

A Water-Soluble Phospholipid Polymer as a New Biocompatible Synthetic DNA Carrier

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The purpose of this work was the preparation of a water-soluble DNA complex with a biocompatible phospholipid polymer for delivering DNA into a target cell. The phospholipid polymer, poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-2-aminoethyl methacrylate (AEMA)] (poly(MPC-*co*-AEMA)), was synthesized and the functions of poly(MPC-*co*-AEMA) as a DNA carrier were compared with those of a conventional cationic DNA carrier such as poly(L-lysine). We investigated the toxicity of these polymers and the water solubility, structures, and nuclease resistance of the associated DNA/polymer complexes. An evaluation of toxicity in a colony forming test using Chinese hamster fibroblast cells (V-79 cells) indicated that the toxicity of poly(MPC-*co*-AEMA) is about 40% of that of poly(L-lysine). In the case of a salmon sperm DNA/poly(L-lysine) complex, a water-insoluble precipitate formed. In contrast, the DNA/poly(MPC-*co*-AEMA) complex was completely water-soluble. Although the DNA/poly(L-lysine) complex did not provide a CD spectrum, the DNA/poly(MPC-*co*-AEMA) complex did. DNase I hardly degraded the DNA in the DNA/poly(MPC-*co*-AEMA) complex. To demonstrate the transient expression of β-galactosidase, a plasmid DNA/poly(MPC-*co*-AEMA) complex was incubated with V-79 cells with chloroquine treatment. Under a phase-contrast microscope after 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, more stained cells due to the transient expression of β-galactosidase were observed in a sample with the poly(MPC-*co*-AEMA) complex than in a sample with the poly(L-lysine) complex. From these results came the following conclusions: poly(MPC-*co*-AEMA) is a useful water-soluble polymer that can form a complex with DNA, and DNA/poly(MPC-*co*-AEMA) has good potential for DNA delivery into cells.

Much attention has been paid to developing effective DNA carriers for cells in gene therapy.[1–4] This is because the utilization of DNA presents the following major problems: (i) rapid degradation in vitro and in vivo and (ii) rapid elimination from living organisms. One general approach to avoiding these problems is using complexes with polycations such as poly(L-lysine).[5–11] Other cationic polymers that have been studied include polyethylenimine (PEI),[12] poly-L-ornithine,[13] poly(*N*-ethyl-4-vinylpyridiumbromide),[14] poly[2-(dimethylamino)ethyl methacrylate],[15] and the poly(amidoamine) dendrimers.[16,17] The polycations, however, induced uncomfortable bioresponses in living organisms.[18–20] Moreover, a DNA/polycation complex usually has poor water-solubility and tends to aggregate in an aqueous medium. For DNA delivery, a polymer having biocompatibility, water-solubility, and the capability to form a complex with DNA is necessary. 2-Methacryloyloxyethyl phosphorylcholine (MPC) is a methacrylate with a phosphorylcholine group in the side chain.[21] Since MPC is easily polymerized with another monomer by a conventional radical polymerization method, the molecular design of an MPC polymer with suitable functions can be achieved.[21–24] The biocompatibility and blood compatibility of MPC polymers have been investigated. The surface of an MPC polymer having a 0.70 mole fraction of butyl methacrylate (BMA) units coated on the polymer substrate can inhibit coagulation of human whole blood without an anticoagulant.[25] Another finding was that plasma proteins barely adsorbed onto the MPC polymer surface.[26,27] These findings clearly show that MPC polymers have excellent blood compatibility and can be applied to prepare new biomedical devices that are safer to use in vivo.[28] Syntheses and evaluations of water-soluble MPC polymers having various functional groups have been reported.[29,30] The MPC unit provides high water-solubility to a polymer even when many hydrophobic units are contained in it.[31] The water-soluble MPC polymers can solubilize hydrophobic compounds in aqueous media. Therefore, as new drug carriers, they could be used instead of phospholipid assemblies, liposomes, or lipid microspheres.

In this study, we synthesized a water-soluble MPC polymer with primary amino groups to bind DNA molecules and investigated the toxicity of the polymer. After complexation between the MPC polymer and DNA, the water solubility, structure, and nuclease resistance of the DNA/MPC polymer complex were investigated. Finally, we demonstrated transfection of cells using the DNA/MPC polymer complex.

