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In vitro transfection of plasmid DNA by cationized gelatin prepared from different amine compounds

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Abstract—The objective of this paper is to compare the *in vitro* transfection efficiency of a luciferase plasmid DNA using cationized gelatin prepared from different amine compounds. The compounds used here were ethylenediamine, putrescine, spermidine and spermine, chemically introduced to the carboxyl group of gelatin for the cationization. Complexation of the cationized gelatin with the plasmid DNA was performed by simply mixing the two materials at various N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) in aqueous solution. Gel retardation studies revealed that the formation of cationized-gelatin–plasmid DNA complexes depended on the N^+/P^- mixing ratio. The stronger interaction of plasmid DNA with the cationized gelatin of spermine compared to the other cationized gelatins was observed by an ethidium bromide intercalation assay and Scatchard binding analysis. When the transfection efficiency of plasmid DNA complexed with the various cationized gelatins at different N^+/P^- mixing ratios was evaluated for mouse L929 fibroblasts, the highest transfection efficiency was observed for the complex prepared from the cationized gelatin of spermine at a N^+/P^- mixing ratio of 2. The present study indicates that there is an optimal N^+/P^- mixing ratio and a type of amine compound or cationization extent of cationized gelatin to enhance the transfection efficiency of plasmid DNA.

Key words: Cationized gelatin; gene transfection; ethylenediamine; putrescine; spermidine; spermine.

INTRODUCTION

Gene therapy involves the introduction of exogenous genes into target cells where production of the protein encoded is expected. In the case of acquired or inherited

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genetic disorders, this enables the replacement of a missing or defective gene, leading to normalization of cell functions [1–8].

DNA has been delivered or internalized into cells for gene transfection either by a viral or non-viral system. Viral carriers include adenoviruses, retroviruses, adeno-associated viruses and lentivirus [9]. Although the viral carriers possess the inherently high efficiency of gene transfection, there are some issues to be resolved for the clinical trials, such as immunological and/or toxic responses toward the carriers themselves. To tackle these problems, several genetic modulations of biological approach have been attempted for viral carriers. As another approach, non-viral carriers have been investigated to demonstrate several advantages over the viral carriers. For the non-viral transfection system, naked DNA alone or the complex of DNA with cationic liposomes, cationic lipids and cationic polymers have been utilized [10–12]. Their nature to induce toxicity and immune responses is low compared with that of viral carriers. Macromolecules, such as poly(ethylenimine) and poly(lysine), and lipids of positive charges have been designed to demonstrate their feasibility as the non-viral carrier in enhancing gene expression [13–15]. However, some points of low transfection efficacy and cytotoxicity should be improved [16].

Gelatin has extensively used for industrial, pharmaceutical and medical applications and the bio-safety has been proven through its long clinical use as biomaterial and drug ingredient [17]. Other advantages of gelatin include the availability of samples with various physicochemical natures and the simple chemical modification. For example, the positively charged, cationized gelatin can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. We previously reported that when a plasmid DNA was complexed with the ethylenediamine-introduced cationized gelatin and applied to cells, the transfection efficiency of plasmid DNA was increased significantly [18–22]. However, there are only few reports on the effect of diamine compounds to be introduced on the gene expression.

The present study was undertaken to investigate the *in vitro* transfection efficiency of plasmid DNA by the cationized gelatin of non-viral carriers prepared from different types of amine compounds. Ethylenediamine, putrescine, spermidine and spermine were chemically introduced into the carboxyl groups of gelatin to prepare cationized gelatins. A plasmid DNA was mixed with the cationized gelatin at different ratios to form the complexes while their physicochemical properties were evaluated in terms of the apparent molecular size, the zeta potential and the interaction affinity for the plasmid DNA. We examined the *in vitro* transfection of plasmid DNA for mouse fibroblasts by the cationized gelatin of different amine compounds at various mixing ratios and evaluated the cytotoxicity.

MATERIALS AND METHODS

Materials

A gelatin sample (molecular weight 1×10^5), prepared through acid treatment of pigskin type-I collagen, was kindly supplied by Nitta Gelatin. Ethylenediamine was

purchased from Wako. Putrescine, spermidine and spermine were purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC), 2,4,6-trinitrobenzensulfonic acid, β-alanine and ethidium bromide (EtBr) were purchased from Nacalai Tesque.

