In Vitro Transfection of Plasmid DNA by Amine Derivatives of Gelatin Accompanied with Ultrasound Irradiation

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Purpose. The purpose of this study is to examine the ultrasound (US)-enhanced gene expression by the complexes of a plasmid DNA with gelatin derivatives of aminization.

Methods. Gelatin derivatives with different introduced extents of ethylenediamine (Ed), spermidine (Sd), and spermine (Sm) were prepared with a water-soluble carbodiimide. The molecular size and zeta potential of the gelatin derivatives before and after complexation with the plasmid DNA were examined. After incubation with the complexes with or without US exposure, the DNA expression of rat gastric mucosal cells was measured to evaluate the effect of the type of gelatin derivatives on their gene expression. The cell uptake of the complexes, the cell viability, and the buffering effect of gelatin derivatives were examined.

Results. The apparent molecular size and zeta potential of gelatin derivatives became larger as their aminization extent increased although the Sm gelatin derivative of higher aminization showed a larger value than other corresponding derivatives. Irrespective of the type of gelatin derivatives, the apparent molecular size of plasmid DNA was reduced by increasing the gelatin-DNA mixing ratio to attain a saturated value of about 150 nm. The condensed gelatin-DNA complexes showed the zeta potential of 10–15 mV. The cells incubated with the complex exhibited significantly stronger luciferase activities than free plasmid DNA, and the activity was further enhanced by US irradiation. The enhancement was significant for the Sm derivative compared with the corresponding Ed and Sd derivatives. The amount of plasmid DNA internalized into the cells was significantly increased by the complexation with every gelatin derivative, whereas US irradiation did not significantly increase the DNA internalization. US irradiation had no effect on the viability of cells incubated with every gelatin derivative-plasmid DNA complex, although the viability was decreased by the complex incubation. The buffering capacity of Sm derivative was higher than that of Ed and Sd derivatives and comparable with that of polyethylene amine.

Conclusion. Among amine derivatives of gelatin, the Sm derivative enabled the plasmid DNA to induce the US-enhanced gene expression of cells *in vitro* most effectively because of the superior buffering effect.

KEY WORDS: plasmid DNA; *in vitro* transfection; amine derivatives of gelatin; ultrasound; buffering capacity.

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INTRODUCTION

Recently, a number of delivery systems of DNA were investigated to improve the efficacy of gene therapy (1). They are designed to introduce genes into eukaryotic cells aiming at the efficient stimulation of protein production for genetic marking and gene therapy or replacement (2,3). It is no doubt that success of *in vitro* and *in vivo* gene therapy greatly depends on the delivery system of genes. There have been two major approaches proposed for gene delivery: the viral-mediated and non-viral-mediated gene transfer (4). However, from the viewpoint of immunologic and safety issues of viral vectors, necessity in the development of non-viral vector systems has been increasingly magnified. Several attempts for the vector development have been made with cationic lipids and macromolecules (5–8). Generally, because plasmid DNA is a large, negatively charged molecule up to 1 μm long (9), it is impossible to make the plasmid DNA to internalize into cells after the attachment onto the negatively charged cell membrane. The cationic vectors all ionically complex the plasmid DNA of negative charge, which enables the plasmid DNA to condense the molecular size and to give it a positive charge (6). This complexation enhances the *in vitro* cell uptake of plasmid DNA, resulting in promoted gene transfection. However, the extent is usually much less than that by the viral vector system.

There are some research strategies to enhance the transfection efficiency of plasmid DNA. Among them are to modify the uptake process of plasmid DNA into cells by external physical stimuli. So far, electroporation has been applied to modify the gene transfection of plasmid DNA and found to be effective in enhancing the efficiency *in vitro* and *in vivo* (10). However, because the electric power to be applied is so high, the problematic cytotoxicity and tissue damage are sometimes observed. As another stimulus, ultrasound (US) irradiation is being noted recently (11,12). Indeed, it has been shown that the transfection efficiency of plasmid DNA *in vitro* and *in vivo* was enhanced by the US irradiation (12). US irradiation in the presence of a plasmid DNA enables cells cultured *in vitro* to show the enhanced gene expression, irrespective of the cell type, such as plant and animal cells (12,13–16). When applied to some cells, followed by the subsequent US irradiation, the plasmid DNA complexed with a cationized liposome exhibited promoted gene expression *in vitro* to a significantly higher extent than that without US irradiation (17). We recently showed that US exposure was effective in enhancing the gene expression of a plasmid DNA even when the plasmid given was being complexed with a cationized gelatin prepared by ethylene diamine introduction (18). However, there are some trials that introduced other molecules, such as spermine, to another gene carrier (6).

