Biodegradable Polyester, Poly[α-(4-Aminobutyl)-L-Glycolic Acid], as a Non-Toxic Gene Carrier

Yong-Beom Lim,[1] Sang-Oh Han,[1] Han-Uk Kong,[1] Yan Lee,[1] Jong-Sang Park,[1] Byeongmoon Jeong,[2] and Sung Wan Kim[2,3]

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Purpose. The aim of this study was to develop a non-toxic polymeric gene carrier. For this purpose, biodegradable cationic polymer, poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) was synthesized. PAGA was designed to have ester linkage because polyesters usually show biodegradability.

Methods. Degradation of PAGA in an aqueous solution was followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). PAGA/DNA complexes were characterized by gel electrophoresis, atomic force microscopy (AFM), dynamic light scattering (DLS). The transfection was measured by using the β-galactosidase reporter gene.

Results. PAGA was degraded in aqueous solution very quickly and the final degradation product was a monomer (L-oxylysine). Formation of self-assembling biodegradable complexes between PAGA and DNA at a charge ratio 1:1 (+/−) was confirmed by gel band shift assay and AFM. In these studies, controlled release of DNA from the complexes could be seen. The complexes showed about 2-fold higher transfection efficiency than DNA complexes of poly-L-lysine (PLL), a structural analogue of PAGA, which is the most commonly used poly-cation for gene delivery. The polymer did not show cytotoxicity, possibly because of its degradability and the biocompatibility of the monomer.

Conclusions. The use of the biodegradable poly-cation, PAGA, as a DNA condensing agent will be useful in safe gene delivery.

KEY WORDS: PAGA; gene delivery; biodegradable; ester linkage; non-toxic gene carrier; controlled release.

INTRODUCTION

In recent years, gene therapy has become an important area, as a new therapeutic method (1,2). Several human clinical trials are under way to treat various diseases utilizing viral vector gene carriers (3). Viral vectors include adenoviruses and retroviruses that demonstrated high transfection efficiency but are limited due to adverse effects such as immunogenicity, toxicity, and mutagenesis caused by the cell-infected viruses (4). Synthetic gene carriers, poly-cationic polymers (5–9) or cationic liposomes (10) form complexes with plasmid DNA or antisense oligonucleotide, which then can be carried into cell compartments.

PLL is the polymer most frequently used as a gene carrier (11,12). However, it is degraded slowly and is toxic as shown in cell culture studies (13,14). Although biodegradable polymer was used previously as a nanoparticle DNA carrier (15), a soluble and charged biodegradable polymer has not yet been used as a gene carrier.

We synthesized a novel PLL analogue, PAGA, containing both a degradable ester linkage and a positively charged backbone. The polymer was degraded very quickly in aqueous solution. This polymer possessed the capability of condensing DNA into compact complexes and showed fast hydrolytic degradation after cellular uptake. Furthermore, PAGA/DNA complexes exhibited higher transfection efficiency in cells than did PLL/DNA complexes. The PAGA also showed much less toxicity than did the PLL. Therefore, the newly designed PAGA can be substituted for PLL gene carriers and other positively charged polymers giving increased transfection and non-toxicity.

MATERIALS AND METHODS

Materials

PLL (mol. wt. 4,000) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). pSV-β-gal plasmid vector (6,821 bp) from Promega (Madison, WI). The matrix, α-CHCA was from Aldrich (Milwaukee, WI) and used without further purification.

PAGA Degradation Study by Matrix-Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)

PAGA was dissolved in 25 mM Hepes, pH = 7.3 at a concentration of 5 mg/ml and was incubated at 37°C. α-cyano-4-hydroxy cinnamic acid (αCHCA) was used as the matrix, which was prepared at a concentration of 10 mg/ml in water/3% TFA/acetonitrile, 4:1:6 (v/v). At an appropriate time interval, a 1 μl aliquot of PAGA solution was added to the 9 μl of the matrix solution in a microcentrifuge tube. One μl aliquot was applied to the MALDI sample plate and dried in vacuum. A N_2 laser radiating at 337 nm wavelength with 3 ns pulses was used in a Voyager Biospectrometry Workstation (Perseptive Biosystems, Framingham, MA). The ions generated by the laser pulses were accelerated to 29 kV energy in a positive mode. All the spectra were obtained at the same laser power. Molecular weight (M_p, the most probable peak molecular weight determined from the highest peak) of PAGA determined was 3,200.

