Synthesis of Biodegradable Cross-Linked Poly(*β*-amino ester) for Gene Delivery and Its Modification, Inducing Enhanced Transfection Efficiency and Stepwise Degradation

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Biodegradable cross-linked poly(*β*-amino ester) (CLPAE) was synthesized by Michael addition of pentaerythritol triacrylate and *N*,*N*-dimethylethylenediamine and modified with aminohexanoic acid and lysine to CLPAE-Ahx and CLPAE-Lys, respectively, for a gene delivery system. They could self-assemble with plasmid DNA, forming nanosized polyplexes, and CLPAE-Ahx polyplex released plasmid DNA slowly during a week through stepwise degradation. The polymers showed minimal cytotoxicity on 293 cells due to their biodegradability and biocompatibility. Transfection efficiencies of CLPAE-Ahx and CLPAE-Lys were comparable to that of PEI in 293 cells and C2C12 cells. Additionally, high transfection of CLPAE-Ahx on primary rat aorta vascular smooth muscle cells (SMC) and primary mouse embryonic fibroblast cells (MEF) shows a potential for a gene delivery system on primary cells, restenosis treatment of human SMC, and MEF cell function research. In conclusion, CLPAE-Ahx could be used as a nontoxic and highly efficient gene delivery system.

INTRODUCTION

During the past decade, intensive research and development in multidisciplinary fields have been directed toward developing safer and more efficient methods for transferring therapeutic genes into cells and for eventual use in human gene therapy (*1*).

Several successful clinical trials reported so far involved viral vector systems (retroviruses, adenoviruses) that provide efficient transduction and high levels of gene expression. However, their severe immunogenicity and toxicity are still major obstacles for viral vector-mediated gene therapy (*2*). For these reasons, as alternative tools to such risky viral vectors, nonviral vectors have been introduced and studied for their potential to provide safer, more desirable methods for gene delivery and clinical gene therapy (*3*−*6*).

Studies have been performed to improve gene delivery efficiency of polymeric gene carriers and to reduce their cytotoxicity in many labs. Among them, the introduction of biodegradability such as ester, phosphoester, or disulfide bonds to the polymeric carriers was proved to be very successful for lowering cytotoxicity (*7*−*10*). However, the results from these approaches were dependent on specific conditions, so structure−activity relationships could not be obtained easily. Recently, Langer et al. constructed structurally manifold polymer libraries by using combinatorial reactions of amine derivatives and diacrylate monomers and screened them by high-throughput cell-based screening assay (*11*−*13*). Through this strategy, they discovered a few candidates showing very high gene delivery efficiency.

In general, linear cationic polyester polymers are reported to be degraded very fast in aqueous solution and to release DNA from polyplexes within a few hours (*14*). This means that the application of linear polyester polymers could be limited in the long term because of its fast degradation rate. So, we designed a novel cross-linked poly(*β*-amino ester) polymer (CLPAE) to control its degradation rate and deliver DNA for a longer period.

In this report, we present the synthesis, characterization of CLPAE, and further modification of the polymer for application to gene delivery. Also, self-assembly of polymers with plasmid DNA, in vitro DNA release, and cell-based experiments investigating cytotoxicity and transfection efficiency are performed and presented.

EXPERIMENTAL PROCEDURES

Materials. Poly(ethylenimine) (25 kDa), pentaerythritol triacrylate, *N*,*N*-dimethylethylenediamine (95%), *N*,*N*-diisopropylethylamine (DIPEA), piperidine, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Anaspec, Inc. (San Jose, CA). Fmoc-6-aminohexanoic acid (Fmoc-*ϵ*-Ahx-OH) and Fmoc-Lys(Fmoc)-OH were purchased from Novabiochem (Laufelfingen, Switzerland). pGL3-control vector, Luciferase 1000 Assay System, and Reporter Lysis Buffer were purchased from Promega (Madison, WI). The luciferase expression plasmid (pCN-Luci) was a kind gift from Hyung-suk Jang in Samsung Medical Center (Seoul, Korea). pCN-Luci was constructed by subcloning cDNA of *Photinus pyralis* luciferase with a 21-amino acid nuclear localization signal from SV40 large T antigen to pCN (*15*). PicoGreen was purchased from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS), Minimal Essential Medium (MEM), and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD). Micro BCA protein assay kit was purchased from Pierce (Rockford, IL). All other chemicals were purchased and used without any further purification.

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Polymer Synthesis. *N*,*N*-Dimethylethylenediamine (0.481 mL, 4.16 mmol) and pentaerythritol triacrylate (0.526 mL, 2.08 mmol) were dissolved in methylene chloride (3 mL), respectively. Then, diamine solution was added to triacrylate solution dropwise, keeping it at 50 °C. After 2 days when a yellow precipitate was found in the reaction mixture, it was cooled to room temperature and evaporated under vacuum. The remnant was precipitated into diethyl ether two times to remove residual amine, leaving a light yellow solid, CLPAE.

Also, CLPAE was further modified to acquire the enhanced properties of gene delivery by the introduction of aminohexanoic acid (Ahx) or lysine (Lys) residues. These conjugation reactions were conducted by coupling the carboxylic group of the acid to the hydroxyl group of the triacrylate with HOBt/HBTU. Fmoc-protected Ahx and Lys were used to prevent side reactions.

(1) Aminohexanoic Acid-Conjugated CLPAE (CLPAE-Ahx). First, 100 mg of CLPAE was mixed in DMF with a large excess of Fmoc-ϵ-Ahx-OH (248 mg, 0.702 mmol), HOBt (94.8 mg, 0.702 mmol), and HBTU (266 mg, 0.702 mmol). At this time, a large excess of coupling reagents was used to complete the conjugation because we did not know the exact amount of hydroxyl groups in CLPAE. Then, DIPEA (245 μL, 1.40 mmol) was added to the reaction mixture. The reaction was allowed to proceed at room temperature for 2 days to complete the reaction. After the coupling reaction was completed, the product was precipitated with an excess of diethyl ether three times to remove the excess of coupling reagents and DMF. Dried under a vacuum, the product was dissolved in DMF and mixed with an equal volume of piperidine (30% in DMF) at room temperature for 20 min to remove the Fmoc group of aminohexanoic acid. The reaction mixture was precipitated again with diethyl ether to remove the piperidine and reaction remnants and dried under vacuum, leaving a sticky yellow solid, CLPAE-Ahx. Each coupling reaction and Fmoc-deprotection reaction was confirmed by ^{1}H NMR.

