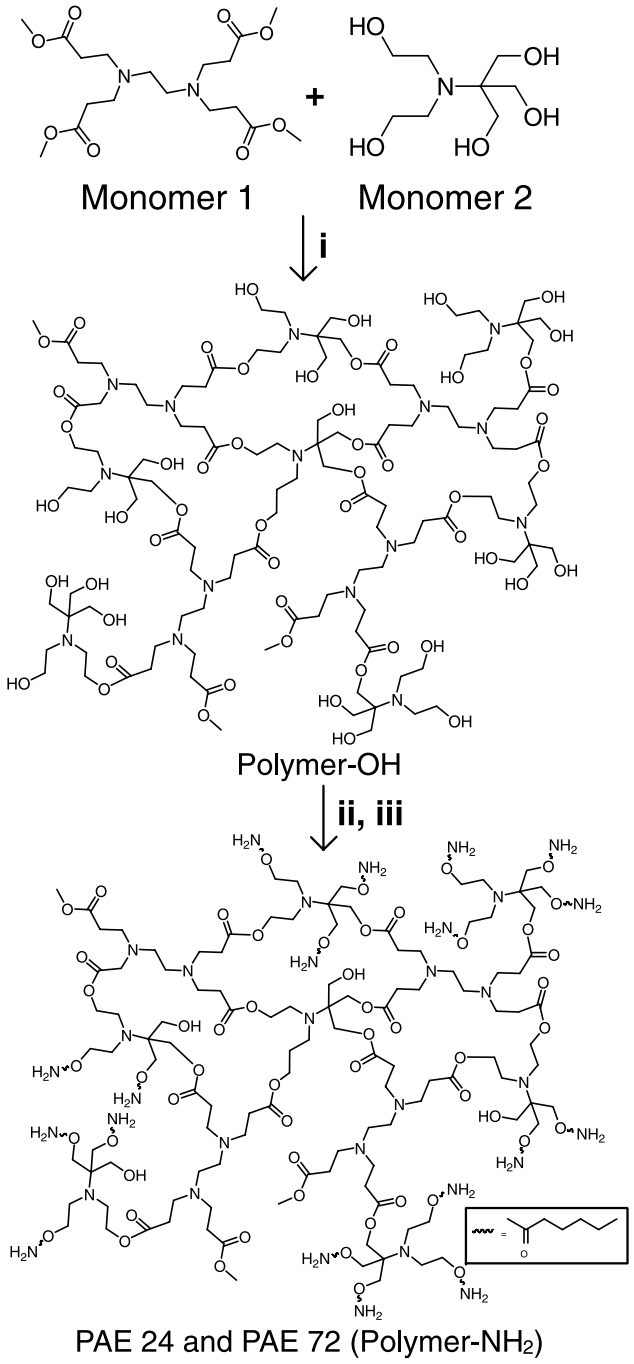

Figure 1. (i) Bulk polycondensation. Two polymers obtained by different reaction times: 24 and 72 h for PAE 24 and PAE 72, respectively; (ii) Fmoc–ε-Ahx–OH, HOBt, HBTU, DIPEA; (iii) 15% piperidine/DMF (v/v).

2.2. Size of PAE 24 and PAE 72 polyplexes

The *z*-weighted hydrodynamic polyplex radius (R_h) distributions were obtained by the DLS method in order of increasing weight ratio (Fig. 3) as 1:1, 1:5, 1:10, 1:20, and 1:40. The initial step, at 1:1, revealed a rather heterogeneous size distribution of average 470 and 390 nm for PAE 24 and PAE 72, respectively. At 1:5, where a stable polyplex was formed according to the PicoGreen results (Fig. 2), narrower size distributions than 1:1 were observed. But stable and regular size polyplex was formed at 1:10. As the weight ratio increased, the radius decreased from 260 nm (1:10) to 200 nm (1:40) for PAE 24 and from 200 nm (1:10) to 140 nm (1:40) for PAE 72.