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Materials and Methods

Synthesis of MPC Polymer with a Primary Amino Group. MPC was synthesized with a previously reported method and purified by recrystallization from acetonitrile.[21] All other reagents were of an extra-pure reagent grade. A random copolymer of MPC and 2-aminoethyl methacrylate (AEMA) hydrochloride (Polysciences, Inc., PA, U.S.A.) was prepared with a conventional radical polymerization technique in H_2O using 2,2′-azobis(2-methylpropionamide) dihydrochloride (V-50) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as the initiator. The chemical structure of poly(MPC-*co*-AEMA) was confirmed with ^{1}H NMR (JNM-EX270, JEOL, Tokyo, Japan) in D_2O.

Gel permeation chromatography (MIXED-C, Polymer Laboratories Ltd., Shropshire, U.K.) was used to determine the number-average molecular weight (M_n) and the weight-average molecular weight (M_w) of the poly(MPC-*co*-AEMA) in $CHCl_3$/MeOH (6:4, v/v) containing 0.5 wt % LiCl with poly(methyl methacrylate) standards. It was necessary in this step to protect the amino groups by inducing a reaction with a carboxylic acid to form an amide group. To prepare propionic acid succinimideester, 0.005 mmol of propionic acid, 0.006 mmol of *N*,*N*′-carbonyldiimidazole and 0.006 mmol of *N*-hydroxysuccinimide were dissolved in 4 mL of *N*,*N*-dimethylformamide and incubated at room temperature for 3 h. Ten mg of poly(MPC-*co*-AEMA) was dissolved in 0.6 mL of the propionic acid succinimide ester solution and incubated overnight at 4 °C to allow for a reaction between the amino group and propionic acid succinimide ester. The MPC polymer with the amide group was purified by dialysis against H_2O, and in the final step, it was freeze-dried. Figure 1 shows the chemical structure of the poly(MPC-*co*-AEMA), and Table 1 gives the synthetic results.

Evaluation of Cytotoxicity of Poly(MPC-*co*-AEMA). The V-79 cell line (fibroblast cells from Chinese hamsters, Riken, Saitama, Japan) was used to evaluate cytotoxicity in a colony forming test (to determine the IC_{50} value).[32] Fifty cells per well were seeded into a 24-well culture dish and incubated at 37 °C for 16 h in a 5% CO_2 atmosphere and in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (FCS) (final concentration: 5% (v/v)) and antibiotic-antimycotic (Gibco BRL, CA, U.S.A.). For each of the following polymer solutions, 50 μL of the solution was pipetted into individual wells: the poly(MPC-*co*-AEMA), poly(L-lysine) hydrobromide (P-7890, Sigma, MO, U.S.A.), and H_2O (as the control). The concentrations of the polymers (the poly(MPC-*co*-AEMA) and poly(L-lysine) hydrobromide) ranged between 0.1 wt % and 4.88×10^{-5} wt %. After incubation at 37 °C for 7 days in a 5% CO_2 atmosphere, each well was washed three times with Dulbecco's phosphate buffered saline (D-PBS), and the cells were fixed with EtOH. To stain the formed colony, 100 μL of the Gimsa solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was pipetted into each well, which was then incubated at room temperature for 10 min and washed three times with H_2O. The IC_{50} value was determined by counting the formed colonies.

Agarose Gel Electrophoresis. A sample was prepared by direct mixing of a solution of the poly(MPC-*co*-AEMA) and salmon sperm DNA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a 40 mM Tris–acetate buffer containing 1 mM EDTA (pH 8.3) (TAE). The mixed solution was kept at 25 °C for 30 min. The salmon sperm DNA to poly(MPC-*co*-AEMA) ratio ranged between 1:0 (wt/wt) and 1:2 (wt/wt) in TAE. Each complex was electro-