(2) Lysine-Conjugated CLPAE (CLPAE-Lys). CLPAE (100 mg) was mixed in DMF. Then an equimolar amount of Fmoc-Lys-(Fmoc)-OH (415 mg, 0.702 mmol), HOBt, HBTU, and DIPEA in accordance with the above procedure was dissolved in DMF and added to the CLPAE solution. Afterward, following the coupling reaction, the Fmoc-deprotection and diethyl ether precipitation steps were carried out in the same way as in the above-mentioned CLPAE-Ahx synthesis.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra of the polymers were obtained using a Bruker DPX-300 NMR spectrometer (300 MHz). For analysis, the polymer samples were dissolved in D_2O or MeOD containing 0.05% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt as an internal reference (0 ppm). A known molar quantitiy of benzoic acid was added to sample solutions as a NMR standard to quantify the degree of modification with Ahx and Lys for further experiments.

Molecular Weight Analysis. The average molecular weight of polymers was determined by multiangle laser light scattering (MALLS) in combination with size exclusion chromatography (SEC). The SEC system included a P680 HPLC pump from Dionex Corp. (Sunnyvale, CA). Polymer samples were detected by a three-angle laser light scattering detector (miniDAWN Tristar, 30 mW GaAs laser, 690 nm, K5 cell) and an interferometric refractometer (Optilab DSP, P10 cell) from Wyatt Technologies (Santa Barbara, CA). The MALLS system was operated by Astra software version 4.90. DMF (HPLC grade, Mallinckrodt) was used as an eluent after being filtered through a 0.22 μm filter and degassed before use. A Styragel HR 3 column from Waters (Milford, MA) was used. All sample solutions were filtered through a 0.45 μm filter again. The solution was delivered to a flow cell inlet at a constant flow rate of 0.5 mL/min.

Quantitative Analysis of Primary Amine Numbers of CLPAE-Ahx and CLPAE-Lys. A series of ethanol amine solutions of various concentrations was mixed with 50 mM borate buffer to 800 μL of the total volume to construct the standard calibration curve. Fluorescamine solution (1 mg/mL in DMF) (200 μL) was mixed with the amine solution. After 12 min incubation, 1.5 mL of stop solution (DMF/water, 50% v/v) was added to quench the reaction. Fluorescence measurements were carried out with a FP-750 spectrofluorometer (Jasco) at room temperature. Excitation and emission wavelengths were 360 and 465 nm. In the case of polymer samples, fluorescence was measured in the same way. A standard curve prepared in this way was used to analyze the amount of primary amines/μg polymer quantitatively.

Gel Retardation Assays. Polymer/plasmid complexes (polyplexes) at various weight ratios ranging from 0.5 to 7 were prepared in HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4). After 30 min incubation at room temperature for complex formation, the samples were electrophoresed on a 0.7% (w/v) agarose gel and then stained in a 1% (w/v) ethidium bromide solution. After 40 min staining, the gel was analyzed on a UV illuminator to show the location of the DNA.

Polymer/DNA Self-Assembly Analysis by PicoGreen Assay. PicoGreen assay was carried out to analyze the self-assembly of polymer/plasmid DNA quantitatively as reported recently (*16*). Briefly, PicoGreen reagent (200X) was diluted to 200-fold in TE buffer before the experiment. The diluted PicoGreen stock solution (200 μL) was mixed with the same volume of blank solution or polyplex solution (1 μg DNA, 1X HBS) prepared at various weight ratios ranging from 0 to 10. After 2 min incubation, each solution was added to 1.6 mL of TE buffer in a test tube. Fluorescence was measured with a FP-750 spectrofluorometer (Jasco) at room temperature. Excitation and emission wavelength were set at 480 and 520 nm, respectively.

Polyplex Size Measurements. The hydrodynamic diameters of the polymer/plasmid DNA complexes were determined by light scattering. Polyplex solutions (500 μL) containing 10 μg of DNA were prepared at various weight ratios ranging from 0.5 to 40. After 30 min incubation, polyplex solutions were diluted to a final volume of 2 mL prior to measurements. Polyplex sizes were measured using a Zetasizer 3000HS (Malvern Instruments, UK). The laser used is a nominal 5 mW HeNe laser having a 633 nm wavelength. Scattered light was detected at a 90° angle. The refractive index (1.33) and the viscosity (0.89) of ultrapure water were used at 25 °C for measurements. Zetasizer 3000 (Advanced) Size mode v1.61 software was used for data acquisition. Data analysis was performed in automatic mode. Measured sizes were presented as the average values of 10 runs.

Atomic Force Microscopy (AFM). The particle shapes and sizes of the polymer/plasmid DNA complexes were analyzed using atomic force microscopy (Nanoscope IIIa system, Digital Instruments, Inc., Santa Barbara. CA). The samples were prepared by mixing 0.1 μg of plasmid DNA with aqueous polymer solution at various weight ratios to obtain a final DNA concentration of 10 ng/μL. After 30 min incubation, 1 μL aliquots of the complex solutions were placed on freshly cleaved mica surface and allowed to stick for 1−2 min. Excess solution was removed by careful absorption onto filter paper, and the mica surface was further dried at room temperature for 24 h. The image mode was set to tapping mode, and the scanning speed was 1−5 Hz.

Zeta-Potential Measurements. One hundred microliter quantities of polyplex solutions containing 2 μg of DNA were prepared at various weight ratios ranging from 0.5 to 10. After 30 min incubation, 25 μL quantities of polyplex solutions were diluted to 10 mL of the final volume prior to measurements. Zeta-potential measurements were carried out using a Zetasizer 3000HS (Malvern Instruments, UK) at 25 °C. Zetasizer 3000 (Advanced) Zeta mode v1.61 software was used for data acquisition. The sampling time was set to automatic. Potential values were presented as the average value of 5 runs.