2.3. Degradation

The design of an efficient and safe gene delivery polymer is an important concern. Both PAE 24 and PAE 72 contain ester bonds in their backbone structure to induce biodegradability under aqueous conditions. The degradation profiles of polymers, as degradation continued

Table 1. Averaged molecular weight (g/mol) and polydispersity index obtained by MALLS method at a laser wavelength of 690 nm

	M_n	M_w	M_z	Polydispersity (M_w/M_n)	Polydispersity (M_z/M_n)
PAE 24	2.70×10^4	3.04×10^4	4.65×10^4	1.125	1.722
PAE 72	4.45×10^4	4.59×10^4	5.83×10^4	1.033	1.311

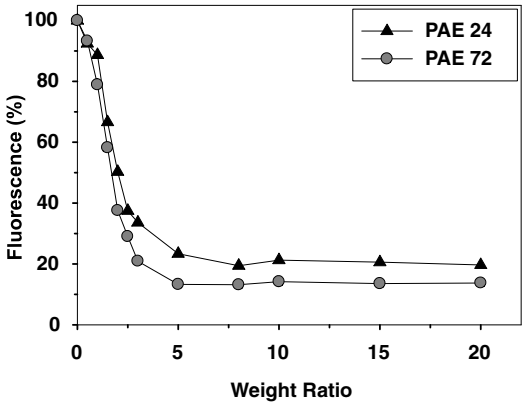

Figure 2. Polyplex formation of PAE 24 and PAE 72 with plasmid DNA at increasing weight ratios by PicoGreen method. The weight ratios 5, 10, 15, 20, 30, and 40 are calculated to the charge N/P ratios of 6.3, 12.6, 18.9, 26.6, 37.8, and 50.5, respectively, for PAE 24. Also the weight ratios 5, 10, 15, 20, 30, and 40 are 4.2, 8.4, 12.6, 16.8, 25.2, and 33.6, for N/P ratios for PAE 72, respectively.

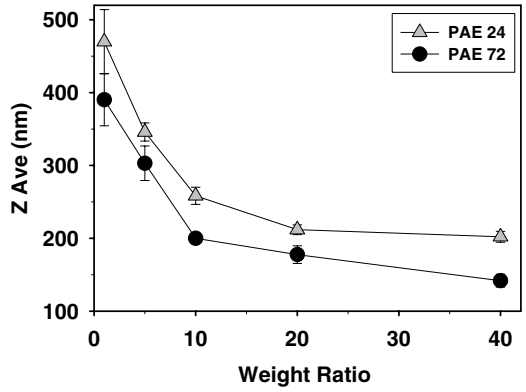

Figure 3. Polyplex sizes of PAE 24 and PAE 72 by DLS.

at pH 7.4 in an incubator at 37 °C, were observed by fluorescence using the PicoGreen method (Fig. 4A and B). At a certain time point, the polymers started to loose DNA complexing ability as a result of degradation, and the partly revealed or free DNA from polyplex was detected as a strong fluorescence signal. A total of 4 polymer conditions were studied for degradation; two weight ratios (1:5 and 1:40, DNA/polymer), each by polymer and polyplex. The ratio 1:5 was used for the lowest, but stable polyplex forming ratio from the results by PicoGreen (Fig. 2), and the ratio 1:40 was chosen from the overall best transfection results by two cell lines (Fig. 7). Fast initial DNA release from both PAE 24 and PAE 72 was obstructed for 3–4 days with a ratio of 1:40 compared with 1:5. Improved DNA release prevention was observed when the form of polyplex was used. Also slower release was obtained by the PAE 72