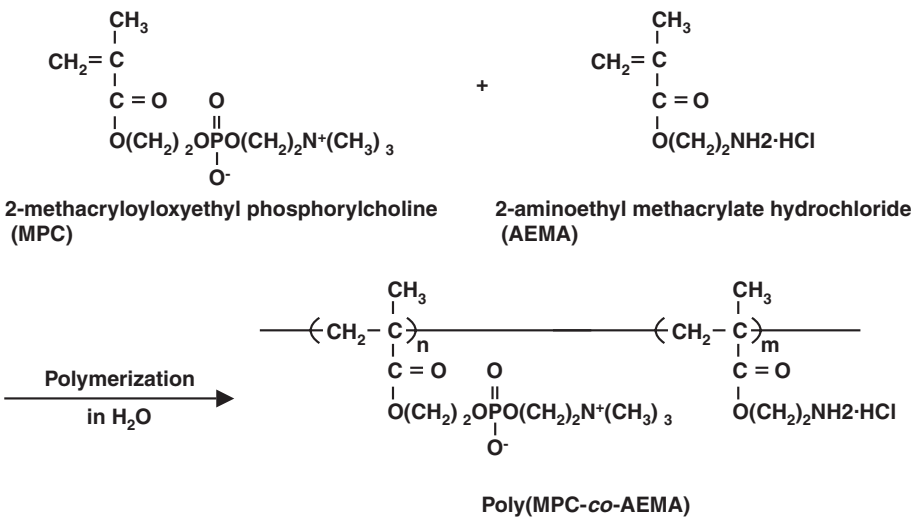

Fig. 1. Chemical structure of poly(MPC-*co*-AEMA).

Table 1. Synthetic Results of Poly(MPC-*co*-AEMA)

Mole fraction of MPC		Yield/%[b]	$M_n \times 10^{-4}$ [c]	$M_w \times 10^{-4}$ [c]	M_w/M_n [c]
In feed	In polymer[a]				
0.50	0.65	65.5	3.9	5.1	1.3

a) Unit of mole fraction of MPC in the copolymer was determined by ^{1}H NMR in D_2O. b) [monomer] = 0.5 mol/L, [2,2′-azobis(2-methylpropionamide)dihydrochloride (V-50)] = 0.01 mol/L. Polymerization was carried out at 60 °C for 8 h in H_2O. c) Determined by GPC with the poly(methyl-methacrylate) standard, M_n and M_w represent the number- and weight-average molecular weight, respectively.

phoresed on agarose gel (0.6% wt/vol) for 30 min at 100 V. Ethidium bromide was added into the gel so that the location of the DNA could be determined with a UV transilluminator.

Dynamic Light Scattering (DLS). The diameter of the complex was determined by dynamic light scattering (DLS) measurement with a Nicomp Model 370 (Particle Sizing Systems, CA, U.S.A.). The weight ratio of the salmon sperm DNA to poly(MPC-*co*-AEMA) was 1:1 (each at a concentration of 125 μg/mL) in a 10 mM Tris–HCl buffer containing 1 mM EDTA (TE). The complex was kept at 25 °C for 30 min and then measured.

Evaluation of the Stability of the DNA/Poly(MPC-*co*-AEMA) Complex. The stability of the salmon sperm DNA/poly(MPC-*co*-AEMA) complex in an aqueous medium was determined by UV spectroscopy with a UV–vis spectrophotometer V-560 (JASCO, Tokyo, Japan). The secondary structure of the DNA after complexation with the poly(MPC-*co*-AEMA) was determined by circular dichroism (CD) measurement with a CD spectropolarimeter J-720W (JASCO, Tokyo, Japan). The weight ratio of the salmon sperm DNA to poly(MPC-*co*-AEMA) was 1:1 (each at a concentration of 45 μg/mL) in TE. The complex was kept at 25 °C for 30 min and then measured.

Degradation of DNA by DNase I. Increments of absorbance at 260 nm were measured to evaluate the DNA degradation caused by DNase I.[33] The weight ratio of the salmon sperm DNA to poly(MPC-*co*-AEMA) was 1:1 (each at a concentration of 20 μg/mL) in a 100 mM sodium acetate buffer containing 5 mM $MgSO_4$. The complex was kept at 25 °C for 30 min. Then, 160 U of DNase I (Takara Shuzo Co., Ltd., Tokyo, Japan) were added, and the complex was kept at 25 °C. Absorbance at 260 nm was measured with the UV–vis spectrophotometer.