Preparation of cationized gelatin with different amine compounds

Ethylenediamine ($NH_2(CH_2)_2NH_2$), putrescine ($NH_2(CH_2)_4NH_2$), spermidine ($NH_2(CH_2)_4NH(CH_2)_3NH_2$) or spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) was added together with 3 g EDC into 250 ml 100 mM phosphate-buffered solution (pH 5.0) containing 5 g gelatin at different molar ratios to the carboxyl groups of gelatin (0, 1, 10, 25, 50 and 100). Immediately after that, the pH of solution was adjusted at 5.0 by adding 5 M of HCl aqueous solution. The reaction mixture was agitated at 37°C for 18 h and then dialyzed (cut-off molecular weight (12–14) $\times$ 10^3, Viskase) against double-distilled water (DDW) for 48 h at 25°C. The dialyzed solution was freeze-dried to obtain cationized gelatin samples. The percentage of amino groups introduced into gelatin (the cationization extent of gelatin) was determined by the conventional trinitrobenzene sulfonate method [23] based on the calibration curve prepared by using β-alanine at the pre-determined concentration. The molecular weight of all the cationized gelatins was evaluated by gel-permeation chromatography (UV-8000 system, Tosoh).

DNA isolation

The pGL3 vector (5.26 kb) encoding firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega) was propagated in *Escherichia coli* (strain DH5α) and purified with the EndoFree™ plasmid kit (Qiagen) according to the manufacturer's instruction. The yield and purity of plasmid DNA obtained were ascertained by UV spectroscopy and the $E_{260\ \mathrm{nm}}/E_{280\ \mathrm{nm}}$ absorption ratio ranged between 1.8 and 1.9.

Preparation of plasmid DNA-cationized gelatin complexes

Complexation of the cationized gelatin with the luciferase plasmid DNA was performed by simply mixing the two materials at various N^+/P^- mixing ratios in aqueous solution. Briefly, 50 μl 100 mM phosphate-buffered saline solution (PBS, pH 7.4) containing 12.5, 25, 50, 100, 250 and 500 μg of cationized gelatin was slowly added to the same volume of PBS containing 10 μg of luciferase plasmid DNA at N^+/P^- mixing ratios of 0.25, 0.5, 1, 2, 5 and 10. The mixed solution was gently agitated at 37°C for 30 min to form cationized-gelatin–plasmid DNA complexes.

Dynamic and electrophoretic light scattering measurements

To investigate the hydrodynamic radius of cationized-gelatin–plasmid DNA complexes, the dynamic light scattering (DLS) measurement was carried out on a DLS

700 machine (Otsuka Electronics) equipped with a He-Ne^{-} laser at detection angles of 30, 90 and 120° at room temperature. The cationized-gelatin–plasmid DNA complex solution was filtered by a disposable syringe filter (pore size 0.8 μm; Millipore) for DLS measurement. The hydrodynamic diameter of cationized gelatin complexed with the plasmid DNA was analyzed based on the cumulants method and calculated automatically by computer software equipped to express the values as the apparent molecular size. Each experiment was done 5–10 times independently. Electrophoretic light scattering (ELS) measurement was carried on a ELS-7000 machine (Otsuka Electronic) at room temperature and an electric field strength of 100 V/cm. The complex samples were prepared similarly using 10 mM phosphate buffer (pH 7.4). The solution was filtered through a disposable syringe filter (pore size 0.8 μm; Millipore) for ELS measurement. The zeta potential was automatically calculated using the Smoluchowski equation. Each experiment was done 10–20 times independently unless stated otherwise.

Electrophoresis of cationized-gelatin–plasmid DNA complexes

Formation of cationized-gelatin–plasmid DNA complexes was confirmed by the gel retardation assay [24]. An aliquot (5 μl) of plasmid DNA complex solution (0.1 μg/μl) was loaded into a well of an 0.8% agarose gel containing ethidium bromide and electrophoresed at 100 V for 15 min in Tris-borate-EDTA (TBE) buffer. Bands corresponding to plasmid DNA were detected under UV light and photographed.

Ethidium bromide (EtBr) intercalation assay

An EtBr intercalation assay was carried out according to the method of Xu and Szoka [25]. In brief, cationized gelatin solution (50 μl) and 20 μg/ml plasmid DNA solution (50 μl) were mixed in PBS (pH 7.4) in various N^{+}/P^{-} mixing ratios. EtBr (1.2 μg/ml, 50 μl) was added to these mixtures, and the mixed aqueous solutions (150 μl) were placed in a 96-well flat-bottomed microassay plate (Becton Dickinson). The fluorescence of EtBr intercalated to DNA was specifically monitored using a Gemini EM fluorescent microplate reader (excitation 510 nm, emission 590 nm; Molecular Devices). Free EtBr was not detected under these conditions.