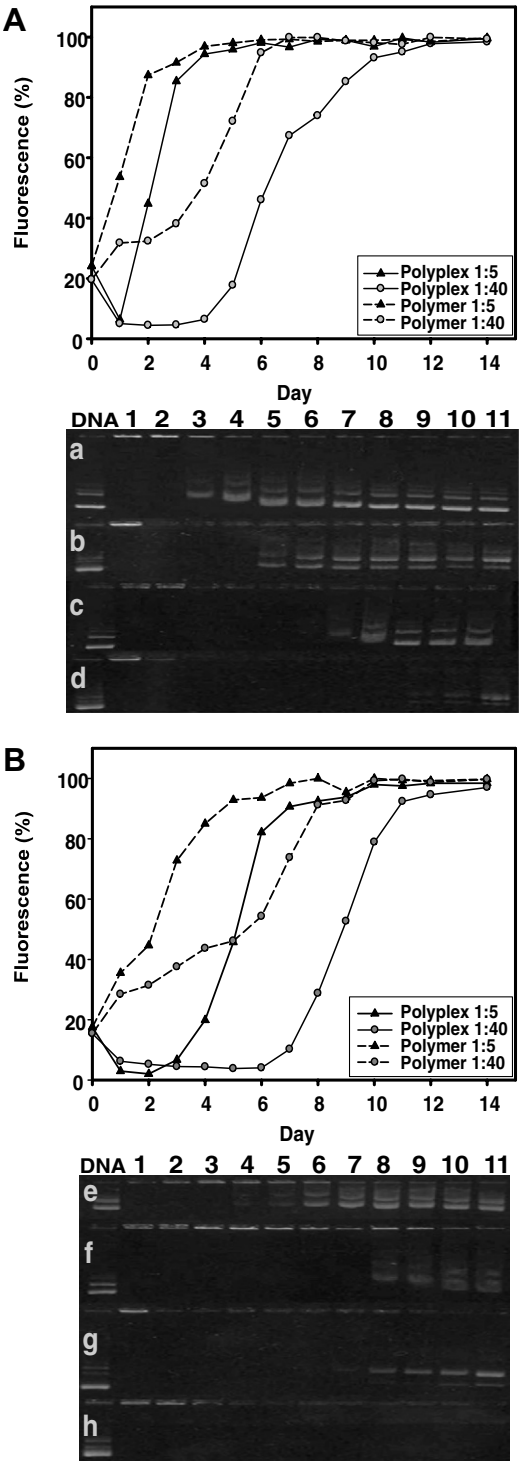

Figure 4. Degradation profiles of PAE 24 (A), PAE 72 (B) and PAE 24 (a–d), PAE 72 (e–h) using PicoGreen (A and B) and gel retardation (a–h), respectively, in terms of time (day). (a–d: PAE 24) and (e–h: PAE 72) are polymer 1:5, polyplex 1:5, polymer 1:40, polyplex 1:40, each in order.

polyplex than the PAE 24 polyplex at the same ratio. Lower fluorescence was obtained after 24 h than at 0 h, only by the polyplexed forms by surface degradation (Fig. 5). These surfaces seemed to cover DNA more effectively at 24 h than at 0 h and the maximum protection lasted 3 and 5 more days under the same conditions at 1:40 for PAE 24 and PAE 72 polyplexes, respectively, until DNA release started. Unlike the compactly self-assembled polyplexes, the uncomplexed polymers showed simple increasing degradation profiles. Steady and simple degradation of the polymers resulted from a loose, incompact polymer scaffold in aqueous condition, leading to easier access and cleavage at the core site of the structure. These results were also confirmed by the gel retardation assay (Fig. 4a–h).