Transient Expression. The transient expression of β-galactosidase using DNA/poly(MPC-*co*-AEMA) and DNA/poly(L-lysine) complexes was investigated. A plasmid DNA, plasmid pCMV SPOR-βgal (Gibco BRL, CA, U.S.A.), and the V-79 cell line (fibroblast cells from Chinese hamsters, Riken, Saitama, Japan) were used in this case.[34] Twenty thousand cells per well were seeded into a 96-well culture dish and incubated at 37 °C for 10 h in a 5% CO_2 atmosphere and in DMEM supplemented with FCS (final concentration: 10% (v/v)). The medium was removed after the incubation. To promote improvement in endocytosis of the cells, 100 μL of 100 μM chloroquine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in DMEM supplemented with FCS (final concentration: 2% (v/v)) was added into each well, which was then incubated at 37 °C for 30 min in a 5% CO_2 atmosphere. The medium was removed after the incubation. To prepare a plasmid pCMV SPOR-βgal/poly(MPC-*co*-AEMA) complex and SPOR-βgal/poly(L-lysine) complex, a solution consisting of one of the polymers and plasmid pCMV SPOR-βgal was mixed in DMEM without FCS. The DNA/polymer complex charge ratio ([amino group] from the poly(MPC-*co*-AEMA) or poly(L-lysine)/[phosphate group] from plasmid pCMV SPOR-βgal) was 4:1. Each complex was kept at 25 °C for 30 min. One hundred μL of each complex was pipetted into individual wells, whose cells were then cultured and incubated at 37 °C for 16 h in a 5% CO_2 atmosphere. The medium was removed after the incubation. For expression of β-galactosidase, DMEM supplemented with FCS (final concentration: 10% (v/v)) was pipetted into each well, which were then incubated at 37 °C for 16 h in a 5% CO_2 atmosphere. The medium was removed after the incubation. Each well was washed three times with D-PBS. To fix the cells, 100 μL of a 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added into each well, which was then incubated overnight at 4 °C. The fixing solution was removed after the incubation, and then each well was washed three times with D-PBS. To prepare the staining solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 400 mg of X-gal were dissolved in 10 mL of D-PBS containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, and 2 mM $MgCl_2$.[35] One hundred μL of the X-gal solution was pipetted into each well, which was then incubated overnight at 25 °C to stain the expressed β-galactosidase. The staining solution was removed after the incubation, and then each well was washed three times with D-PBS. Finally, the cells were observed through a phase-contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Results and Discussion

Characteristics of the MPC Polymer as a Complex Agent with DNA. The copolymerization of MPC and AEMA HCl proceeded homogeneously in H_2O. Table 1 summarizes the results of the copolymerization of MPC with AEMA. The chemical structure of poly(MPC-*co*-AEMA) was confirmed by ^{1}H NMR, that is, peaks assigned to α-CH_3 (0.95 ppm), $-N^+(CH_3)_3$ (3.1 ppm) and $-CH_2N-$ (3.3 ppm) were observed. The mole fraction of the MPC unit in the polymer was 0.65, which was determined from NMR spectroscopy, specifically the ratio of integration of the $-N^+(CH_3)_3$ peak and $-CH_2N-$ peak. M_n and M_w were 3.9×10^4 and 5.1×10^4, respectively. The obtained poly(MPC-*co*-AEMA) was soluble in water, PBS, and ethanol.