Sorption experiment with Scatchard binding analysis

For the immobilization of gelatin to agarose beads, a HiTrap™ NHS-activated HP column (Amersham Bioscience) containing *N*-hydroxysuccinimide (NHS)-immobilized agarose beads in ethanol (1.0 ml) was washed three times with 1 mM cold HCl solution (5 ml). Next, 1.0 ml cationized gelatin solution (1.0 mg/ml) in a coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl aqueous solution, pH 7) was added to the column. Then, the column was washed three times with 2 ml NHS

deactivation buffer (0.5 M ethanolamine and 0.5 M NaCl aqueous solution, pH 8.3) three times, neutralized by 2 ml acidic buffer (0.1 M acetic acid and 0.5 M NaCl, pH 4.0) and 2 ml PBS (pH 7.4) five times. The gelatin-immobilized column prepared was stored in 2 ml PBS (pH 7.4). The amount of cationized gelatin immobilized was determined by protein assay using a Lowry kit (Nacalai Tesque) by measuring the protein amount eluted from the column and calculated from the amount of gelatin in the solution before and after immobilization reaction. Non-immobilized and NHS-deactivated columns were prepared as controls.

^{125}I-radiolabeled plasmid DNA was prepared according to the chloramine-T method reported by Chan *et al*. [26]. The sorption test of ^{125}I-radiolabeled plasmid DNA to the cationized gelatin-immobilized column was performed. Briefly, aqueous solution containing different amounts of ^{125}I-radiolabeled plasmid DNA (100 μl) was added to the cationized gelatin-immobilized column and left for 15 min at 37°C. Then, the column was washed with 2 ml PBS (pH 7.4) three times to remove non-complexed ^{125}I-radiolabeled plasmid DNA. The radioactivity of ^{125}I-radiolabeled plasmid DNA bound to the cationized gelatin-immobilized column was measured by a gamma counter. The equilibrium concentration of non-complexed ^{125}I-radiolabeled plasmid DNA cationized gelatin eluted (C_f) was also determined by measuring the radioactivity. The molar ratio of complexed plasmid DNA to cationized gelatin (r) was calculated using 3.5×10^6 and 1×10^5 as the molecular weight of plasmid DNA and gelatin. The r/C_f was plotted according to the Scatchard binding model [27]. The dissociation constant (K_d) was obtained from the slope and the intercept at $r = 0$ of the $(r/C_f) - r$ line.

In vitro *transfection study*

L929 cells of a murine fibroblast cell line were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). The cells were seeded, 24 h prior to transfection, into each well of a 6-well cell-culture plate (Corning) at a density of 2.5×10^4 cells/well in 2 ml of the culture medium. For transfection, the culture medium was replaced with 1.9 ml of FCS-free culture medium, and then the solution of cationized gelatin-plasmid DNA complexes prepared at different N^+/P^- mixing ratios or free plasmid DNA in PBS (100 μl) was added to the well, following by incubation for 6 h. After the medium was exchanged to that containing 10% (v/v) FCS, the cells were incubated for a further 24 h. The luciferase gene expression was quantified using a commercial kit (Luciferase Assay System, Promega) and the relative light units (RLU) were determined by a luminometer (MicroLumatPlus LB 96V, Berthold). The total protein of each well was determined using the BCA Protein Assay Reagent (Pierce) in order to normalize the luciferase activity for cell number. A lipid-based reagent (Lipofectamine, Invitrogen) commercially available was used for comparison. Transfection study was carried out three times independently for every experimental group.

Evaluation of cytotoxicity

The cytotoxicity of cationized-gelatin–plasmid DNA complexes was evaluated. Briefly, L929 cells were cultured with the complexes prepared at different N^+/P^- mixing ratios for 24 h. After that, the culture medium was discarded and 1 ml fresh culture medium containing 100 μl of a 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, followed by 4 h incubation. Next, the MTT-containing medium was replaced with 200 μl isopropanol-HCl (0.1 M), keeping it at 37°C for 10 min to solubilize the formazan crystals. The sample solution was transferred to each well of a 96-well cell-culture plate (Corning) and the absorbance was measured at a wavelength of 570 nm. The percent cell viability of the control (non-treated) cells was taken as 100%.