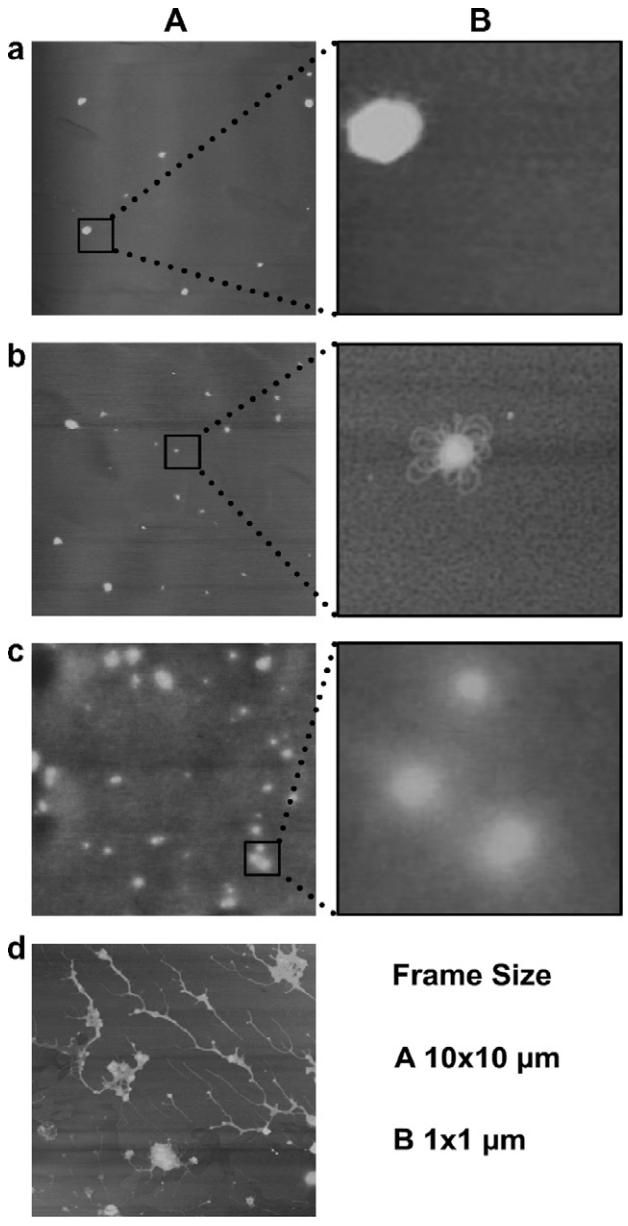

Figure 5. AFM images of polyplex degradation in HBS buffer. Frames a–d show the image of the polyplexes by 0 h, 6 h, 24 h, and DNA control, respectively.

Previous reports have shown that linear polyesters bearing primary and/or secondary amines in their structure degraded quickly by a process of self-destruction in aqueous conditions, which could not exceed 8 h at pH 7.4, 37 °C.[7,8] However, an extended degradation rate is valuable for in vivo experiments, in order to protect the DNA when a low cytotoxicity is confirmed. The cross-linked, complex backbone structure of PAE 24 and PAE 72 lengthened the degradation rate to several days. Once a polyplex was formed, the initial degradation occurred at the surface of a compact spherical polyplex and protected the inner structure from cleavage, which prolonged the degradation rate and complexing ability up to 5 and 10 days for PAE 24 and PAE 72, respectively (Fig. 4a–h).

2.4. AFM images of polyplex and its degradation

Atomic force microscopy (AFM) was used to investigate the morphology of a DNA/polymer particle of PAE 24 at the weight ratio of 1:40 (DNA/polymer) depending on the degree of degradation in HBS buffer, stored at 37 °C. Normally, divalent metal cations, such as Mg^{2+}, are used for enhanced binding of the plasmid DNA to the surface of the mica. Instead, in this experiment, we mixed plasmid DNA with a small amount of the polymer below the weight ratio (1:0.5, DNA/polymer) needed to form compact complexes and the image is presented as a control DNA image in Figure 5d. The self-assembled particles were spherical in shape through 0–24 h. Compared to the clean globular particles at 0 h (Fig. 5a), protruding plasmid DNA could be seen after the incubation of the complexes at 37 °C for 6 h by some polyplexes (Fig. 5b). Longer maintenance in buffer conditions revealed bigger polyplex images with blur rimed shape at 24 h (Fig. 5c). This morphological change was a consequence of polymer degradation at the surface of the polyplex, resulting in a bigger shape.

2.5. Cytotoxicity measurement

High molecular weight and charge contents of polycations might cause hemolytic effects including the toxicity mediated by cellular uptake through the negatively charged cell membrane by polymer interaction.[19–21] The polyester-based PAE 24 and PAE 72 were designed to lower the cellular toxicity by biodegradation. The cell cytotoxicity results of PAE 24 and PAE 72 using PEI as a control were examined by the MTT method (Fig. 6), and both polymers showed similar profiles. HepG2 cells at very high polymer concentration (200 μg/mL) displayed 83% and 82% viability for PAE 24 and PAE 72, respectively. Slightly lower viabilities of 78% and 73% were obtained for C2C12 cells at the same high polymer concentration for PAE 24 and PAE 72, respectively. To observe degradation effects, both polymers were incubated in PBS buffer at 37 °C for 24 h and applied to the cells at the same protocol. The polymer degradation leads to higher cell viabilities as 91% and 87% for PAE 24 and PAE 72, respectively, at polymer concentration of 200 μg/mL in HepG2 and also 88% and 85% for PAE 24 and PAE 72 in C2C12. The viability clearly elevated at low polymer concentrations up to