In this study, gelatin was chemically modified with ethylene diamine (Ed), spermidine (Sd), and spermine (Sm) to prepare gelatin derivatives of cationic charge with different extents of aminization. Gelatin was selected because its biosafety has been proved through long medical and pharmaceutical usage in clinics. The different gelatin derivatives were mixed with a plasmid DNA-encoding luciferase in aqueous solution to prepare various gelatin derivative-plasmid DNA complexes. After rat gastric mucosal cells were incubated

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with the complexes and subsequently exposed to US, their gene expression was compared with that without US irradiation to evaluate the effect of the US irradiation and the type of gelatin derivatives on the gene expression. We also examine internalization of the gelatin derivative-plasmid DNA complexes into the cells and the cell viability with or without US irradiation as well as the buffering effect of gelatin derivatives.

MATERIALS AND METHODS

Materials

Gelatin, prepared through an acid process of pig skin, type I collagen, was kindly supplied from Nitta Gelatin Co., Osaka, Japan. Ethylenediamine (Ed), 2,4,6-trinitrobezenesulfonic acid (TNBS), β-alanine, and protein assay kit (lot no. L8900) were purchased from Nakalai Tesque Inc. (Kyoto, Japan) and were used as obtained. As a coupling agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiamide hydrochloride salt (EDC) and DNA MW Standard Marker (1-kb DNA ladder) were obtained from Dojindo Laboratories, Kumamoto and Takara Shuzo Co. Ltd., Shiga, Japan, respectively. Spermidine (Sd) and spermine (Sm) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were used as obtained. Rodamine B isothiocyanate (RITC) was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan).

Preparation of Gelatin Derivatives of Ed, Sd, and Sm and Their Complexation with Plasmid DNA

Gelatin derivatives with different extents of aminization were prepared by introduction of Ed, Sd, and Sm into the carboxyl groups of gelatin based on the conventional of EDC method (19). Briefly, Ed, Sd, and Sm, together with 21.39 mg of EDC, were added into 2200 mL of double-distilled water (DDW) containing 44 mg of gelatin at different molar ratios of the amine compounds to the carboxyl groups of gelatin, followed by immediate adjustment of the solution pH at 5.0 by HCl addition. The reaction mixture was agitated at 37°C for 18 h and then dialyzed against DDW for 48 h at 25°C. The dialyzed solutions were freeze-dried to obtain powdered different gelatin derivatives. The percentage of amino groups introduced into the carboxyl groups of gelatin (the aminization of gelatin) was determined by the TNBS method (20) based on the calibration curve prepared by using β-alanine.

The plasmid DNA used is the DNA construct (12.5-kb DNA), which contains a cytomegalovirus (CMV) promoter inserted at the upstream region of sequence coding the firefly (Photinus Pyralis) luciferase. The plasmid DNA was amplified in an *E. coli* bacteria transformant and isolated from the bacteria by Qiagen Maxi kit-25 (Qiagen K.K., Tokyo, Japan). The absorbance ratio at the wavelength of 260 to 280 nm for purity assessment of plasmid DNA obtained was measured to be between 1.8 and 2.0.

Complexation of the gelatin derivatives with the plasmid DNA was performed by simply mixing the two materials at various mixing weight ratios in aqueous solution. Briefly, 150 μL of 0.1 M phosphate-buffered saline solution (PBS, pH 7.4) containing 2.5, 5, 10, 25, 50, and 100 μg of gelatin derivatives was added to the same volume of PBS containing 10 μg of plasmid DNA. The solution was gently agitated at 37°C for 30 min to form gelatin derivatives-plasmid DNA complexes.