Figure 2 shows the relationship between polymer concentration and the ratio of colony formation. As is well known, amino are harmful to cells. The amino groups sometimes induced cell death. Thus, we determined the IC_{50} value of the poly(MPC-*co*-AEMA) and compared it with that of the poly(L-lysine). Poly(L-lysine) is commonly used as the polycation for a complex with DNA.[5–11] After incubation with the polymers, the cell colonies were counted. In other words, if 50 cells made up 50 cell colonies in the presence of one of the polymers, that polymer has no cytotoxicity. When 50% of the cells could not form a cell colony, the IC_{50} value of the poly(MPC-*co*-AEMA) was 0.005 wt %, whereas that of the poly(L-lysine) was 0.019 wt %. The ratio of these values is 2.6, which means the poly-

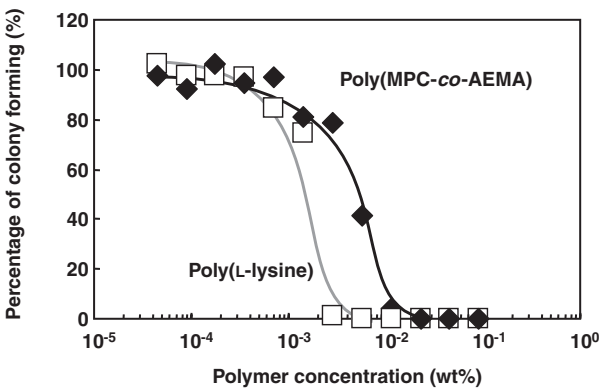

Fig. 2. Relation between polymer concentration and percentage of colony forming. ◆ poly(MPC-*co*-AEMA), □ poly(L-lysine).

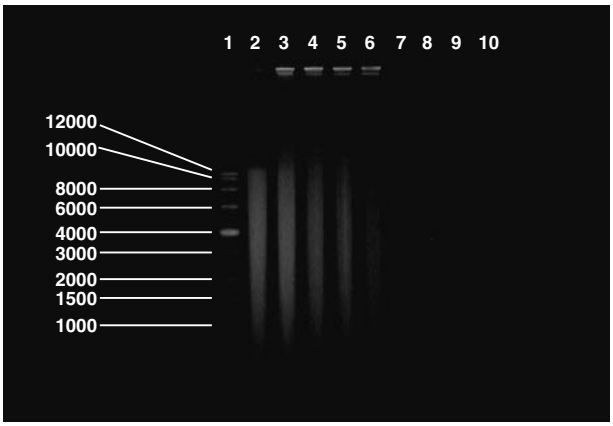

Fig. 3. Agarose gel electrophoresis of salmon sperm DNA and its complex with poly(MPC-*co*-AEMA). [agarose]: 0.6%, 2 mg DNA/lane, lane 1: molecular weight indicator, lane 2–lane 10: DNA/poly(MPC-*co*-AEMA) (wt/wt) 1/0, 1/0.25, 1/0.5, 1/0.75, 1/1, 1/2.5, 1/5, 1/7.5, 1/2.

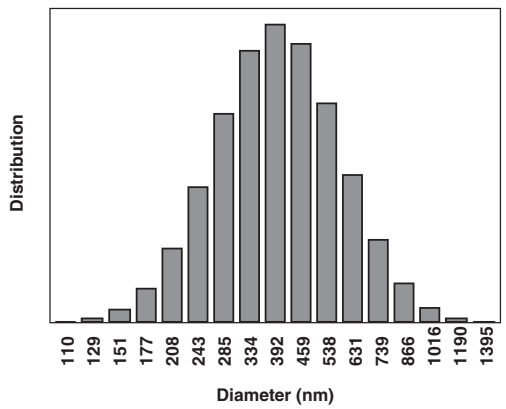

Fig. 4. Size distribution of salmon sperm DNA/poly(MPC-*co*-AEMA) complex.

(MPC-*co*-AEMA) has a much milder interaction with cells as compared with the poly(L-lysine).

Complex of the MPC Polymer with DNA. In fundamental complex experiments, we used salmon sperm DNA, which is well understood and easily obtained. Figure 3 shows the results of agarose gel electrophoresis of the salmon sperm DNA/poly(MPC-*co*-AEMA) complex. When the percentage of the poly(MPC-*co*-AEMA) in the complex was increased, complexation proceeded. When the DNA/MPC polymer ratio was 1:1, a complete complex was observed. Because the cationic amino groups of the MPC polymer and anionic phosphoric acid of the DNA were equal in number, the charge of the complete complex was neutral, so the complex did not move in the agarose gel. When the DNA/MPC polymer ratio was greater than 1:1.25, the complex was not detected. This is because the charge of the complex was cationic and the complex moved to the opposite side.