In Vitro DNA Release Measurements. In vitro DNA release from polyplex according to the degradation of polymer in aqueous solution was analyzed by PicoGreen assay. Each procedure was identical to that in the experiments described above except that the weight ratio of polyplex used was 20 where polymers formed stable complexes with plasmid DNA. The polyplexes formed in HBS (pH 7.4) were incubated at 37 °C and collected for assay at various times ranging from the first to the 10th day. Results were expressed in % fluorescence relative to control plasmid DNA.

Cell Lines. HepG2 human hepatocellular carcinoma cells were maintained in MEM, and 293 human kidney transformed

cells and C2C12 mouse myoblast cells were maintained in DMEM medium with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. The primary rat aorta vascular smooth muscle cells (SMC) were a kind gift from Hyung-suk Jang in Samsung Medical Center (Seoul, Korea) and maintained in DMEM/F12 medium with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. The primary mouse embryonic fibroblast cells (MEF) were a kind gift from Prof. Han-Woong Lee in Sungkyunkwan University, School of Medicine. MEFs were prepared from 13.5 day postcoitus mouse (FVB/NJ strain) embryos and maintained in DMEM medium with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. All media contained 1% antibiotic agent.

Cytotoxicity Assay. The cytotoxicity of the polymers was measured by MTT assay. 293 cells were seeded in a 96-well tissue culture plate at 10^4 cells per well in 90 μL of DMEM medium containing 10% FBS. Cells achieving 70−80% confluence after 24 h were exposed to 10 μL of the polymer solutions having various concentrations for 24 h. Then, 25 μL of stock solution of MTT (2 mg/mL in DPBS) were added to each well. After 4 h of incubation at 37 °C, each medium was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystal formed by proliferating cells. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA) and recorded as a percentage relative to untreated control cells.

In Vitro Transfection. HepG2 and 293 cells were seeded at a density of 1.5×10^5 cells/well in a 24-well plate in medium containing 10% FBS and grown to reach 70−80% confluence prior to transfection. C2C12 (7×10^4 cells/well) cells, SMC (4×10^4 cells/well) cells, and MEF (6×10^4 cells/well) cells were seeded in the same way. Before transfection, the cells were rinsed with DPBS, and serum-free or serum-containing medium was added to each well. The cells were treated with polyplex solutions containing 2 μg of plasmid DNA at different weight ratios for 4 h at 37 °C. All the cells were tested with the control pGL3 plasmid except MEF cells for which pCN-Luci plasmid was used. In the presence of serum, the cells were incubated for 24 h after transfection. After exchange of medium, cells were further incubated for 2 days after transfection. Then the growth medium was removed, and the cells were rinsed with DPBS and shaken for 30 min at room temperature in 120 μL of Reporter Lysis Buffer. Luciferase activity was measured by a luminescence assay, and a protein assay was performed using a Micro BCA Protein Assay Reagent Kit. Ten microliters of the lysate was dispensed into a luminometer tube, and luciferase activity was integrated over 10 s with a 2 s measurement delay in a Lumat LB 9507 luminometer (Berthold, Germany) with an automatic injection of 50 μL of Luciferase Assay Reagent. The final results were reported in terms of RLU/mg cellular protein.

RESULTS AND DISCUSSION

Synthesis of CLPAE, CLPAE-Ahx, and CLPAE-Lys. It was reported by Ferruti et al. in 1970 (*17*) that linear poly(amido amine) was synthesized by simple Michael addition of bifunctional amines and bisacrylamides. Poly(amido amine)s of both linear and dendritic structure have been investigated and applied in various fields including gene delivery (*18*−*20*).

Recently, Langer et al. reported a series of achievements in relation to synthesis of poly(amino ester) polymers, their identification, and application to gene transfer by a high-throughput cell-based screening assay (*11*−*13*). They possessed several advantages for gene transfer such as a simple and inexpensive polymerization step and low cytotoxicity due to the biodegradability of the polymer. However, considerable screening is required to find nontoxic efficient gene delivery carriers among the library of randomly synthesized candidate polymers. Moreover, it is well-known that linear polyester polymers tend to be degraded within several hours in aqueous solution (*7*, *14*). The instability and fast degradation of polyplexes formed from these linear polymers limit their potential for long-term application including in vivo gene delivery.

So, we designed cross-linked poly(amino ester) polymers to improve stability and to reduce the fast degradation rate of the polymer. It was reported that cross-linked ester polymer was degraded more slowly under physiological conditions than linear polymer and possessed the endosome buffering effect due to its three-dimensional structure and internal tertiary amines, yielding high transfection efficiency (TE) in mammalian cells (*21*). To synthesize cross-linked polymers, triacrylate was used as a trivalent linker. Pentaerythritol triacrylate was chosen because it has the shortest carbon backbone length among analogous acrylate monomers for high charge density, which is known to generally lead to high TE in cells. Moreover, it has a hydroxyl group, which would enhance water solubility of the polymer and enable further modification of the polymer with other functional groups after polymerization. In the case of the amine monomer, we used *N*,*N*-dimethylethylenediamine that has only one primary amine which could react with acrylates, thus avoiding the formation of excessively complex polymer networks insoluble in aqueous solution.

Scheme 1 shows the synthesis of CLPAE and its modification procedures to prepare CLPAE-Ahx and CLPAE-Lys. CLPAE was synthesized from the nucleophilic Michael addition of *N*,*N*-dimethylethylenediamine with pentaerythritol triacrylate in MC at 50 °C for 2 days. Attempts to synthesize CLPAE under other solvent conditions (DMF, THF etc.) resulted in polymers that could not condense DNA efficiently and showed a very low TE in 293 cells through preliminary experiments (data not shown) So, MC was selected as the optimal solvent for the preparation of CLPAE.