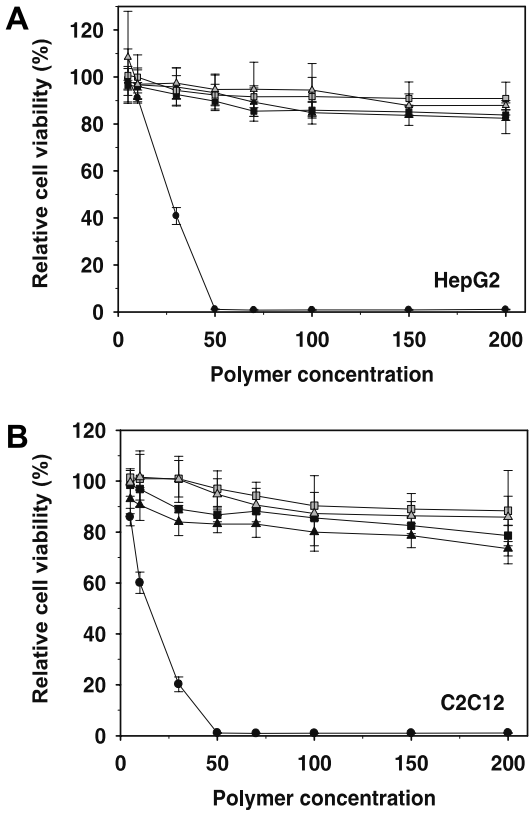

Figure 6. Cell cytotoxicity assay results in HepG2 (A) and C2C12 (B) cells. The colorimetric MTT method was used to calculate the percentage of viable cells compared with untreated control cells. PEI (●), PAE 24 (■), and PAE 72 (▲) at various polymer concentrations (μg/mL) were added to cells and incubated further before measurements. PAE 24 (□) and PAE 72 (△) were added to the cells after 24 h polymer incubation at 37 °C for degradation effects.

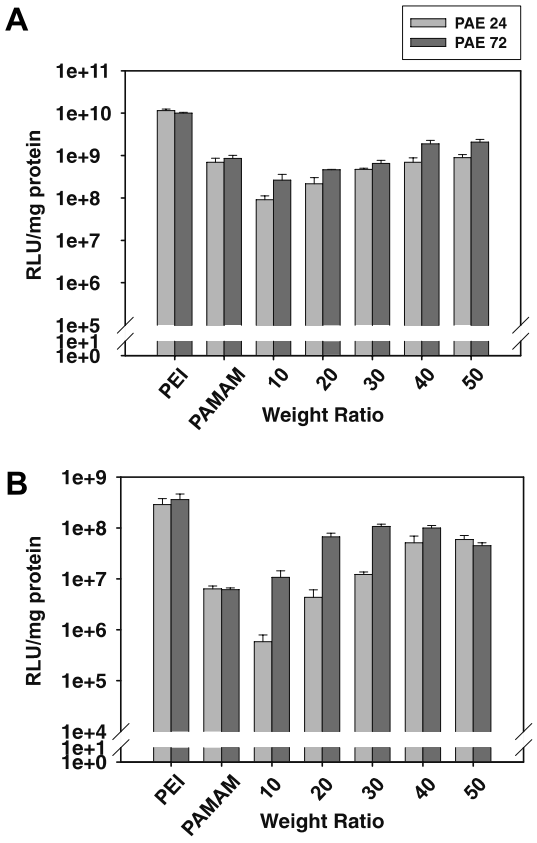

Figure 7. Cell transfection results of PAE 24 and PAE 72 in HepG2 (A) and C2C12 (B) cells.

30 μg/mL in the C2C12 cells, as both polymers showed no toxicity. Since PAE 72 had more primary amines per weight than PAE 24, it revealed mainly lower viability for both types of cells. As we expected, the polymers displayed low toxicity even at a very high concentrations compared with PEI and good biocompatibility resulted from the biodegradability of the polymer, which is in good accordance with previous reports.[7,11,22]

2.6. Transfection

The positively charged polycationic polymers might bind to a negatively charged cell membrane, inducing non-specific cellular uptake. PEI has been one of the most efficient polymeric gene carriers. But the high cytotoxicity of PEI, caused by its high charge and non-degradability, is of serious concern and hinders its application and use in human gene therapy.[23]

