Development of a Safe Gene Delivery System Using Biodegradable Polymer, Poly[α-(4-aminobutyl)-L-glycolic acid]

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Received March 24, 2000

In recent years, there has been considerable interest in biodegradable polymers that can be used as biomedical materials.[1,2] Much progress has been made in the field of drug delivery systems in which many polymeric materials such as poly(lactide-*co*-glycolide) showed promising properties as a controlled drug reservoir.[3] But the utilization of biodegradable cationic polymers as nonviral gene carriers has been limited. Backbone linkages of most current gene carriers are composed of amide or vinyl bonds, which hardly degrade spontaneously in an aqueous solution. For this reason, the potential for current nonbiodegradable nonviral gene carriers to accumulate in an endosomal compartment or cell nucleus and adversely interact with the host gene exists.[4,5] These issues present a problem with regard to using them to treat human disease with gene therapy. Our ongoing research is focused on the development of biodegradable and nontoxic cationic polymers, the first of which was achieved by synthesizing poly(4-hydroxy-L-proline ester).[6] The polymer showed relatively weak binding ability to DNA, but cationic polymers binding strongly to anionic DNA are required to condense DNA into a geometrically compact shape. Here we report the synthesis of a novel biodegradable cationic polymer, poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), which forms DNA complexes strongly below a charge ratio of 2(±).

First, monomer **1** (N_ϵ-cbz-L-oxylysine) was prepared by converting an α-amino group of N_ϵ-cbz-L-lysine into a hydroxyl group (Scheme 1). Polymerization of the monomer was conducted in the melt to make polymer **2**. The number- and weight-average degrees of polymerization determined by gel permeation chromatography were 14 and 32, respectively. The polydispersity index (2.3) obtained was rather broad, but reasonable as the polymer was synthesized in the melt. Cbz groups protecting ϵ-amines were removed and the resulting primary amine groups of PAGA were obtained as a hydrochloride salt. The molecular weight distribution (MWD) of PAGA was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as the MWD of charged, water soluble, and fast degrading polymer is very difficult to measure by other methods.[7] The molecular weight of PAGA represented as an M_p (the most probable peak molecular weight determined from the highest peak intensity in the MALDI spectrum)[6] was 3300 (Figure 1), which is an appropriate size for DNA condensation.

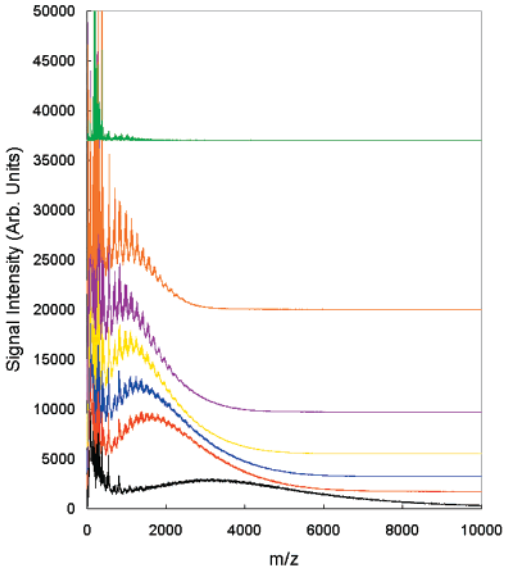
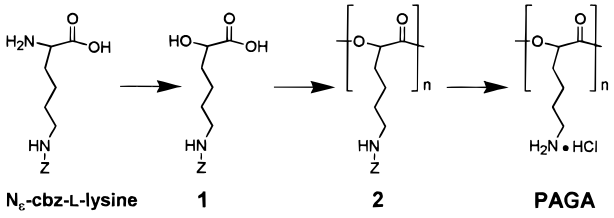


Figure 1. Degradation profiles of PAGA incubated at 37 °C, pH 7.3. Aliquots were taken at appropriate time intervals and MALDI spectra were measured. M_p at 0 min (black line), 3300; 30 min (red line), 1600; 1 h (blue line), 1330; 3 h (yellow line), 1100; 5 h (violet line), 1050; 3 days (orange line), 990; 6 months (green line), NA. (NA = not applicable).