Gel Band Shift Assay

PAGA/DNA complexes were formed by mixing pSV-β-gal plasmid solution (20 μg/ml in H_2O) with an equal volume of PAGA in Hepes buffered saline (15 mM Hepes, 150 mM NaCl, pH 7.3) (HBS) at an appropriate charge ratio. After allowing 1 h for the complexes formation, samples were electrophoresed through a 0.8 % agarose gel at 70 V for 40 min and stained with ethidium bromide to visualize the DNA.

Atomic Force Microscopy

DNA (pSV-β-gal) was dissolved in Hepes-Mg (25 mM Hepes, 10 mM $MgCl_2$, pH 7.6) buffer (16) at a concentration

[1] School of Chemistry & Molecular Engineering, Seoul National University, Seoul 151-742, Korea.

[2] Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, BPRB, Room 205, Salt Lake City, Utah 84112.

[3] To whom all correspondence should be addressed. (e-mail: rburns@pharm.utah.edu)

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of 1 μg/ml to obtain a DNA image. The 2 μl of the solution was deposited onto a freshly cleaved mica substrate. The solution was allowed to adsorb for 2 min, washed with 1 ml of distilled water, and rapidly dried in a stream of N_2 gas. For PAGA/DNA complexes and the degraded complexes images, complexes with 5:1 (+/−) charge ratio were made by mixing pSV-β-gal plasmid solution (5 μg/ml in H_2O) with an equal volume of PAGA solution in H_2O. The complexes solution was incubated at 37°C to allow the degradation of the complexes to occur. At an appropriate time interval, 2 μl of the complexes solution was deposited onto a freshly cleaved mica substrate. The solution was allowed to dry for 2 min and then excess fluid was wicked off with filter paper. The solution was dried at room temperature before imaging. AFM was performed using a Nanoscope IIIa equipped with a E scanner (Digital Instruments, Santa Barbara, CA). All AFM imaging was conventional ambient tapping mode AFM with scan speeds of about 5 Hz and data collection at 512 × 512 pixels.

Dynamic Light Scattering and Zeta Potential

The Z-average particle size and polydispersity index of the PAGA/DNA complexes were determined by DLS at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (λ = 633 nm) as the incident beam at a scattering angle of 90° and the Automeasure version 3.2 software (Malvern Instrument Ltd, UK). For data analysis, the viscosity (0.8905 mPa.s) and refractive index (1.333) of pure water at 25°C were used. PAGA/DNA complexes were prepared as described above.

Transfection and Cytotoxicity Assay

293 cells were seeded at a density of 6×10^4 cells/well in 24-well plate, 24 h prior to transfection. Due to its rapid degradation in aqueous conditions, PAGA was dissolved in water just prior to mixing with plasmid DNA. Plasmid pSV-β-gal/PAGA complexes were prepared by mixing pSV-β-gal (10 μg/ml) and PAGA (100 μg/ml) in an FBS-free cell culture medium at an appropriate charge ratio, and incubated for 20 min at room temperature to achieve complexes formation. The medium in the 24-well plate was replaced with transfection mixture followed by 4 h of incubation at 37°C. Then the transfection mixture was replaced with fresh medium. Cells were further incubated for 48 h at 37°C. The β-galactosidase activity in transfected cells was determined using a colorimetric ONPG (o-nitrophenyl β-D-galactopyranoside) assay system (Promega, Madison, WI). Absorbance at 405 nm was normalized to the protein concentration determined using the Pierce BCA protein assay reagent (Pierce, Rockford, IL).

For the cytotoxicity assay, 293 cells were treated with PAGA or PLL in a 96-well plate, followed by 24 h incubation at 37°C. Then the cytotoxicity was measured by MTT assay (17). Briefly, 25 μl of the 5 mg/ml stock solution of MTT were added to each well, and after 2 h of incubation at 37°C, 100 μl of the extraction buffer (20% w/v of SDS in a solution of 50% of DMF, pH 4.7) were added. After an overnight incubation at 37°C, absorbance at 570 nm was measured.

RESULTS

Synthesis of PAGA

For the synthesis of the PLL analogue having an ester backbone, a new monomer, L-oxylysine, was made from L-lysine by converting the α-amino group into a hydroxyl group.