Statistical analysis

All the data were expressed as the mean ± standard deviation of the mean. Statistical analysis was performed based on the unpaired Student's t-test (two-tailed) and significance was accepted at $P < 0.05$.

RESULTS

Preparation and characterization of cationized gelatin with different amine compounds

Table 1 shows the cationization extent of gelatin samples prepared through the chemical introduction of different amine compounds. The cationization extent could be controlled by changing the molar ratio of amine compounds added. GPC measurement revealed that the molecular weight of all the cationized gelatins used was approximately 1×10^5, irrespective of the type of amine groups introduced.

Table 1.
Cationization extent of gelatin prepared at different molar ratios of amine compounds added to the carboxyl groups of gelatin

Gelatin (g/ml)	Molar ratio[a]	Amino compound (mol%/mol%)[b]			
		Ethylenediamine	Putrescine	Spermidine	Spermine
2×10^{-2}	1	10.4 ± 1.2	11.5 ± 1.0	12.5 ± 1.1	14.1 ± 0.6
2×10^{-2}	10	33.0 ± 1.1	29.6 ± 1.0	32.6 ± 1.2	34.0 ± 1.3
2×10^{-2}	25	42.6 ± 0.8	40.9 ± 1.2	47.6 ± 1.1	47.0 ± 1.2
2×10^{-2}	50	50.9 ± 1.1	45.9 ± 1.0	48.1 ± 1.0	49.0 ± 1.1
2×10^{-2}	100	50.2 ± 1.5	49.8 ± 1.1	54.1 ± 1.3	55.6 ± 1.2

Values are mean ± SD.

[a] The molar ratio of ethylenediamine, putrescine, spermidine and spermine added to the carboxyl groups of gelatin.

[b] The molar percentage of amino residues introduced to the carboxyl groups of gelatin.

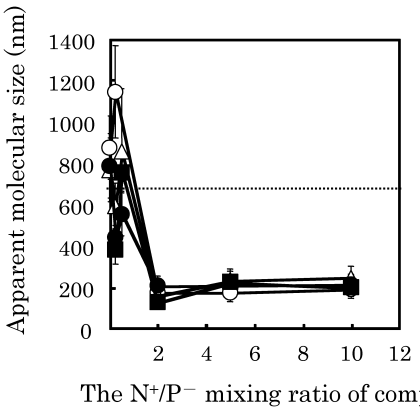

Figure 1. The apparent molecular size of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA). The amine compound used for gelatin cationization is ethylenediamine (○), putrescine (△), spermidine (■) and spermine (●). A dotted line indicates the apparent molecular size of naked plasmid DNA.

Our preliminary study revealed that the gene expression level of cationized gelatin-plasmid DNA complexes depended on the cationization extent. Based on this result, the cationization extent of 50 was used for the following experiments unless mentioned otherwise.

Figure 1 shows the apparent molecular size of cationized gelatin-plasmid DNA complexes prepared at different N^+/P^- mixing ratios. The apparent molecular size of plasmid DNA decreased by mixing with the cationized gelatin to around 200 nm, irrespective of the amine compounds introduced, when the N^+/P^- mixing ratio was 1 or higher. The apparent molecular size did not depend on the measurement angle. The zeta potential of cationized gelatin-plasmid DNA complexes prepared at different N^+/P^- mixing ratios tended to increase with increasing the N^+/P^- mixing ratio to obtain a certain zeta potential at the ratio of 2.0, irrespective of the cationized gelatin type (Fig. 2).

Figure 3 shows the electrophoretic patterns of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios. The band of plasmid DNA did not migrate for every type of cationized gelatin at the N^+/P^- mixing ratio of 1 or higher. However, the plasmid DNA band was migrated similarly to the original plasmid DNA at N^+/P^- mixing ratios of 0.5 or lower.

Figure 4 shows the fluorescence assay results of EtBr intercalation for various cationized-gelatin–plasmid DNA complexes in aqueous solution. At N^+/P^- mixing ratios of 1 or higher, a significant decrease in the percent fluorescent intensity was detected, irrespective of the cationized gelatin type. In addition, the percentage was significantly lower for the cationized gelatin of spermidine or spermine compared to ethylenediamine or putrescine.