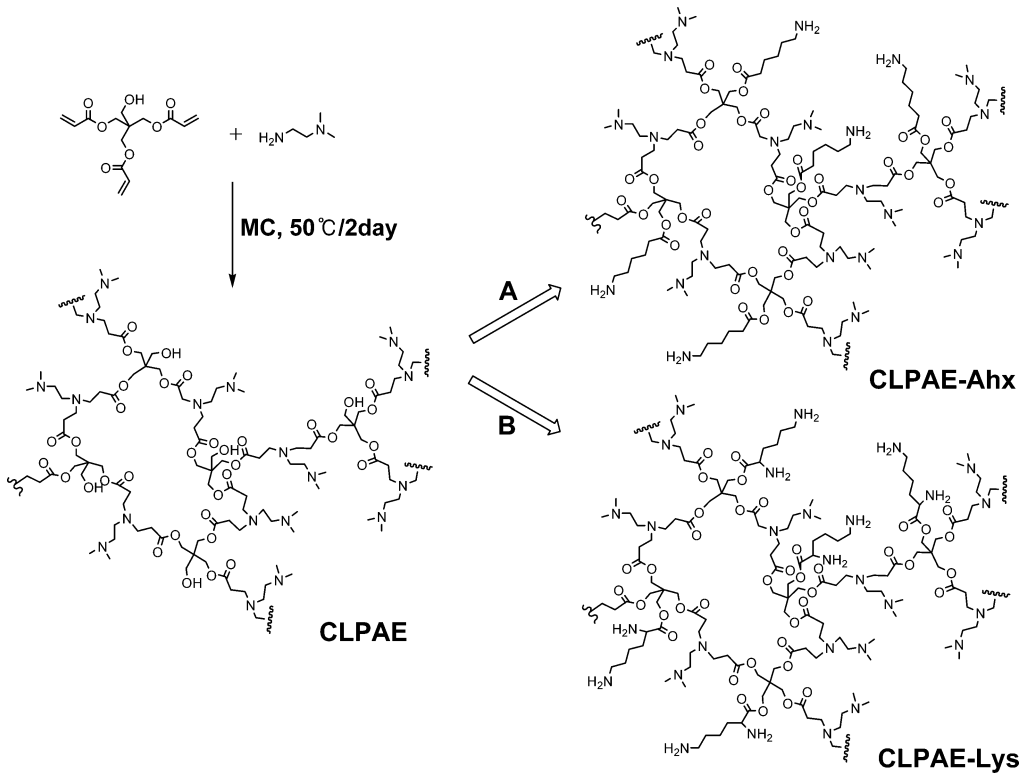
In the case of linear poly(amino ester) polymers, the molar reaction ratio between amine and diacrylate must be equal for a complete stoichiometric reaction yielding the highest molecular weight of the polymer. However, for cross-linked polymers, investigation of the optimal mole ratio was required because triacrylate has three functional groups and diamine has two. CLPAE was synthesized at 1, 2, and 3 mole ratios (amine/acrylate). To determine the optimal ratio, they were analyzed by ^{1}H NMR. From the ^{1}H NMR spectra, we found that CLPAE showed residual unreacted acrylate groups at a mole ratio of 1, and the peaks of acrylate groups had vanished completely at a mole ratio of 2 (data not shown). Therefore, the reaction mole ratio was set to 2 throughout the experiments.

After 2 days of polymerization, a weak yellow precipitate was observed in the reaction mixture. The product was precipitated in an excess volume of diethyl ether two times, leaving a yellow solid. CLPAE was soluble in water and methanol but not in DMF.

Then, CLPAE was further modified by conjugating with aminohexanoic acid (Ahx) and lysine (Lys). Ahx and Lys residues were introduced in order to enhance the DNA-condensing ability of the polymers. The conjugation reaction was performed by coupling the hydroxyl groups of the triacrylate backbone in CLPAE with carboxylic acid using HOBt and HBTU as coupling agents. Because it was complicated work to quantify the amount of hydroxyl groups in CLPAE, we used excessive molar quantities of Fmoc-protected acids and coupling agents for complete conjugation. Also, the reaction time was prolonged to 2 days for the same reason.

After 2 days of coupling in DMF at room temperature, DMF-insoluble CLPAE was dissolved in the reaction mixture in DMF, suggesting that Fmoc-Ahx or Fmoc-Lys was coupled to CLPAE, increasing its solubility in DMF. The reaction solution was evaporated and then precipi-

Scheme 1. The Synthetic Scheme of CLPAE, CLPAE-Ahx, and CLPAE-Lys



tated in diethyl ether three times to remove the excess of residual coupling agents and starting materials (Ahx or Lys). Then, Fmoc groups of aminohexanoic acid or lysine were removed by 15% piperidine solution at room temperature for 20 min to expose primary amines.

Characterization of CLPAE, CLPAE-Ahx, and CLPAE-Lys. The molecular weights (MWs) of the polymers were determined by multiangle laser light scattering (MALLS) combined with size exclusion chromatography. This method is known to be able to determine the absolute MWs of polymers without the need for reference standard polymers (*22*). Molecular weights were calculated by Astra software version 4.90. The eluent solvent was DMF.

The molecular weights of the polymers were as follows. CLPAE-Ahx: $M_n = 2.54 \times 10^4$ g/mol, $M_w = 3.40 \times 10^4$ g/mol (PDI = 1.34), dn/dc = 0.0498; CLPAE-Lys: $M_n = 2.18 \times 10^4$ g/mol, $M_w = 3.28 \times 10^4$ g/mol (PDI = 1.50), dn/dc = 0.0548. The MW of CLPAE could not be obtained by MALLS because it was not soluble in DMF. The increased MW of CLPAE-Ahx is thought to be due to the number of the coupled Ahx being greater than for Lys, a view that corresponds with the following ^{1}H NMR spectra and primary amine quantification results.

We obtained ^{1}H NMR spectra of CLPAE-Ahx and CLPAE-Lys to examine the average degree of modification of aminohexanoic acid or lysine, and the molecular weight of only CLPAE based on the NMR results. A known molar quantity of benzoic acid was added as an internal standard because its proton peaks do not overlap with those of CLPAE and it does not react with CLPAE. We could obtain the number of conjugated aminohexanoic acid or lysine molecules per polymer by calculating the ratio between the number of moles of benzoic acid protons and the NMR peak intensities.

Table 1 shows the average degree of Ahx or Lys modification and the molecular weights of the polymers. We found that an average of 77 molecules of Ahx were conjugated to CLPAE-Ahx and 66 molecules of Lys to CLPAE-Lys. The molecular weight of only CLPAE can be inversely calculated because the value after subtraction of the total MW of conjugated Ahx or Lys from the MW of modified CLPAEs equals the MW of unmodified CLPAE. The molecular weight of only CLPAE was determined to be 2.52×10^4 g/mol and 2.43×10^4 g/mol based on calculations from CLPAE-Ahx and CLPAE-Lys, respectively. The lower degree of modification of Lys compared to Ahx is thought to be due to steric hindrance of di-Fmoc-lysine and this result agrees well with primary amine quantification assay as follows.