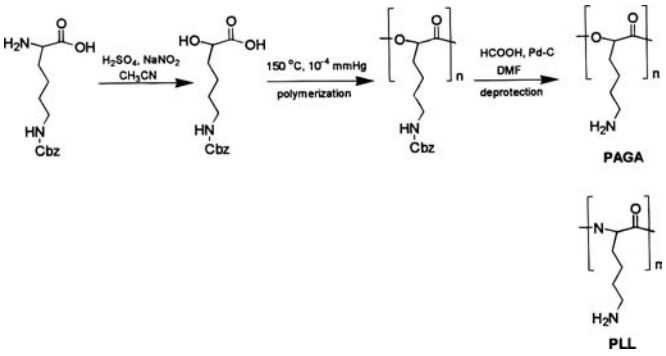

Fig. 1. The synthetic scheme of PAGA and the structure of PLL.

The monomer was polymerized by melting condensation as shown in Fig. 1. Any side products or products from the polymerization should not be toxic to cells. The difference between PAGA and PLL is only in the backbone linkage, an ester bond in PAGA versus an amide bond in PLL.

Degradation of PAGA in an Aqueous Solution

Molecular weight and degradation of PAGA in an aqueous solution were investigated by using MALDI-TOF MS (Fig. 2). It was shown that M_p, the most probable peak molecular weight determined from the highest peak from the MALDI-TOF MS spectrum, could be used to estimate the molecular weight of polymer (18). So we followed M_p change of PAGA from the MALDI-TOF MS spectra for degradation study. The degradation of PAGA was characterized by rapid initial degradation within 100 min and gradual degradation until it is degraded to monomer (L-oxylysine). The fast degradation of PAGA seems to come mainly from self-degradation via hydrolysis of it own ester linkage by the ε-amino groups. PAGA degraded completely to monomer within 6 month at 37°C. The degradation study was also performed on PLL under the same conditions

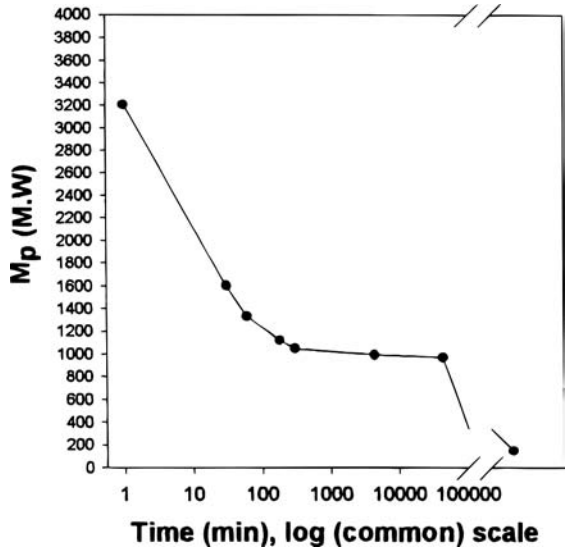

Fig. 2. Degradation study of PAGA in aqueous solution by MALDI-TOF MS. M_p: the most probable peak molecular weight determined from the highest peak. M_p can be interpreted as a molecular weight of PAGA. The spectrum was the sum of 128 laser shots.

or in a pseudo-extracellular milieu. Even after 3 months, PLL hardly degraded (data not shown).

Gel Band Shift Assay

The DNA condensing ability of PAGA was determined by gel band shift assay. The positively charged PAGA makes strong complexes with the negatively charged phosphate backbone of DNA. When the charge ratio (+/−) reaches 1:1, no free DNA is in solution (Fig. 3a). Since the degradation of PAGA is fast, as shown in Fig. 2, it is crucial to verify the stability of the PAGA/DNA complexes over the time needed for transfection. Stability studies were done by measuring dissociated DNA, in the form of a band, from the complexes at pH = 7.3 and 37°C (Fig. 3b–d). We observed slower degradation rate of the complexes than PAGA alone. The complexes dissociated completely within one day, while PLL/DNA complexes were stable for four days (Fig. 3e).