Electrophoresis of Gelatin Derivatives-Plasmid DNA Complexes

Various gelatin derivatives-plasmid DNA complexes were prepared at various mixing weight ratios of gelatin derivatives to plasmid DNA (0.1 μg) according to the same procedure as described above. The complex samples were electrophoresed for 40 min at 100 V in 0.75 wt % of agarose gel by 45 mM Tris-Borate and 1 mM EDTA buffer (pH 8.0). The gel was stained with 0.5 mg/mL ethidium bromide solution for 30 min to visualize the location of plasmid DNA with a Gel Doc 2000 (Bio-Rad Laboratories, Tokyo, Japan).

Measurement of Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS)

For complex preparation, PBS containing 25 mg of gelatin derivatives (2.5 mL) was mixed with 2.5 mL of PBS containing 5 mg of plasmid DNA. The mixed gelatin derivative and plasmid DNA solution was subjected to DLS measurement on a DLS-DPA-60HD instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) equipped with an Ar^+ laser at a detection angle of 90° at 37°C for 30 min and performed three times for every sample. The corresponding hydrodynamic radius, R_s, can be calculated from Einstein-Stokes' equation: $R_s = kT/3\pi\eta D$, where k is the Boltzman constant, T is the absolute temperature, η is the solvent viscosity, and D is translational diffusion coefficient obtained from the DLS measurements. In the present study, the autocorrelation function of samples was analyzed on the basis of the cumulants method, and the R_s value was automatically calculated by the equipped computer software and expressed as the apparent molecular size of samples. ELS measurement was performed on an ELS-7000AS instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) for the mixed gelatin derivative and plasmid DNA solution at 37°C and an electric field strength of 100 V/cm. The ELS measurement was done three times for every sample.

The zeta potential (ζ) was automatically calculated by using the Smoluchouski equation based on the elecrtrophoretic mobility measured u:$\zeta = 4\pi\eta u/\varepsilon$, where η and ε are the viscosity and the dielectric constant of the solvent, respectively.

Transfection Experiment by Gelatin Derivatives-Plasmid DNA Complexes

Rat gastric mucosal (RGM)-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, lot no. 1073750; Gibco BRL, Life Technology Inc., NY, USA) supplemented with 10 wt % fetal calf serum, 0.12 wt % sodium bicarbonate, and 100 units/mL mixed penicilline-streptomycin solution. The cell suspension (1×10^5 cells/2 mL) was plated into each well of six-well multi-well culture plates (Code 3800-6100, Iwaki brand, Scitech Div.; Asahi Techno Glass Corp., Chiba, Japan) and cultured at 37°C for 1 day in a 95% air-5% CO_2 atmosphere to reach the cell confluency of about 70%. After the gelatin derivatives-plasmid DNA complexes were added to each well, followed by incubation for 1 h, the cells were exposed to US by an US machine (Intertron R 6100 US,

Nihon Medix; Ultrasound Williams Health Care Systems Ltd., Kyoto, Japan) for 60 s at the frequency of 3 MHz, the intensity of 1 W/cm^2, and the duty cycle of 10%. The head of the US transducer was inserted into a sterile bag, whereas the top portion of the bag was filled with an US gel (Aquastic R100, Ultrasound Transmission Gel; Parker Laboratories Inc., NJ, USA) for complete US coupling. The transducer was fixed by a stand cramp at the distance of 5 mm between the head and the cell monolayer surface. The culture plate was placed on a polystyrene foam mat to reduce the reflection of US waves in a sterile air cabinet at room temperature. During insonation, the temperature of the transducer head ranged from 26 to 30°C. Each experimental group was performed for three wells. As controls, the cells were only incubated with the complex, and US irradiation was or not performed to the cells incubated with the cultured medium alone or that containing free plasmid DNA or the original gelatin-plasmid DNA complex. After incubation for 4 h, the culture medium was exchanged to exclude the gelatin derivative, free plasmid DNA, and gelatin derivative-plasmid DNA complexes added. Then, the cells were incubated for an additional 48 h, washed twice with 1 mL of PBS, and lysed by 100 μL of a lysis buffer (Luciferase Assay System, Cat# E 1500; Promega Co., WI, USA). The cell lysate was centrifuged at 12,000 rpm for 5 s at 4°C, and the supernatant was carefully collected and kept in the ice. The supernatant sample (16 μL) was mixed with 80 μL of a reconstituted luciferase assay solution (Lucifearse Assay System, Cat# E 1500; Promega Co.), and the relative light unit (RLU) of the solution mixture was determined by a luminometer (Lumat Lb 9507, Wallac-Berthold Inc., Bad Wildban, Germany). The protein concentration of the lysate was also assayed by the Lowry kit (lot. no. L8900; Nakalai Tesque Inc., Kyoto, Japan). Briefly, 50 μL of lysate was mixed with the 1 mL of the copper solution, followed by leaving for 10 min at 25°C. After addition of 0.1 mL of 1 N phenol aqueous solution, the solution mixture was incubated for 30 min at 25°C and the absorbance was determined at the wavelength of 750 nm. The protein concentration was calculated on the basis of the calibration curve prepared with use of a standard albumin solution.