Table 1. Molecular Weights of the Polymers and the Degree of Modification with Ahx or Lys[a]

	M_n from MALLS (g/mol)	M_w from MALLS (g/mol)	degree of modification from NMR	M_w of CLPAE from calculation (g/mol)
CLPAE-Ahx	2.54×10^4	3.40×10^4	77	2.52×10^4
CLPAE-Lys	2.18×10^4	3.28×10^4	66	2.43×10^4

[a] Molecular weights of CLPAE-Ahx and CLPAE-Lys were obtained from MALLS.

Quantification of primary amine amount by fluorescamine assay showed that CLPAE-Ahx had 2.0 nmol primary amines/μg of polymer and CLPAE-Lys had 1.5 nmol/μg. Only CLPAE showed a negligible value, confirming that CLPAE itself had no effect on amine quantification.

Formation of Polymer/DNA Polyplexes. The self-assemblies of each polymer and plasmid DNA were observed by using agarose gel electrophoresis and PicoGreen assay, respectively. Figure 1 shows the gel retardation results of each polymer with plasmid DNA. CLPAE was found not to retard plasmid DNA until the weight ratio reached 3. In contrast to CLPAE, the polyplexes of CLPAE-Ahx and CLPAE-Lys with DNA showed complete retardation even at a weight ratio of 1.

These results were compared with PicoGreen exclusion

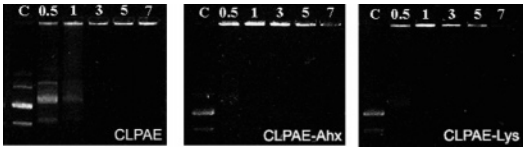


Figure 1. Gel retardation assay. C: DNA only. Numbers represent the weight ratio of the polyplexes.

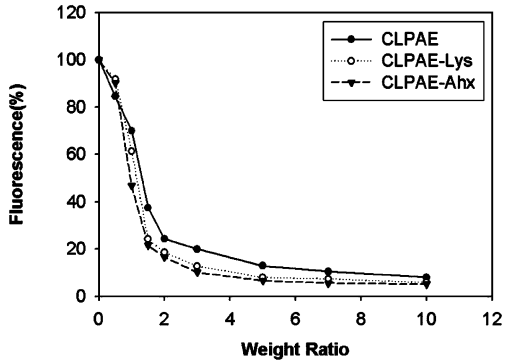


Figure 2. DNA complexation assay using PicoGreen reagent. Excitation and emission wavelength were set at 480 and 520 nm, respectively.

assay results (Figure 2). PicoGreen reagent is a DNA intercalating dye like ethidium bromide (*23*). So, the fluorescence of PicoGreen reagent is inhibited when the DNA is condensed into particles by self-assembly with cationic polymers. CLPAE/DNA polyplex showed an 85% relative fluorescence at a weight ratio of 0.5, which decreased sharply to 8% at a weight ratio of 10. CLPAE-Ahx and CLPAE-Lys were observed to show a greater DNA condensing ability than CLPAE. CLPAE-Ahx polyplex showed a 90% fluorescence value at a weight ratio of 0.5 and this decreased abruptly to 5% at a weight ratio of 10. Although the fluorescence of CLPAE-Lys polyplex was slightly higher than that of CLPAE-Ahx for all the weight ratios examined, the profile was similar to that of CLPAE-Ahx polyplex. These results correspond with gel retardation results and mean that a series of newly synthesized polymers could condense plasmid DNA efficiently with differences of a degree. Polymers condensed DNA compactly in order of CLPAE-Ahx > CLPAE-Lys > CLPAE. This may be due to the differences in the amount of primary amine or amine density, according to amine quantification results. However, at a weight ratio of 1 each polymer showed no less than 47, 61, 70% fluorescence in order of CLPAE-Ahx, CLPAE-Lys, CLPAE, respectively. This is thought to be due to the high sensitivity of the PicoGreen reagent.

Average Size Measurements of Polyplexes. The polyplex sizes and their distributions were determined by a Zetasizer. Each polyplex was prepared at various weight ratios ranging from 0.5 to 40 at room temperature. As shown in Figure 3, the polymers were found to have a similar tendency for average sizes of polyplexes to be about 120−180 nm over a weight ratio of 2, which were thought to be appropriate sizes for gene transfer. Abrupt size increments of polyplexes due to hydrophobic interaction between polyplexes arising from charge neutralization were observed at a low weight ratio. Interestingly, although CLPAE and CLPAE-Lys polyplexes showed maximum sizes (370, 352 nm respectively) at a weight ratio of 1, CLPAE-Ahx was observed to have a maximum size (334 nm) at a lower weight ratio of 0.7, not 1. This means CLPAE-Ahx possessed a higher amine density and was able to condense DNA more compactly than the other

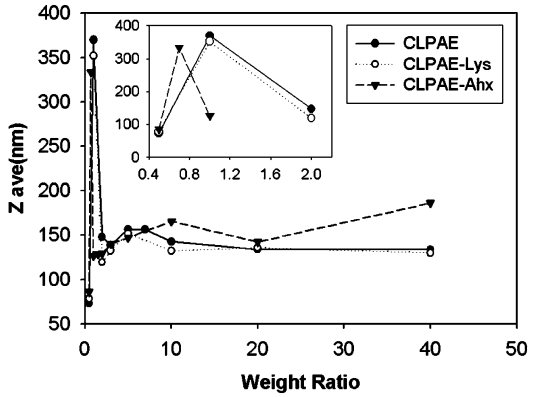


Figure 3. Average size measurements of polyplexes by Zetasizer. The inside box shows values at subdivided weight ratios ranging from 0.5 to 2.0.