Atomic Force Microscopic Image of PAGA/DNA Complexes During Degradation

All the possible forms of plasmid DNA, super-coiled, nicked circular, and linear plasmid DNA can be seen in the AFM image (Fig. 4a). Mg^{2+} was used to obtain a plasmid DNA image because DNA binding to mica has been shown to be enhanced in the presence of divalent metal counterions (19). But in obtaining PAGA/DNA complexes images, a divalent cation was not needed. Formation of self-assembling complexes between PAGA and DNA can be seen in Fig. 4b at charge ratio (+/−) of 5:1. The shapes of the complexes were largely spherical but rather heterogeneous. The toroidal shape can be seen in Fig. 4b. The sizes of the complexes range from 100 to 400 nm. After incubation of the complexes at 37°C for 4 h, degradation was apparent (Fig. 4c). A protruding DNA strand from the complexes could be seen with the shape of the complexes becoming homogeneous and globular. After 8 h incubation, more of the DNA was released from the complexes (Fig. 4d). Two apparent characteristics of 24 h incubation images were that most of the DNA was released from the complexes and that the density of the complexes was significantly reduced (Fig. 4e). From gel band shift assay, PAGA lost its ability to make the complexes when the complexes were incubated for 24 h at 37°C (Fig. 3).

Size Distribution of the Complexes by Dynamic Light Scattering

Size distribution of PAGA/DNA complexes measured by dynamic light scattering shows that the complexes had average size of 326 nm at a charge ratio of 5:1 (+/−) (Fig. 5). The size distribution was rather heterogeneous as demonstrated by the polydispersity index (0.252). A tendency for the complexes size to increase was found as the charge ratio (+/−) was increased (data not shown).

Transfection and Cytotoxicity

The transfection efficiency of PAGA was investigated and compared with its structural analogue, PLL. The transfection efficiency was measured using colorimetric β-galactosidase enzyme activity assay (ONPG assay). The transfection protocol was optimized for each of the polymers and done in the presence of chloroquine (100 μM). It was found that optimum transfection of PAGA and PLL was obtained at charge ratios (+/−) of 40:1 and 5:1, respectively. The transfection efficiency of PAGA/DNA complexes on 293 cells under optimized condition was nearly twice that of PLL/DNA complexes as shown in Fig. 6.

The toxicity of PAGA on the cells was assayed using a MTT assay (Fig. 7). After incubating 293 cells for 24 h with 100 μg/ml PAGA, the cells showed neither a decrease in population nor a change in morphology indicative of minimal toxicity. In contrast, PLL exhibited much increased toxicity when compared to PAGA (about 25%). The PLL treated cells granulated, and the cell population decreased under the same concentrations as PAGA. At higher concentrations of PAGA, up to 300 μg/ml, there was no significant cytotoxicity observed (data not shown).

DISCUSSION

The present article deals with the use of a novel biodegradable polymer as a gene carrier. The design principle that we used for the synthesis of PAGA was that the polymer should be biodegradable. Currently, most non-viral gene carriers are subdivided into cationic polymers and lipids. Most of them have amide bonds (e.g., PLL) or vinyl bonds (e.g., poly(ethyleneimine)). These bonds are very stable in aqueous solution and there is no direct evidence of their degradation in body fluid. They are known to be cytotoxic (20). Also, most of the lipids used as gene carriers are known to be cytotoxic to some

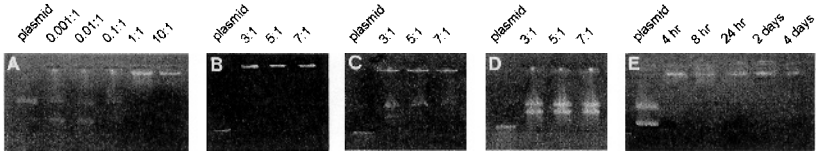

Fig. 3. Formation of PAGA/DNA complexes and the degradation of the complexes. (A) The effect of increasing proportions of PAGA on plasmid DNA (pSV-β-gal) electrophoretic migration; (B) The complexes were incubated at 37°C for 4 h; (C) 8 h; (D) 24 h. The charge ratio between PAGA (+) and DNA (−) is shown in each lane. (E) The degradation study of the PLL/DNA complexes at the same condition as PAGA/DNA complexes. The molecular weight of PLL (about 3500) used in this and the other experiment was similar to the PAGA, and it was determined by MALDI-TOF MS. Samples were electrophoresed through 0.8% agarose gel and stained with ethidium bromide to visualize DNA.