The transfection efficiency was investigated by the firefly luciferase reporter gene expression in two cells, HepG2 and C2C12. PEI and PAMAM G4 were used as control reagents (Fig. 7). Both cells were tested under serum-containing conditions at various polymer/DNA weight ratios. Generally cationic polymeric gene delivery materials usually display lower gene expressions in serum-containing conditions than in serum-free conditions, since the charged polymers are known to interact with negatively charged serum proteins to form aggregates resulting in lower transfection efficiencies.[24] Relatively high levels of luciferase activity were confirmed by PEI in both HepG2 and C2C12 compared to other polymers, but PEI was found to induce a 1.5 order lower luciferase activity for C2C12 than for HepG2, proving C2C12 cells, one of the fibroblast cells, were more difficult to transfect with PEI than HepG2. One microgram DNA was condensed with PAE 24 and PAE 72 at various weight ratios for both cell lines. PAE 24 showed maximum transfection efficiencies at ratio 50, as 1/12- and 1/5-fold in HepG2 and C2C12 cells compared with the results using PEI, respectively. Higher maximum values were gained using PAE 72, yielding 1/5-fold transfection levels in HepG2 and 1/3-fold in C2C12 cells compared to the levels of PEI at ratio 50 and 30, respectively. C2C12 resulted in over one order or less lower overall transfection efficiencies than those of HepG2 by all polymers. Interestingly, C2C12 cell transfection by PAE 72 showed relatively better efficiencies at lower weight ratio than PAE 24, as a plateau was reached at the ratio 20, whereas HepG2 cell transfection revealed a similar transfection tendency for both PAE 72 and PAE 24. Higher transfection results were obtained at higher weight ratios by both PAE 24 and PAE 72, as some synthetic polymers showed their best transfection results at high ratios.[25,26] Among the results, PAE 72 displayed overall better transfection efficiencies for both cell lines. Cationic gene carrier polymers need a certain extent of size and amine quantities

for efficient transfection results, and PAE 72 had a 1.51-fold higher molecular weight and 1.5-fold more primary surface amines than PAE 24, which would affect the final transfection results.

3. Conclusion

In this research, two amino ester polymers were synthesized in a relatively simple process without harsh conditions by a double-monomer method, leading to a slow degradation due to their structural complexity using the multiple reactive arms of two monomers. We have shown that they effectively form spherical nano-sized polyplexes with DNA, providing good transfection efficiency under the serum-containing condition with relatively low cytotoxicity and a slow degradation rate, having the essential potentials as a gene delivery carrier for long-term in vivo experiments.

4. Experimental

4.1. Materials

Ethylene diamine, Fmoc–ε-Ahx–OH, ethidium bromide, methyl acrylate, tris(2-hydroxyethyl)amine, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), *N*,*N*-diisopropylethylamine (DIPEA), aluminum chloride, piperidine (hexahydropyridine), ethanolamine, PEI (average molecular weight 25 kDa), ethylenediamine core PAMAM G4, and bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane(bis–tris) were purchased from Sigma–Aldrich (St. Louis, MO). Fmoc-6-amino-hexanoic acid (Fmoc–eAhx) was purchased from Novabiochem (Laufelfingen, Switzerland). *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazole-1-yl)uranium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from Anaspec, Inc. (San Jose, CA). Reporter Lysis Buffer, Luciferase 1000 Assay system, is a product of Promega (Madison, WI). Dulbecco's modified Eagle's medium (DMEM), Minimal Essential Medium (MEM), and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD). Micro BCA protein assay kit was purchased from PIERCE (Rockford, Il). PicoGreen was purchased from Molecular Probes (Eugene, OR).

4.2. Synthesis of monomer 1

A methanol solution of ethylenediamine (20 mmol) was added to a stirred solution of methyl acrylate (1.5 mol) at room temperature and continuously stirred for 52 h. The excess methyl acrylate and methanol were evaporated to obtain monomer 1 (99%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.37 (t, 4H, –CH_2CH_2COO–), 2.38 (s, 4H, –CH_2N–), 2.65 (t, 4H, –CH_2CH_2COO–), 3.56 (s, 12H, –CH_3).