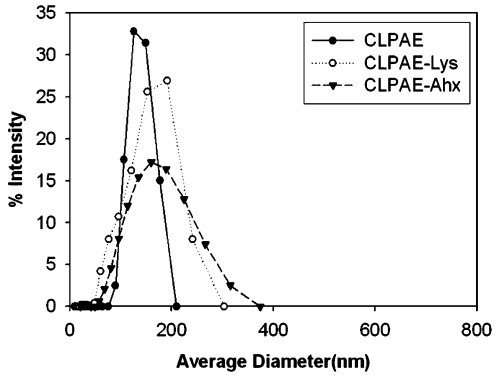


Figure 4. Average size distributions of the polyplexes at weight ratio of 20.

polymers synthesized, agreeing well with the amine quantification results.

Figure 4 shows the size distribution of each polyplex at a weight ratio of 20. CLPAE polyplexes were found to have a 97% population for a size range from 106 to 177 nm. Aggregates larger than 1 μm were not observed in any complexes. However, CLPAE-Lys and CLPAE-Ahx polyplexes were observed to have broader size distributions. It is considered that the irregularly modified polymer structure could lead to the formation of less homogeneous complexes with plasmid DNA. DLS results show that the polymers could form nanosized complexes with plasmid DNA having narrow size distributions.

Morphology of Polyplexes. The morphologies of polyplexes of the polymers were observed by AFM. Figure 5 shows AFM images of CLPAE polyplexes at various weight ratios ranging from 1 to 20. At a weight ratio of 1, CLPAE could not condense DNA completely (Figure 5A). Aggregates of 388−424 nm diameter and partially condensed DNA of irregular form were found. However, as the weight ratio of polymer increased to 10, they disappeared and compact sphere polyplexes of 125−190 nm size were observed (Figure 5B). Like CLPAE, CLPAE-Lys and CLPAE-Ahx showed compact spherical polyplex particles of about 80−200 nm size at a weight ratio of 20 (Figure 5C, 5D). These results agreed well with DLS data. The height of each polyplex was also determined to be 7−15 nm. As the weight ratio of polymer increased, the heights of polyplexes decreased. In general, a convolution of the imaging-tip's shape with the object's actual shape is known to affect the width of the object, while in such convolutions the height of the object is affected only by the compressibility of the object (*24*). So, it was

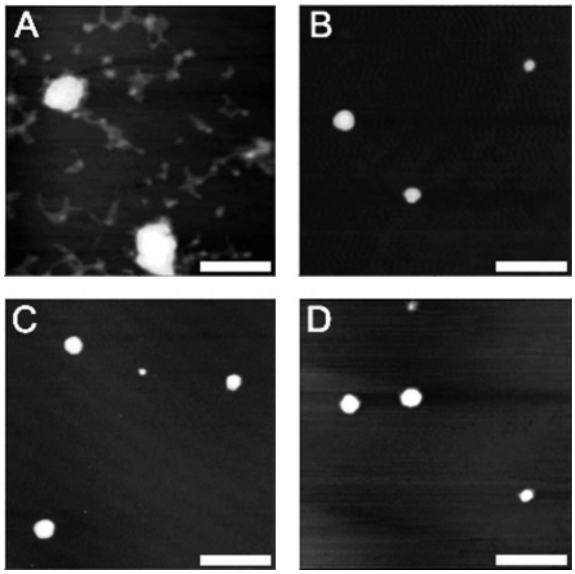


Figure 5. AFM images of the polyplexes. CLPAE: (A) weight ratio of 1, (B) 10, CLPAE-Lys: (C) 20, CLPAE-Ahx: (D) 20. The scale bars represent 1 μm.

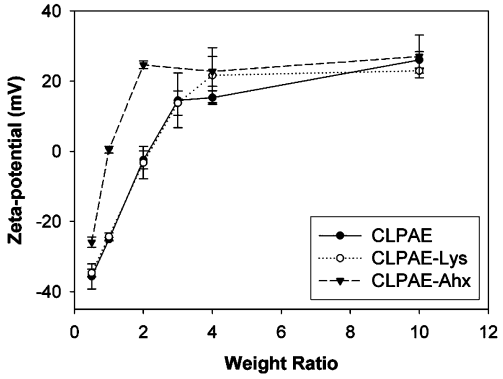


Figure 6. Zeta-potential measurements. Potential values are presented as the average value of 5 runs at 25 °C.

revealed that the polyplexes formed smaller and more compact particles as the weight ratio of polymer to DNA increased.

Zeta-Potential Measurements. Zeta-potentials of each polyplex were determined at various weight ratios. As shown in Figure 6, CLPAE and CLPAE-Lys polyplexes were found to have similar zeta-potential values at all weight ratios examined. They did not show positive values until the weight ratio was increased up to 2. However, the zeta-potential value of CLPAE-Ahx polyplex rose to almost the zero point even at a weight ratio of 1, and it showed a value of 24.6 $\pm$ 1.1 mV at 2. Because negative zeta-potential values of polyplexes were induced by phosphate anions of DNA of complex surfaces at low weight ratios where cationic polymers could not condense DNA completely, positive values of complexes mean the formation of stably compact complexes. Positively charged polyplexes are known to bind cellular membranes well, inducing endocytosis of the polyplexes (*25*). This result agreed well with our expectation and previous reports that a large number of CLPAE-Ahx-conjugated primary amines would lead to polyplex formation earlier than for tertiary amines of CLPAE.

In Vitro DNA Release Study. Linear types of biodegradable ester backbone polymers (PHPE, PAGA, etc.) were found to be decomposed in pH 7.4 solution within a few hours (*7*, *14*). This means biodegradable linear

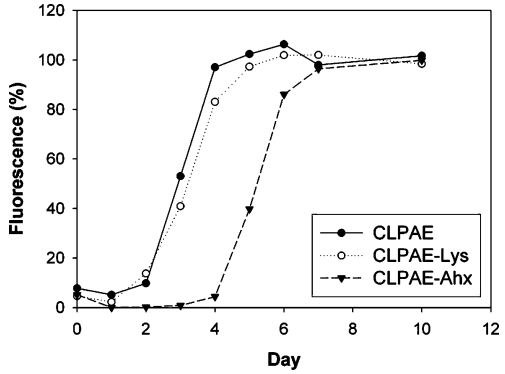


Figure 7. In vitro release profile of DNA from polyplexes by Picogreen reagent assay. Results are expressed in % fluorescence relative to control plasmid DNA.