Scheme 1. Synthetic Scheme of PAGA



The structure of PAGA is similar to one of the polyamino acids, poly-L-lysine (PLL), except for the backbone linkages. The backbone linkages of PAGA and PLL are ester and peptide bonds, respectively. PLL has been one of the most intensively studied gene delivery carriers.[8] Although peptide linkage is a natural bond found in proteins and L-lysine, a monomeric unit of PLL, is an amino acid, a homopolymer of L-lysine is not found in nature. As a result, PLL is known to have cytotoxicity[9] that possibly arises from its slow degradation in vivo. We thought that the ester backbone linkages in PAGA would make this polymer spontaneously degradable in an aqueous solution.

As a model of biodegradation, we incubated PAGA at 37 °C, pH 7.3, and aliquots taken at appropriate time intervals were subjected to MALDI-TOF MS. From the MALDI spectra shown in Figure 1, it is evident that the degradation of PAGA occurred very quickly in an aqueous solution. It needs only 30 min for M_p for the intact polymer (0 min) to halve. When the time reached 5 h, M_p had reached 1050, which is a third of the polymer intact. Fast degradation of PAGA implies that the polymer is probably another example of "a self-destroying polymer" in which the main chain cleavages occur by the nucleophilic attack of amine groups of the polymer itself or of the nearby polymer molecules.[6,10] The degradation slowed after 1 day passed. Taking into account the

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10.1021/ja001033h CCC: $19.00
Published on Web 06/23/2000

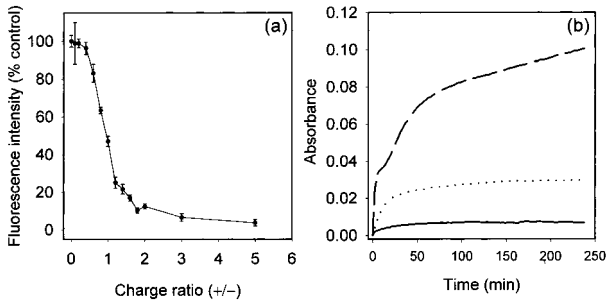

Figure 2. (a) Fluorescence intensity change of DNA intercalated with ethidium bromide. Mean ± standard deviation ($n = 3$). (b) Absorbance change profiles of DNA against nuclease (DNase I) attack. DNA alone (dashed line), PAGA/DNA complexes at a charge ratio (±) of 3 (dotted line) and 5 (solid line). Absorbance was measured at 260 nm.

formation of amide bonds after the nucleophilic attack of ϵ-amines on backbone ester bonds, a slower degradation after 1 day can be rationalized. The final degradation product of the polymer was a degraded monomer (L-oxylysine) and required approximately 6 months.

Intercalated ethidium bromide between the base pairs of DNA is excluded when the interpolyelectrolyte complexes of polymer and DNA formed.[11] The fluorescence quenching which occurred for a given concentration of DNA segments exposed to increasing concentrations of PAGA is shown in Figure 2a as a function of the positive to negative charge ratio. A sharp decrease in fluorescence intensity occurred when the charge ratio (±) reached 0.4, and it leveled off at a charge ratio of about 2. This result is an indication of the strong binding of PAGA to DNA molecules and the formation of PAGA/DNA complexes below a charge ratio of 2.