Evaluation of Cell Survival

The gelatin derivative-plasmid DNA complexes were added to RGM-1 cells at the confluency of 70%. After a 1-h incubation, the cells were exposed to US for 60 s at the intensity of 1 W/cm^2, the frequency of 3 MHz, and the duty cycles of 10%, followed by further incubation for 48 h Then, the cells were detached by trypsinization, and the number of cells was counted with use of a hemocytometer to calculate the number percentage to the cells incubated without the complex nor the subsequent US exposure (percent survival). Each experiment was performed independently for three wells. As controls, US irradiation was or not performed in cells incubated in the cultured medium with or without free plasmid DNA.

Internalization Assay of Gelatin Derivatives-Plasmid-DNA Complexes into Cells

For the fluorescent labeling of plasmid DNA, the pCMV-Luciferase and RITC were mixed in 0.2 M of sodium carbonate-buffered solution (pH 9.7) at 4°C for 12 h at both the concentrations of 1 mg/mL. The residual RITC was separated by gel filtration of a PD 10 column (Amersham Pharmacia Biotech K.K., Tokyo, Japan) and the RITC-labeled pCMV-luciferase was obtained by ethanol precipitation. According to the procedure mentioned above, different gelatin derivatives were mixed with the RITC-labeled pCMV-luciferase in PBS at the gelatin-DNA weight mixing ratio of 5 to prepare various complexes. The gelatin derivative-RITC-labeled plasmid DNA complex was added to each well with RGM-1 cells grown at the 70% confluency. After incubation for 1 h, the cells were exposed to US at the frequency of 3 MHz, the intensity of 1 W/cm^2, and the duty cycle of 10% for 60 s or not. As controls, after a 1-h incubation in the cultured medium with or without RITC-labeled-plasmid DNA, the cells were exposed to US or not under the same conditions. The cells were incubated further for 48 h Then, they were washed carefully three times with 1 mL of PBS to exclude the fluorescent agents added and lysed by 500 μL of a lysis buffer (Luciferase Assay System, Cat# E 1500; Promega Co.). The fluorescent intensity of cell lysates was measured by a fluorescent spectrophotometer (F-2000 Fluorescent Spectrophotometer, Hitachi Ltd., Tokyo, Japan, Ex 570 nm/Em 595 nm) and divided by that initially added to obtain the percent internalized. Each experiment was performed independently for six wells.

Evaluation of Buffering Capacity of Gelatin Derivatives

An aqueous solution containing gelatin derivatives and branched polyethylenimine (PEI) with the molecular weight of 25,000 as a control sample was prepared, and the solution pH was adjusted around 8.0 by 0.1 M NaOH. The resulting solution was titrated by stepwise addition of 0.01 N HCl solution (0.2 mL). The solution pH was measured by Horiba D-22 pH meter (Horiba Ltd., Kyoto, Japan). The experiment was performed independently three times to obtain the average pH value.

Statistical Analysis

All the data were statistically analyzed to express the means ± SD of the mean. Student's *t* test was performed, and $p < 0.05$ was accepted as significance level.

RESULTS

Preparation and Characterization of Gelatin Derivatives

The percentage of Ed, Sd, and Sm residues into the carboxyl groups of gelatin increased with an increase in the amount of respective diamine reagents added up to around 48 mol % per the carboxyl groups of gelatin when the added amount was 50 mol % per the carboxyl groups of gelatin and thereafter leveled off. Our previous study showed that the Ed gelatin derivative prepared at the Ed addition molar % of 50 (Ed-50) was the most effective in enhancing the gene transfection of a plasmid DNA among Ed derivatives with other percentages (21). Based on this result, the gelatin derivatives prepared at the addition molar percentage of 50 were selected. The percentages of Ed, Sd, and Sm residues of Ed-50, Sd-50, and Sm-50 gelatin derivatives used here were 47.8, 48.1, and 49.0 mol % per the carboxyl groups of gelatin, respectively.