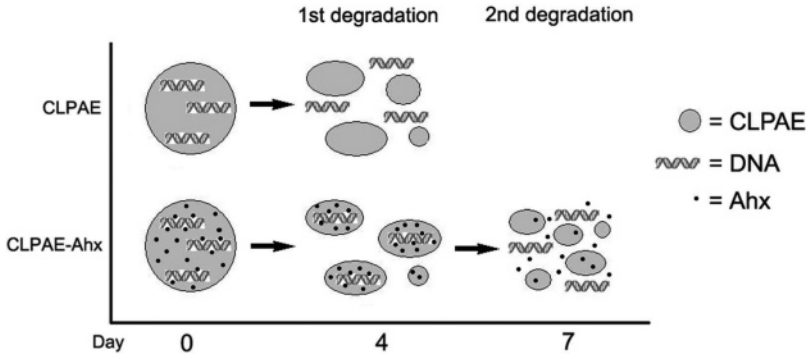
polymeric gene carriers would not be suitable for in vivo gene delivery for long-term applications. On the other hand, a cross-linked polyester polymer (n-PAE, etc.) showed a slower degradation rate and a prolonged DNA release profile in vitro, due to its structural property that one bond break could not cause a whole split of a polymer molecule directly (*21*). So, we designed a cross-linked poly(amino ester) polymer for gene delivery and examined the DNA release from polyplexes to confirm its merit.

In vitro DNA release was observed by PicoGreen assay, measuring the fluorescence quenching of DNA from polyplexes (Figure 7). We could not observe any significant increase in the fluorescence values of all polyplexes during the first 2 days. After 2 days, CLPAE and CLPAE-Lys/plasmid complexes showed gradual increases in DNA release values (53% and 41%, respectively, on day 3). CLPAE polyplex released almost 100% of the DNA by day 4, while, on the other hand DNA release reached 97% on day 5 in the case of CLPAE-Lys polyplex. However, CLPAE-Ahx polyplex was found to show a negligible value for DNA release until even day 4. On day 5, DNA release of CLPAE-Ahx polyplex increased to only 40% and reached 100% by day 7.

We confirm that these new cross-linked polymers are degraded more slowly than linear-type ester polymers and could release DNA outward polyplex in a more retained pattern, so, for long-term in vivo application, this type of polymer would have therapeutic merits. It may be due to the primary amines of the polymer binding with plasmid DNA more effectively that CLPAE-Ahx polyplex showed the most retained release profile among them.

Moreover, this result shows that DNA release due to the degradation of the polymers possibly would follow a stepwise mechanism (Scheme 2). During the time from day 2 to day 4 when the amount of DNA release rose to 100%, CLPAE backbone and tertiary amine moieties might be degraded and so CLPAE could not condense DNA anymore (first degradation step). Also, CLPAE-Ahx polyplex is thought to be under the same 'first degradation step' during this time. However, CLPAE-Ahx could condense plasmid DNA due to the primary amines of aminohexanoic acids after degradation, in contrast to CLPAE. Then, aminohexanoic acids of the polymer would be gradually degraded and cleaved from the polymer, inducing plasmid DNA release from day 4 to day 7 (second degradation step). We think that this stepwise degradation mechanism might result from the differences in the susceptibility to hydrolysis between the ester bonds of the CLPAE backbone and the ester linkages of

Scheme 2. The Degradation Mechanism of CLPAE and CLPAE-Ahx Polyplexes with Plasmid DNA



aminohexanoic acid to CLPAE. In the case of CLPAE-Lys, this effect was slight due to the polymer having fewer primary amines. Because primary amines of these 'self-destroying polymers' are thought to bind to DNA phosphates, not to attack ester bonds after forming polyplexes, the degradation rate of CLPAE-Ahx polyplex would be slower than that of CLPAE polyplex (*7*).

Cytotoxicity. Nontoxicity is one of the most important features for gene delivery carriers. In general, cytotoxicity is known to arise from the accumulation of nondegraded and nondischarged polymers in cells. So, many trials have been performed to reduce the toxicity of the gene carrier until now, which included the introduction of biodegradability to the polymer through such functionalities as ester, phosphoester, or disulfide bonds. Lim et al. reported that cationic polymers composed of ester backbone were barely toxic to mammalian cells (*8*, *21*).

We investigated the cytotoxicity of CLPAE, CLPAE-Ahx, and CLPAE-Lys by using MTT assay in 293 cells (Figure 8). The viability of 293 cells decreased abruptly according to an increase in the concentration of PEI, which shows the well-known cytotoxicity of PEI. CLPAE, CLPAE-Ahx and CLPAE-Lys provided favorable results. They showed about 80% cell viability even at a high concentration (50 μg/mL polymer concentration). This low toxicity may be due to the nontoxic building blocks and degradation products of polymers arising from a backbone of ester bonds. CLPAE-Ahx was found to show the lowest cell viability across the whole concentration range. It is thought that large numbers of primary amines of CLPAE-Ahx damage cells.

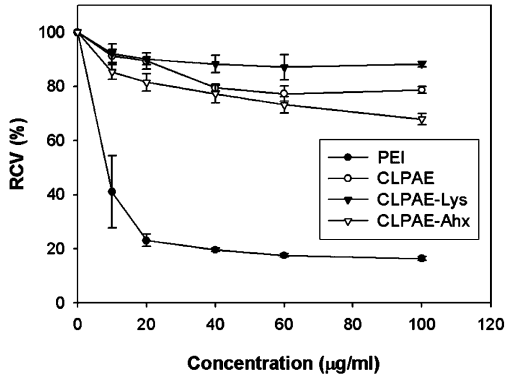


Figure 8. Cytotoxicity in 293 cells by MTT assay. Each data point represents the mean ± standard deviation ($n = 5$).