4.3. PAE (poly amino ester)-OH

The polycondensation of monomer 1 (2 g, 4.94 mmol) and monomer 2 (bis–tris, 1 g, 4.77 mmol) was conducted in a stirred glass vial placed in a silicon bath with increasing temperature from 120 to 180 °C, at a rate of 10 °C/h in the presence of 0.1 mol/L aluminum chloride. When the temperature reached 180 °C, the reaction was held at this temperature for a further 24 or 72 h for PAE-OH 24 or PAE-OH 72, respectively, then dissolved in DMF and centrifuged to eliminate the aluminum chloride. Both polymers were clearly soluble in DMF.

4.4. PAE-NH_2 synthesis

Fmoc–ε-Ahx–OH was used for the surface modification of hydroxyl groups to primary amines. The PAE-OH (PAE-OH 24 or PAE-OH 72 each, 50 mg), Fmoc–ε-Ahx–OH (0.25 g, 0.70 mmol), 1-hydroxybenzotriazole (HOBt, 0.95 g, 0.70 mmol), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazole-1-yl)uranium hexafluorophosphate (HBTU, 0.27 g, 0.70 mmol), and *N*,*N*-diisopropylethylamine (DIPEA, 245 μL, 1.41 mmol) were each dissolved in DMF and mixed in a glass vial for reaction at room temperature for 12 h. Then the reagents were removed by polymer precipitation in diethyl ether four times. The Fmoc group was removed by vortexing the polymer in 15% piperidine in a DMF (v/v) solution for 5 min, followed by purification in diethyl ether precipitation, leaving products of PAE-NH_2 24 and PAE-NH_2 72, which were finally named PAE 24 and PAE 72.

^{1}H NMR (300 MHz, D_2O) δ 1.30–1.75 (br, 6H, –$NH_2CH_2CH_2CH_2CH_2CH_2CO$–), 2.38–2.51 (br, 4H, –$NCH_2CH_2CO$– and –$NH_2CH_2CH_2CH_2CH_2CH_2CO$–), 2.60–2.75 (br, 2H, –$NCH_2CH_2O$–), 2.74–2.86 (br, 2H, –$NH_2CH_2CH_2CH_2CH_2CH_2CO$–), 2.84–3.02 (br, 2H, –$NCH_2CH_2CO$–), 3.52–3.73 (br, 3H, –$OCH_3$), 4.06–4.27 (br, 2H, –$COOCH_2$–).

4.5. Plasmid preparation

The firefly luciferase gene was included in the DNA as a reporter gene constructed by subcloning the cDNA of *Photinus pyralis* luciferase to pCN with a nuclear localization signal consisting of a 21-amino acid.[27] The plasmid was transformed to DH5α competent cells and a Qiagen Mega Kit (Valencia, CA, USA) was used for purification. The final plasmid DNA was stored in distilled, filtered water at −20 °C.

4.6. PicoGreen assay

Polyplexes were prepared in 200 μl HBS buffer (25 mM Hepes, 150 mM NaCl, pH 7.4) with 1.0 μg plasmid DNA and incubated for 30 min at room temperature at various polymer weight ratios ranging from 0.5 to 20 (polymer/DNA), then 200 μl of the working solution was added. The PicoGreen working solution was prepared as 1 μl of PicoGreen reagent diluted in 199 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). After 2-min incubation, the samples were diluted with 1600 μl of TE buffer before fluorescence measurement. A SFM 25 spectrofluorometer (Jasco instruments) was used with the wavelength fixed at 480 nm for excitation (λ_{ex}) and 520 nm for emission (λ_{em}).

4.7. Multi angle laser light scattering (MALLS)

Size-exclusion chromatography (SEC) was performed with a chromatography system (Dionex) composed of Waters Styragel HR3 and Styragel HR5E columns. A MALLS detector (mini DAWN DSP, Wyatt Technology, Santa Barbara, CA) was connected to an SEC system and DMF was used as the mobile phase at a flow rate of 0.5 mL/min. The refractive index increment (d*n*/d*c*) was measured by Optilab DSP (Wyatt Technology, Santa Barbara, CA) with Astra 4.81.07 used as the evaluation software (Wyatt Technology Corp.).