Table 2 shows the K_d values of interaction between the plasmid DNA and various cationized gelatin. The K_d value for the cationized gelatin of spermine

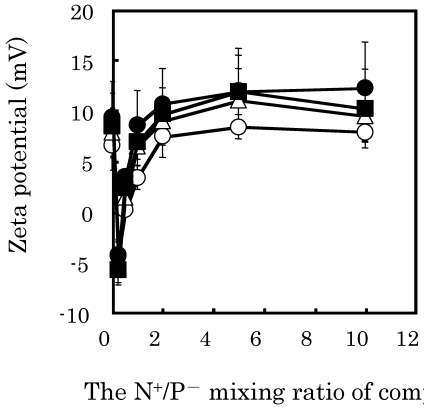

Figure 2. The zeta potential of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA). The amine compound used for gelatin cationization is ethylenediamine (○), putrescine (△), spermidine (■) and spermine (●). The zeta potential of naked plasmid DNA is -14.0 ± 2.2 mV.

was 6.4 μM, which is about 6- and 3-times lower than that of putrescine (41 μM) and ethylenediamine (20 μM), respectively.

In vitro *gene expression in L929 cells by various cationized gelatins*

Figure 5 shows the expression level of luciferase for L929 cells cultured with cationized gelatin-plasmid DNA prepared at various N^+/P^- mixing ratios. Three cationized gelatins of spermine showed higher transfection efficiency than that of other amine compounds. In addition, when the cationized gelatin of spermine was used to complex with the plasmid DNA at a N^+/P^- mixing ratio of 2, the highest transfection was achieved. However, no effect of transfection level on the N^+/P^- mixing ratio was observed for the complexes prepared from the cationized gelatin of ethylenediamine, putrescine or spermidine. Based on this result, the N^+/P^- mixing ratio was fixed at 2 for the following gene-transfection experiments. When Lipofectamine was used for transfection of plasmid DNA, the highest luciferase activity observed was 4.87×10^6 RLU/mg protein, which is significantly lower compared with that of any cationized gelatin.

Figure 6 shows the effect of N^+/P^- mixing ratio on the cytotoxicity of cationized gelatin-plasmid DNA complexes for L929 cells. Cell toxicity was observed for every complex when the N^+/P^- mixing ratio was 5 or higher, although it tended to be higher for the cationized gelatin of spermine compared with that of other cationized gelatins. When Lipofectamine was used for transfection of the plasmid DNA, the viability of cells was $72.1 \pm 5.2\%$, which is significantly higher compared with that of any cationized gelatin.

Figure 7 shows the effect of the cell density on the gene transfection of cationized gelatin derivatized with spermine–luciferase–plasmid DNA complexes for L929