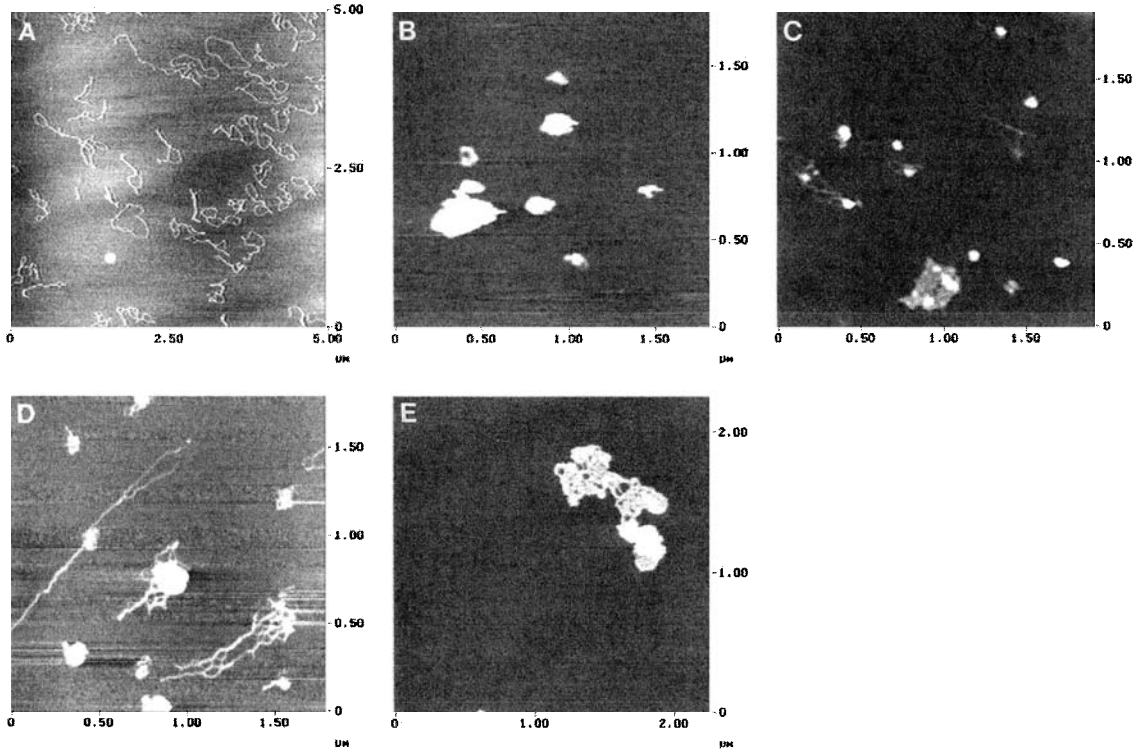

Fig. 4. AFM images. (A) plasmid DNA (pSV-β-gal); (B) PAGA/DNA complexes; (C) after 4 h incubation; (D) after 8 h incubation; (E) after 24 h incubation.

degree. To make non-toxic gene carrier, we designed the polymer with a hydrolytically labile ester bond. Degraded monomer or small oligomers will not elicit an immune response either. The primary design of PAGA was from PLL. The amide backbone linkage of PLL was converted into ester linkage to confer biodegradability upon PAGA.

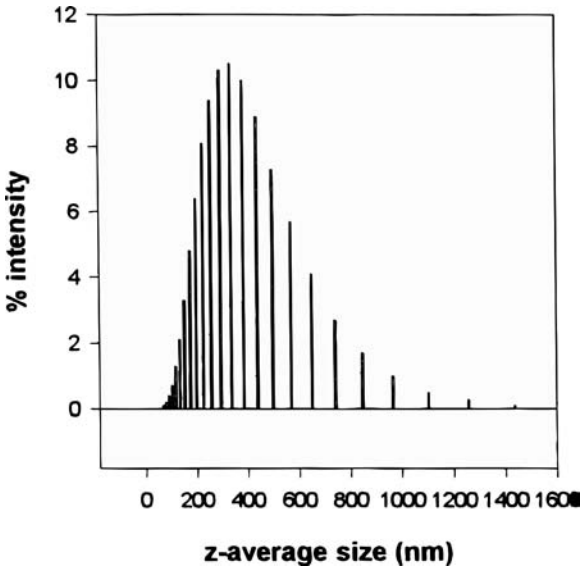

Fig. 5. Dynamic light scattering of PAGA/DNA complexes. Size distribution of PAGA/DNA complexes was measured by DLS. The complexes were formed at 5:1 charge ratio (+/−).