For the DNA complexes of polymer to express the encoded gene in the nucleus of cells, the polymer should protect DNA for a few hours from nuclease attack in the cellular environment.[12] Fragmentation of DNA was found to be fast as manifested by a sudden increase in absorbance at 260 nm, occurring within 1 min after addition of nuclease into DNA solution (Figure 2b). But the DNA was partially protected from nuclease attack at a charge ratio of 3 and complete protection was achieved at a charge ratio of 5. The charge ratio (5, ±) required to protect DNA completely is higher than is needed to form complexes and can be rationalized by the degradation of PAGA. The protection continued throughout an incubation of 4 h with nuclease, implying that the degradation of DNA-complexed PAGA is not so fast compared to free PAGA. We think that this is a result of the masking of the nucleophilic primary amine groups of PAGA by DNA phosphates.

To verify that PAGA is not cytotoxic, the influence of PAGA on cell viability was tested using a tetrazolium-based MTT colorimetric assay (Figure 3a). As we expected, PAGA did not display any detectable cytotoxicity even at high concentrations (300 μg/mL). The cytotoxicity of cationic polymer/DNA complexes is generally lower than that of the polymer alone.[13] In accordance with this, PAGA/DNA complexes did not show cytotoxicity up to high charge ratios (60, ±). Interestingly, about

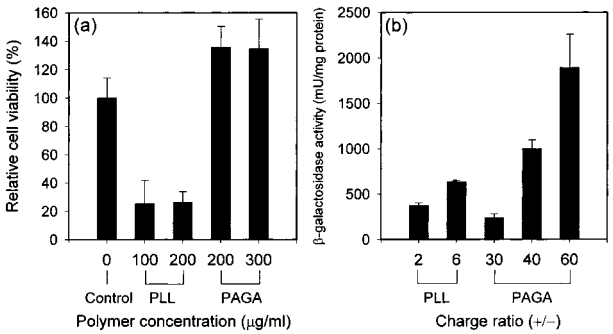

Figure 3. (a) Cytotoxicity assay of PAGA and PLL. (b) Transfection efficiency in 293 cells. PLL with molecular weight of 4000 was used for the experiments. Mean ± standard deviation ($n = 5$).

a 35% increase in cell viability was observed after PAGA treatment. The reason is unclear at this time, but it is certain that PAGA is nontoxic to cells primarily due to its rapid biodegradation. On the other hand, PLL showed significant cytotoxicity in that only 25% of the cells remain viable relative to nontreated cells.

The transfection efficiency of PAGA was compared with that of PLL. Chloroquine, known to disrupt the membrane of the endocytic vesicle, was used in this experiment. Optimal charge ratios showing the highest transfection efficiency were different between the two polymers, 6 and 60 for PLL and PAGA, respectively. Charge ratios for complex formation were similar between PLL and PAGA, between 1 and 2. But the 10-fold charge ratio required for PAGA relative to that of PLL for the highest transfection seems to result from the fact that degradation of PAGA requires more of the polymer for PAGA/DNA complexes to resist the harsh cellular environment. The transfection efficiency of PAGA was about 3-fold higher than that of PLL under optimized transfection conditions. Although small, we ascribe this enhanced transfection efficiency of PAGA/DNA complexes to the nontoxicity of PAGA and faster release of DNA from the complexes due to degradation of PAGA after internalization within cells or nuclei.

In conclusion, we have successfully developed a nontoxic polymeric gene delivery system by using a biodegradable cationic polymer, PAGA. The results indicate that the polymer degraded quickly and completely in an aqueous medium as its backbone linkages are composed of ester bonds. The formation of strong interpolyelectrolyte complexes between the polymer and DNA was observed and the polymer serves to protect DNA from nuclease attack, an example of a harsh cellular environment. Although the instability in aqueous conditions is a weakness of PAGA in the present state, the finding that a biodegradable polymeric gene carrier is nontoxic and shows enhanced transfection is an important step toward making a safe gene carrier.

Acknowledgment. We acknowledge the support of this work from the Brain Korea 21 program, Korea Research Foundation, and Korea Science and Engineering Foundation.

Supporting Information Available: Synthetic details, experimental protocols, and MALDI spectrum (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA001033H

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