Figure 4 shows the size distribution of the salmon sperm DNA/poly(MPC-*co*-AEMA) complex. The average diameter was 365 nm. In this study, we used salmon sperm DNA.

The stability of the salmon sperm DNA/poly(MPC-*co*-AEMA) complex in an aqueous medium was determined by

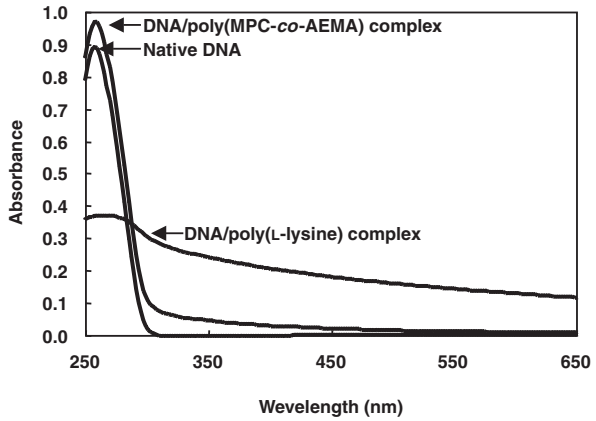

Fig. 5. Absorbance spectra of salmon sperm DNA and its complex with poly(MPC-*co*-AEMA).

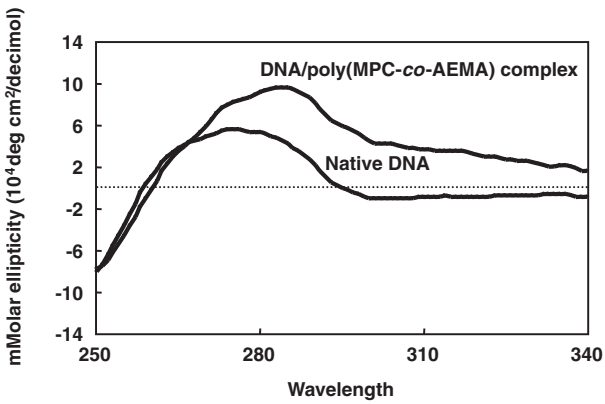

Fig. 6. CD spectra of salmon sperm DNA and its complex with poly(MPC-*co*-AEMA).

UV spectroscopy. Figure 5 shows the absorption spectra of the DNA and its polymer complexes. The poly(MPC-*co*-AEMA) formed a transparent solution even after complexation with the DNA. However, the poly(L-lysine) formed a slightly opaque solution. This is due to aggregation of the DNA/poly(L-lysine) complex. In the case of the poly(L-lysine), cationic amino groups are important for maintaining water-solubility and for complexation with the DNA. As the complexation with DNA proceeded, the number of cationic amino groups decreased dramatically, and the DNA/poly(L-lysine) complex became water-insoluble. In the case of the poly(MPC-*co*-AEMA), water-solubility was maintained by the MPC units even when amino groups were used as the complexation site. That means the MPC units effectively solubilized the DNA through complexation. Moreover, the MPC unit is zwitterionic and electrically neutral thus, the DNA/MPC polymer complex could be made neutral by adjustment of the DNA/MPC polymer ratio.

The secondary structure of the salmon sperm DNA after complexation with the poly(MPC-*co*-AEMA) was determined by CD spectroscopic measurement. Figure 6 indicates the secondary structure of the salmon sperm DNA. Although the poly(L-lysine) complex did not provide a CD spectrum due to aggregation, the complex with poly(MPC-*co*-AEMA) did provide a spectrum.

Figure 7 shows the time dependence of changes in absorbance at 260 nm as attributed to the DNA degradation process caused by the enzyme. The salmon sperm DNA was used as the control. When DNA degrades, the molar absorption coefficient at 260 nm may be higher.[33] The native DNA was immediately degraded and, in this case, the absorbance increased quickly within 10 min. On the other hand, the complex of DNA and poly(MPC-*co*-AEMA) could suppress increments in absorbance. This indicates that DNA degradation was effectively suppressed by the poly(MPC-*co*-AEMA). These results show that the poly(MPC-*co*-AEMA) disrupted enzymatic degradation of the DNA and stabilized the DNA without any conformational change.