4.8. Dynamic light scattering (DLS) size determination

Both polyplexes, PAE 24 and PAE 72, were prepared at increasing weight ratios from 1:1 to 1:40 in HBS buffer. Ten micrograms plasmid DNA was used and the final volume was set to 2 mL with H_2O. A helium–neon lamp was used as the light source (10 mW) on a Malvern Zetasizer 3000HAs system (Malvern Instruments Ltd, Worcestershire, U.K.). Values were obtained 3 cycles per 10 time measurements.

4.9. Degradation studies

The polymers were dissolved in HBS buffer and separated into two groups; polyplex-solution and polymer-solution group, both were kept in an incubator at 37 °C. The polyplex-solution group was prepared in increasing polymer weight ratios, complexed with 1 μg plasmid DNA, and analyzed without further addition of any plasmid DNA. The polymer-solution group, unlike the pre-polyplexed group, was incubated without DNA and 1 μg DNA was added just 30 min before analysis at indicated time intervals, as shown in Figure 4. The analysis continued until the polymer and polyplex groups showed no ability to form complexes. Gel electrophoresis and PicoGreen reagent assays were used for analysis. The PicoGreen samples were analyzed as described above. For the gel electrophoresis method, samples were loaded onto a 0.7% agarose gel at 100 V, and visualized with ethidium bromide for 4 h.

4.10. Atomic force microscopy (AFM)

The polyplex images at the weight ratio of 1:40 (DNA/polymer) were obtained by mixing equal volumes of pCN Luci plasmid vector (5 μg/mL) with polymer solution in HBS. DNA/polymer polyplexes were obtained at various weight ratios by adding 0.1 μg of DNA to polymer solutions, then incubated for 30 min to form self-assembled particles. A small portion (1–2 μL) of the polyplex was dropped onto the middle of a freshly cleaved mica surface and kept for 1 min to adsorb. The remaining solution was removed using a filter paper and dried overnight. The images were obtained on tapping mode at a scanning speed of 5 Hz with a Nanoscope IIIa instrument equipped with an E scanner (Digital Instruments, Santa Barbara, CA).

4.11. Cytotoxicity measurement

HepG2 (human hepatocellular carcinoma cells) and C2C12 (mouse skeletal muscle cells) were grown in 10% FBS (fetal bovine serum) containing MEM and DMEM culture media, respectively, and were seeded in a 96-well plate at a density of 10,000 cells per well. The cells were grown for a further 24 h to reach 70% confluence, then increasing concentrations of polymer solutions were added. After 48-h polymer exposure, the cells were washed with phosphate-buffered saline (PBS) followed by the addition of 26 μL of MTT working solution to each well. The MTT working solution was prepared in PBS at a final concentration of 2 mg/mL and filtered through a 0.2 μm syringe filter before use. After incubation at 37 °C for 4 h, the medium was removed from the well and 150 μL DMSO was added and mixed promptly for measurement by a microplate reader at 570 nm (Molecular Devices).

4.12. Transfection

HepG2 and C2C12 were seeded in a 24-well plate at a density of 50,000 cells per well and maintained in MEM and DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2/95% humidified air at 37 °C until they reached 70% confluence. Then the polyplexes were added to the wells (at the polymer/DNA weight ratios of 5, 10, 15, 20, 30, and 40 for PAE 24 and PAE 72, respectively) and sustained for a further 48 h at 37 °C without removing the polyplexes from the wells. PEI and PAMAM G4 were used at the DNA/polymer weight ratio of 1:1 and 1:8, respectively, with 1 μg of DNA. The growth medium was removed after 48 h and the cells were washed with PBS, followed by lysis for 30 min at room temperature with 120 μL of Reporter lysis buffer (Promega, Madison, WI). A clear lysate was obtained by centrifugation and luciferase activity in the transfected cells was measured with a LB 9507 luminometer (Berthold, Germany). The lysate (10 μL) was loaded onto the luminometer tube and automatic injection of 50 μL Luciferase Assay Reagent (Promega, Madison, WI) measured luciferase activity for 10 s with a 2 s delay. The protein concentration was determined by micro BCA assay reagent (Pierce, Rockford, IL).

Acknowledgments

This work was supported by the Korea Health 21 R&D Project of The Ministry of Health and Welfare, Republic of Korea (A04-0004), and the Gene Therapy Project of The Ministry of Science and Technology (M1053403004-05N3403-00410).

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