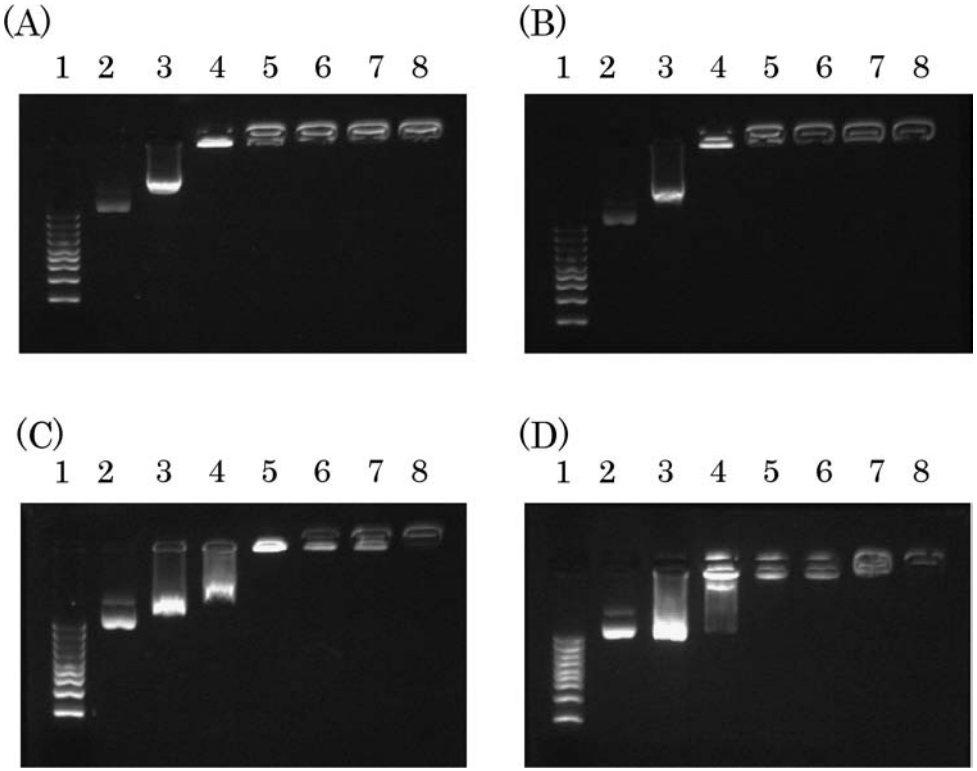

Figure 3. Electrophoretic patterns of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA). Lane 1, molecular marker; lane 2, naked plasmid DNA; lanes 3–8, plasmid DNA complexed with the cationized gelatin at N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) of 0.25, 0.5, 1, 2, 5 and 10, respectively. The amine compound used for gelatin cationization was (A) ethylenediamine, (B) putrescine, (C) spermidine and (D) spermine.

cells. The highest gene expression level was observed at a cell seeding density of 2.5×10^4 cells/well.

DISCUSSION

In the present study, the efficacy of cationized gelatin prepared from different amine compounds as a non-viral gene delivery system was evaluated.

The cationized-gelatin–plasmid DNA complex showed an apparent molecular size in the nanometer range (Fig. 1). The complex size was around 200 nm when the N^+/P^- mixing ratio was over 1, irrespective of the type of amine compound used. It has been demonstrated that the complex with this size range can be favorably taken up by cells [29, 30]. This is also an advantageous feature of the plasmid DNA complex prepared from the cationized gelatin to enhance the transfection efficiency

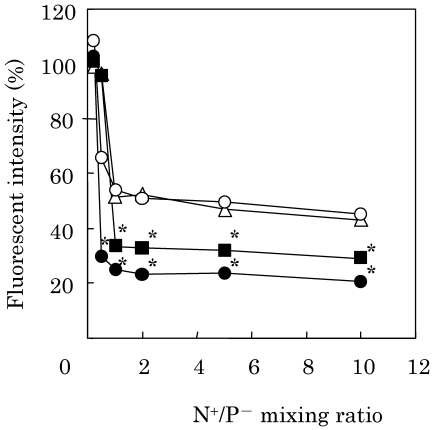

Figure 4. EtBr intercalation fluorescence assay for various cationized-gelatin–plasmid DNA complexes in aqueous solution. The fluorescence of EtBr intercalated into plasmid DNA was exclusively detected. The amine compound used for gelatin cationization is ethylenediamine (○), putrescine (△), spermidine (■) and spermine (●). The fluorescent intensity of EtBr intercalated to free plasmid DNA is defined as 100% for relative fluorescence. $*P < 0.05$, significant difference in the fluorescent intensity against the cationized gelatin prepared from ethylenediamine.

Table 2.
The K_d values of the interaction between plasmid DNA and the cationized gelatins

Amino compound	Temperature (°C)	K_d (μM)	Cationized-gelatin–plasmid DNA binding capacity (mol/mol)
Ethylenediamine	37	20.4	487.9
Putrescine	37	40.5	346.8
Spermidine	37	4.59	240.8
Spermine	37	6.35	210.4

The molar ratio of amino compound added to the carboxyl groups of gelatin was 100.

for gene expression in terms of efficient DNA condensing to a nano-order size. In addition, the surface charge of cationized-gelatin–plasmid DNA complex was tended to increase with increasing N^+/P^- mixing ratio (Fig. 2). These findings strongly suggest that the cationized-gelatin–plasmid DNA complex has a nano-size structure of which surface is covered with cationized gelatin molecules.

The gel retardation assay revealed that the cationized gelatin electrostatically interacted with the plasmid DNA. With an increase in the N^+/P^- mixing ratio, the plasmid DNA band did not migrate in electrophoresis (Fig. 3). It is possible that the negative charge of plasmid DNA is neutralized by complexation with the cationized gelatin and the molecular size of plasmid DNA increases with the cationized gelatin complexation, resulting in reduced electrophoretic migration of plasmid DNA. From the EtBr intercalation assay, relative fluorescence percentage of the complex from cationized gelatin prepared from ethylenediamine or putrescine was larger than that of spermidine or spermine (Fig. 4). The cationized gelatin prepared from