Transfection of Cells Using DNA/MPC Polymer Complex. Figure 8 shows images from a phase-contrast microscope after X-gal staining. We had applied a cationic plasmid DNA/poly(MPC-*co*-AEMA) complex and plasmid DNA/poly(L-lysine) complex, because the polymers could not interact with the cell membrane and the plasmid DNA was not introduced into cells under neutral conditions. About twice as many blue cells were observed in the poly(MPC-*co*-AEMA) complex compared with the poly(L-lysine) complex. Accordingly, more transient expression of β-galactosidase using poly(MPC-*co*-AEMA) compared with that using poly(L-lysine) was observed.

In the samples of these polymers without the plasmid DNA, and the samples of only the plasmid DNA, no cell stained with X-gal was observed (images not provided). Thus, these polymers made complexes with the plasmid DNA, and these complexes interacted with cells and introduced DNA into the cells. Some toxicity or cytostasis may accompany effective DNA delivery. The cationic polymers for DNA delivery can be classified as an "aggregated complex" or a "stable complex (water-soluble complex)", properties that should depend on the individual chemical structures of polymers. On one hand, a poly(L-lysine)/DNA complex is clearly an aggregated complex. The behavior of poly(L-lysine)/DNA complexes have been described by other groups. Kwoh et al. found that a poly(L-lysine)/DNA complex aggregated at all ratios.[36] An aggregation ratio of less than 1:1 was seen by Tang and Szola.[37] On the other hand, the electrically neutral poly(MPC-*co*-AEMA)/DNA complex is a stable complex (water-soluble complex). As has been demonstrated, MPC polymers show excellent biocompatibility and no toxicity, so DNA delivery using a DNA/MPC polymer complex is quite mild compared with that using a conventional DNA/polycation complex. From these results, we concluded that the DNA/poly(MPC-*co*-AEMA) complex is useful for DNA delivery into cells.

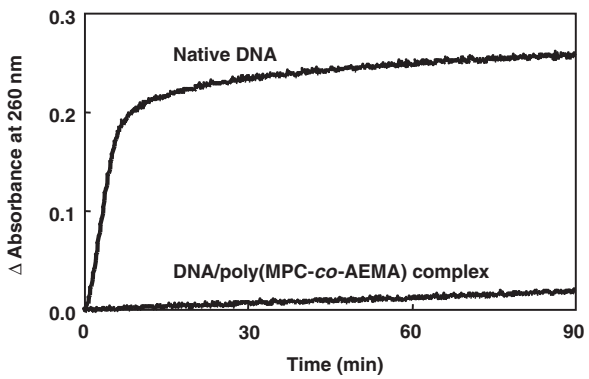

Fig. 7. Degradation of profiles of salmon sperm DNA and its complex with poly(MPC-*co*-AEMA) as cased by DNase I.

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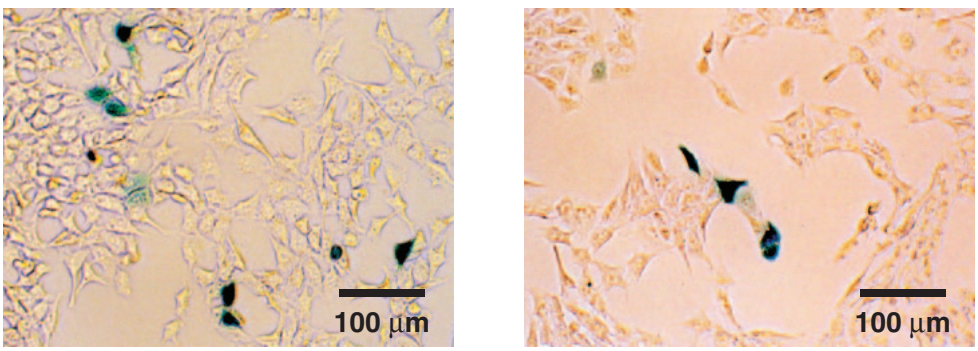

Fig. 8. Phase-contrast microscope images of V79 cells after staining with X-gal.

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