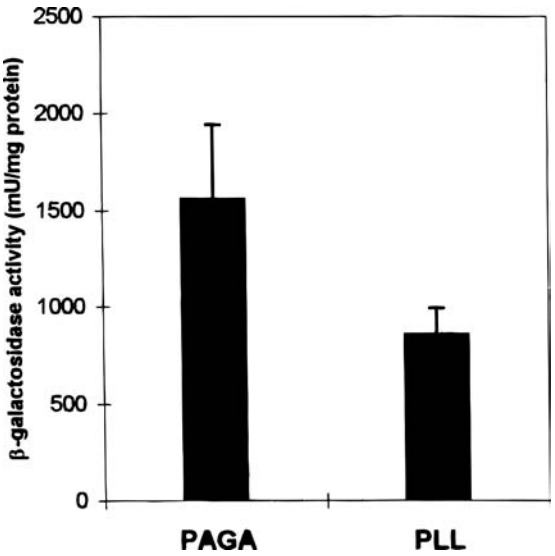

Fig. 6. Transfection of PAGA/pSV-β-gal or PLL/ pSV-β-gal complexes on 293 cells. Transfection efficiency was measured using colorimetric β-galactosidase enzyme activity assay (ONPG assay). Transfection protocol was optimized for each of the polymer and done in the presence of chloroquine (100 μM). The data are expressed as mean values (±SD) of 5 experiments.

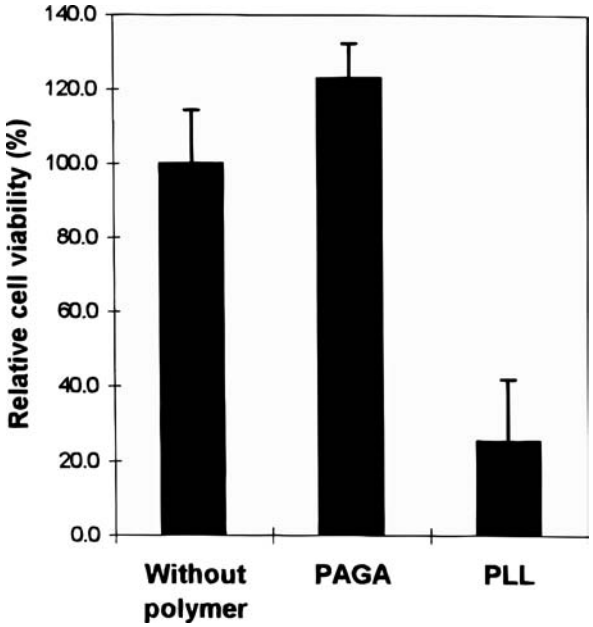

Fig. 7. Cytotoxicity of PAGA and PLL. Cell viability was measured by MTT assay after 24 hr incubation of each polymer (100 μg/ml) with the cells. The viability of cells that was not treated with polymer was taken as 100%. The data are expressed as mean values (±SD) of 5 experiments.

The degradation study we performed in an aqueous solution demonstrates that PAGA is degraded rapidly within 100 min to small oligomers, and then to monomer in a gradual manner. So, we could postulate that degraded PAGA would not have an adverse effect on cell viability and it really didn't (*vide ante*). It seems that degraded fragments and monomer are biocompatible. In addition, the small size of degraded oligomers and monomer will not elicit complement activation that can be a problem when larger ligand-DNA constructs are used (21).

The formation of condensed DNA by PAGA was investigated using several techniques. In gel band shift assay, PAGA was able to retard DNA migration as the charge ratio (+/−) increased. From this result, it was found that formation of the complexes occurred at a near stoichiometric charge ratio (+/−). In the AFM image, the complexes were rather heterogeneous with some large particles around 400 nm. The overall shape of the complexes was microaggregates. Experimental data show certain condensed structures such as toroids and rods can be induced by polyamines in DNA solutions ~1–10 μg/ml (22). We could also see toroidal shaped complex with the DNA concentration we used (5 μg/ml). The average size of the complexes, 326 nm as determined by DLS, is in coherence with that of complexes in the AFM image.