Transfection Efficiency (TE). The transfection abilities of CLPAEs were evaluated on three kinds of cell lines (293 human kidney transformed cells, C2C12 mouse myoblast cells, HepG2 human hepatocellular carcinoma cells), primary rat aorta vascular smooth muscle cells, and primary mouse embryonic fibroblast cells. PEI was used as the control for the standard gene carrier (*26*). As shown in Figure 9A, CLPAE showed very low TE but CLPAE-Ahx and CLPAE-Lys showed considerably enhanced TE compared with CLPAE on 293 cells. At the optimized weight ratio, TE of CLPAE-Ahx increased to 22 and 306 times over that of CLPAE in the absence and presence of serum, respectively. CLPAE-Lys also showed

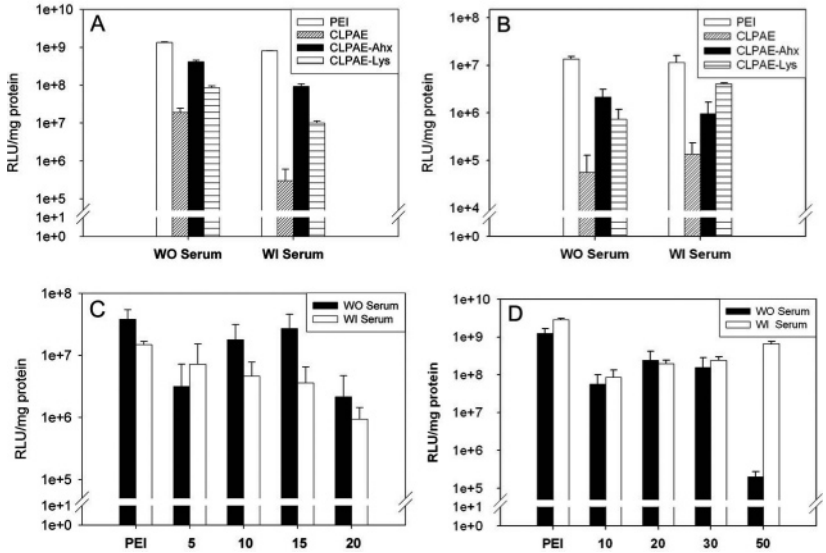


Figure 9. Transfection efficiencies of the polymers on 293 cells (A), C2C12 cells (B). (C and D) Transfection efficiency of CLPAE-Ahx on SMC and MEF cells, respectively. Numbers represent weight ratios.

enhanced TE (4−33 times that of CLPAE) although not by as much as for CLPAE-Ahx. We found similar enhancement effects on TE of CLPAE-Ahx and CLPAE-Lys as for CLPAE against C2C12 cells (Figure 9B). Fibroblast cells such as C2C12 are known to be hardly transfected, so the TE of PEI on C2C12 decreased to about 1% of TE on 293 cells. The TE of CLPAE-Ahx was enhanced 38 times more than that of CLPAE in the absence of serum. Although, interestingly, CLPAE-Lys showed an increase in its TE 30 times greater than CLPAE in the presence of serum, the existence of serum was found to reduce the TE of the polymers generally because cationic gene carriers possessing many positive charges are known to interact with negatively charged serum proteins (*27*, *28*).

The transfection efficiency of CLPAE was found to be much enhanced after coupling with aminohexanoic acid and lysine. Although CLPAE formed nanosized polyplexes having physicochemical properties very similar to those of CLPAE-Ahx and CLPAE-Lys with the exception of their having a slightly lower condensing ability, the TE of CLPAE was very low in the cell lines tested. This result suggests that a self-assembling ability with DNA to the compact nanoparticles simply could not lead to high transfection efficiency on cells. Therefore, primary amine residues such as aminohexanoic acid or lysine residue of CLPAE-Ahx and CLPAE-Lys are thought to certainly participate in the cellular uptake of polyplexes because CLPAE does not have primary amines but rather tertiary amines.

To examine applications on primary cells of CLPAE-Ahx showing generally the highest TE, transfection was carried out on the primary rat aorta vascular smooth muscle cells (SMC) and primary mouse embryonic fibroblast (MEF) cells. The accelerated proliferation of SMCs was reported to be a characteristic feature of atherosclerosis, or renarrowing of the artery (restenosis) after insertion of a stent within the artery wall for coronary artery disease (*29*, *30*). The mechanism of those two syndromes was partially understood but thought to be induced by the manifestation of genetic products such as platelet-derived growth factor (PDGF) (*31*). So, it could probably be a powerful tool for treating these diseases with gene therapy to deliver therapeutic genes with CLPAE-Ahx to vascular smooth muscle cells.

MEF cells are usually used as feeder cells to establish pluripotent embryonic stem (ES) cells. It is important to analyze the real functions of MEF cells and the secreted factors to influence ES cells within the culture system for establishment of a feeder-free culture environment (*32*). To study these through in vitro and ex vivo research, efficient delivery of various functional genes to MEF cells with CLPAE-Ahx could provide a useful tool.

As shown in Figure 9C and 9D, CLPAE-Ahx showed TE comparable to that of PEI on primary SMCs and MEF cells. It is due to higher expression level of pCN-Luci vector than pGL3 vector that TEs of polymers on MEF cells are much enhanced in contrast to those on SMCs. MEF cells showed sensitivity to cytotoxicity of PEI, so the TE of PEI was lower in the absence of serum than in the presence of serum contrary to transfection on other cells. Therefore, CLPAE-Ahx could be used for coronary artery diseases by in vivo gene delivery systems or research of primary MEF cell functions due to its high TE comparable to that of PEI on SMC and MEF cells and its low toxicity would ensure safe gene delivery.

CONCLUSION

We designed and synthesized a cross-linked poly(β-amino ester) for gene delivery to control its degradation rate and conjugated it with aminohexanoic acid or lysine to improve its transfection efficiency on mammalian cells. These CLPAE, CLPAE-Ahx, and CLPAE-Lys polymers showed great ability to form nanosized spherical polyplexes with DNA having narrow size distributions. Fulfilling our expectations, polyplexes of CLPAE-Ahx could release DNA even for a week through stepwise degradation. The polymers had low cytotoxicity in 293 cells. CLPAE-Ahx and CLPAE-Lys showed greatly enhanced transfection efficiencies in comparison to CLPAE on various cell lines, and the TE of CLPAE-Ahx is comparable to that of PEI on primary SMCs and MEF cells. It is evident that we could control the in vitro release rate of DNA by using cross-linked ester polymers, and the TE of polymeric gene carrier could be enhanced by conjugation with aminohexanoic acid or lysine. Therefore, CLPAE-Ahx has much potential as a nontoxic and efficient gene carrier.

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