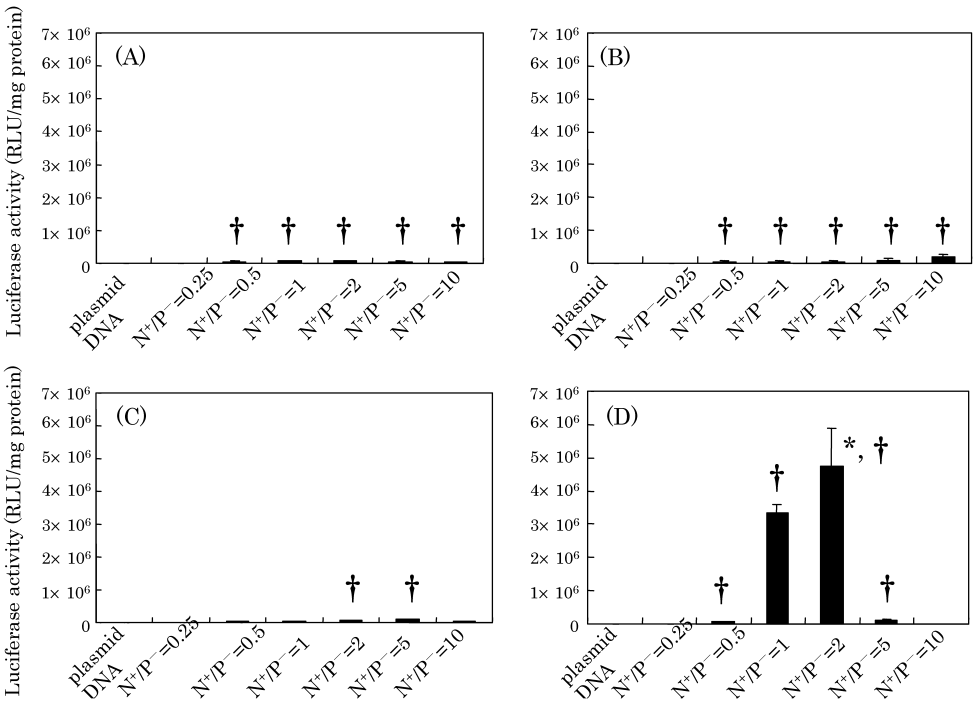

Figure 5. Effect of N^+/P^- mixing ratio (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) on the luciferase activity of L929 cells transfected by cationized-gelatins–luciferase–plasmid DNA complexes. The amine compound used for gelatin cationization was (A) ethylenediamine, (B) putrescine, (C) spermidine and (D) spermine. $*P < 0.05$, significant difference in the luciferase activity against the complexes prepared at other N^+/P^- mixing ratios; † $P < 0.05$, significant difference in the luciferase activity against free plasmid DNA.

ethylenediamine or putrescine was intercalated about 2-fold more easily by EtBr than that of spermidine or spermine at a N^+/P^- mixing ratio of 2, indicating that the affinity of the cationized gelatin prepared from ethylenediamine or putrescine for the plasmid DNA was explicitly smaller than that of spermidine or spermine. For cationic polymers, it is conceivable that electrostatic interaction with the plasmid DNA inhibits the EtBr intercalation, resulting in a large decrease in the fluorescence intensity. The Scatchard plot analysis also demonstrates that the interaction of plasmid DNA with the cationized gelatin derivatized with ethylenediamine or putrescine was weaker than that with spermidine or spermine (Table 2). This experimentally supports the result of EtBr intercalation assay.

The transfection conditions of plasmid-DNA–cationized gelatin complexes for L929 cells, such as N^+/P^- mixing ratio, the cytotoxicity and the cell density, were optimized (Figs 5–7). From these results, the cationized gelatin prepared from spermine was suitable to obtain high transfection efficiency at a N^+/P^- mixing ratio of 2 and cell density of 70%. In addition, the cationized gelatin prepared from spermine showed a large EtBr fluorescence decrease compared with that of