Although PLL/DNA complexes were not decomplexed for several days, PAGA/DNA complexes were gradually decomplexed to release free DNA from the complexes as shown in gel band shift assay. This result indicates that by virtue of degradation, PAGA lose its ability to condense DNA. But almost all the DNA was in a condensed state for over 8 hr. Since only a few hours are required for the maximum transfection of cells (23), PAGA/DNA complexes can be utilized as a stable complex system through cellular uptake. The stability of the complexes investigated by AFM showed protruding DNA strands from the dense core when the complexes were incubated in an aqueous solution. The dense core is likely to be DNA condensed by PAGA. The dense core became more homogeneous than that of intact complexes as the time progressed. This seems to occur because the aggregated complexes were disaggregated as a result of PAGA degradation. Near complete decomplexation and a noticeable decrease in the complexes density after 24 h indicated that almost the entire DNA had been released from the complexes. Mica treated with Mg^{2+} containing buffer or 3-aminopropyltriethoxysilane (APTES) treated mica (AP-mica) (16) is usually used in obtaining AFM images of DNA. Without them, homogeneous spreading of DNA over a mica surface is technically very difficult because DNA gathers together in a very small region. The decrease in complexes density after 24 h seemed to result from the fact that the released DNA gathered together in some very small region making them invisible in the AFM image because the mica used for this experiment was not treated with Mg^{2+} or APTES. From these sets of experiments, we could conclude that PAGA/DNA complexes are stable up to 24 h in an aqueous solution. When we think that transfection mixtures are usually incubated for 4 h with cells for *in vitro* transfection experiment, 24 h stability is sufficient for transfection.

The transfection efficiency of PAGA/DNA complexes on 293 cells under optimized conditions was nearly twice that of PLL/DNA complexes. This result may be explained by the accelerated degradation of PAGA in cytoplasm and nucleus, which enhances the expression of free DNA. Higher polymer/DNA charge ratio for PAGA than PLL required for the optimum transfection could also be explained by the degradation of PAGA, which might demand more PAGA for DNA complexation and negatively charged cell surface binding.

PAGA did not display any detectable cytotoxicity up to high concentrations (300 μg/ml). But PLL had a noticeable cytotoxicity in a concentration of 100 μg/ml. The toxicity of cationic polymers has been a concern in their use as gene carriers. Also, there is a notice of greater toxicity for some of polymers applied in the absence of DNA (24). In this regard, PAGA's lack of toxicity without DNA complexation is a remarkable finding. To our knowledge, there has been no gene carrier that does not display any noticeable cytotoxicity without DNA complexation. The degradable nature of PAGA, with its non-toxic and biocompatible monomer and small oligomers, might be important contributory factor to the non-toxicity of this polymer. The non-toxicity of PAGA will be beneficial for *in vivo* gene therapy.

In conclusion, the newly synthesized PAGA has the following characteristics. First, the polymer has an overall positive charge that forms stable complexes with DNA. Second, the polymer shows accelerated degradation when free and decreased degradation when it forms complexes with DNA. Third, the final degradation product is a degraded monomer, L-oxylysine. Forth, the polymer condenses DNA into a spherical shaped polymer. Fifth, the transfection efficiency of PAGA/DNA complexes is about twice that of PLL/DNA complexes, which possibly arise from the non-toxicity and degradability of the polymer. Sixth, PAGA is non-toxic. We hypothesized the faster degradation of PAGA will increase the release of free DNA to the cytoplasm and /or the nucleus resulting in more efficient expression of the transfected DNA. The degraded PAGA oligomers or monomers will be rapidly removed from cellular compartments followed by metabolism and excretion from the body. PAGA is a promising candidate as a gene carrier

in human gene therapy. Although the transfection efficiency using PAGA carrier may not be higher than that of dendrimers (e.g., starburst PAMAM) or cationic liposomes, PAGA polymer should have advantages due to its high solubility, nontoxic and degradable characteristics when it is used as systemic gene carrier. More efficient Carriers can be designed by specific targeting moieties and/or endosome disruptive peptides to PAGA

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