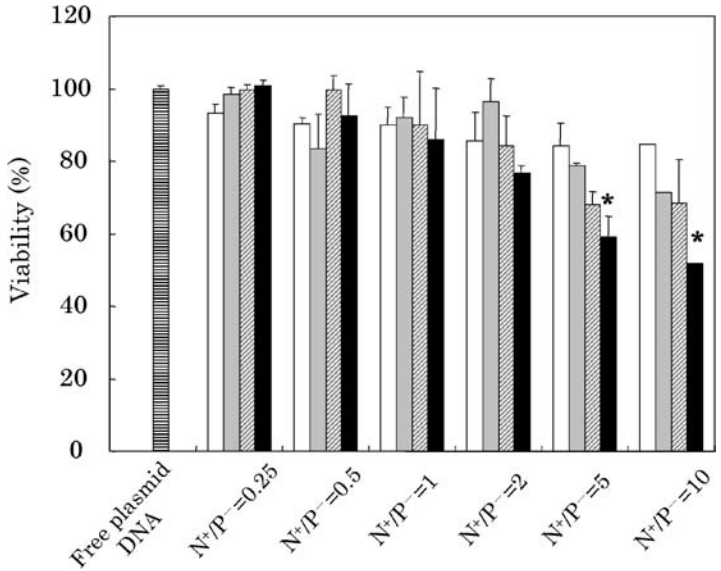

Figure 6. Effect of the N^+/P^- mixing ratio (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) on the cytotoxicity of L929 cells incubated with cationized-gelatin–luciferase–plasmid DNA complexes. The amine compound used for gelatin cationization was ethylenediamine (□), putrescine (▩), spermidine (▨) and spermine (■).

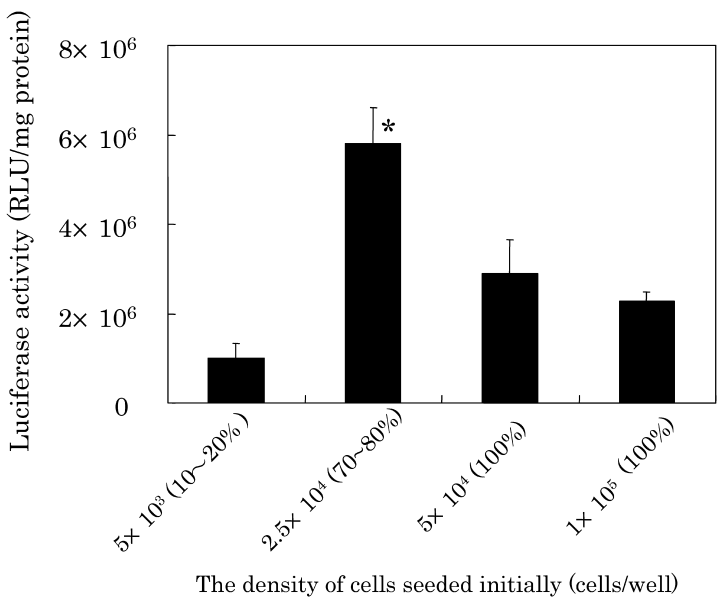

Figure 7. Effect of the cell density on the gene expression of the cationized gelatin derivatized with spermine–luciferase plasmid DNA complex prepared at a N^+/P^- mixing ratio of 2 for L929 cells. Cells were cultured for 24 h before complex addition, while the apparent cell confluency is indicated as the number in parentheses. $*P < 0.05$, significant difference in the luciferase activity against other groups at the corresponding N^+/P^- mixing ratio.

other amine compounds (Fig. 4). The lowest intensity for the cationized gelatin prepared from spermine was observed at a N^+/P^- mixing ratio of 2, indicating the sufficiently interaction with the DNA. The luciferase expression enhancement was higher for the cationized gelatin prepared from spermine than that of other amine compounds (Fig. 5). This can be explained in terms of the buffering capacity of complexes in addition to their molecular size and charge. Our previous study with the same four types of cationized gelatins revealed that the cationized gelatin prepared from spermine possessed the highest buffering effect among all the cationized gelatins used [31]. Both the condensed structure and a net positive charge of cationized-polymer–plasmid DNA complexes have been reported to be key for gene transfection [32]. However, as the N^+/P^- mixing ratio of cationized gelatin prepared from spermine increased, the viability of transfected cells decreased (Fig. 6). There was no difference in the molecular size and charge of complexes between the cationized gelatins of spermine and other amines (Figs 1 and 2). The reason of higher cytotoxicity for spermine-introduced cationized gelatin is not clear at present.

In conclusion, the plasmid DNA was complexed with cationized gelatin prepared from spermine and the *in vitro* expression level of plasmid DNA complexed increased significantly compared with that prepared from other amine compounds at optimal conditions determined. Plasmid DNA is a macromolecule with a negative charge, irrespective of the type of the coded protein. Therefore, it is practically possible to think of the plasmid DNA as one type of biological substance with the similar nature of charge, an anionic macromolecule. This cationized gelatin is being applied as the non-viral carrier for the plasmid DNA of bioactive molecules like growth factor to demonstrate enhancement of the *ex vivo* biological functions at present.

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