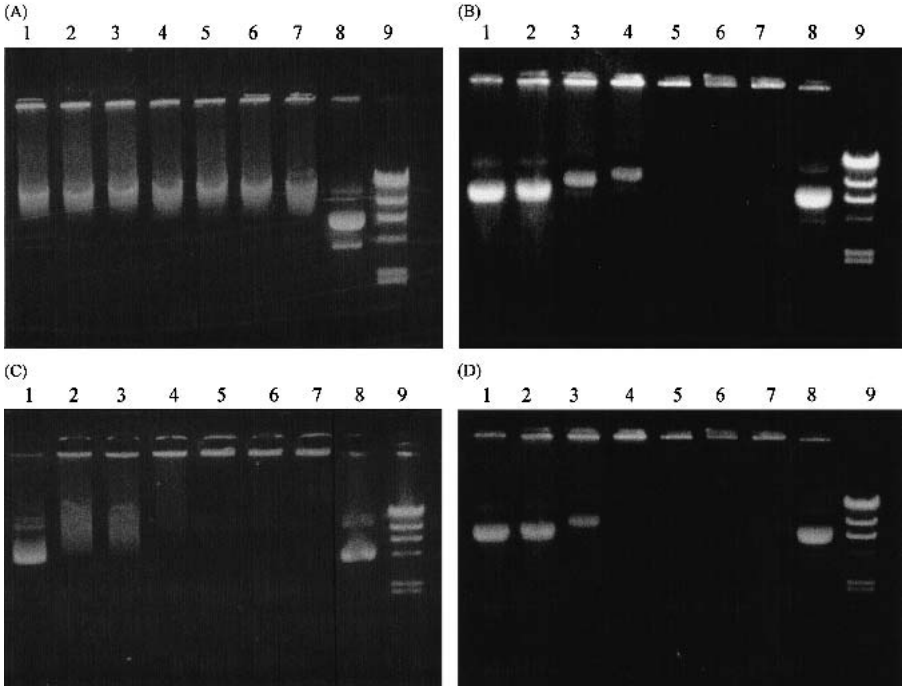

Fig. 1. Electrophoretic patterns of various plasmid DNA complexes with original gelatin and gelatin derivatives of ethylenediamnie, spermidine, and spermine prepared at different mixing weight ratios of gelatin-DNA: (A) Original gelatin; (B) Ed-50; (C) Sd-50; and (D) Sm-50. Lanes 1–7: 0.25, 0.5, 1.0, 1.25, 2.5, 5.0, and 10.0 of the gelatin-DNA mixing ratios; Lane 8: free plasmid DNA; and Lane 9: DNA marker.

The DLS and ELS experiments of gelatin derivatives indicated that the molecular size of the Sm gelatin derivative slightly was larger than the corresponding derivatives when compared at the similar percentage of Ed, Sd, and Sm residues. Irrespective of the diamine type used for derivatization, the zeta potential of gelatin derivatives increased with the increased percentage of amine introduced. The zeta potential of Sm-50 derivatives was significantly higher than that of Ed-50 and Sd-50 derivatives (data not shown).

Characterization of Gelatin Derivatives-Plasmid DNA Complexes

Figure 1 shows the electrophoretic patterns of gelatin derivatives-plasmid DNA complexes prepared at different mixing weight ratios of gelatin-DNA. Irrespective of the derivative type, migration of the plasmid DNA was retarded with an increase in the ratio, but not observed any more at the ratio higher than a certain value. The Sm gelatin derivative enabled the plasmid DNA to firmly prevent the electrophoretic migration at molar ratios lower than the Ed and Sd gelatin derivatives. The original gelatin without any aminization modified the migration pattern of free plasmid DNA but did not achieve the migration prevention of plasmid DNA for every mixing ratio. Figures 2 and 3 show the apparent molecular size and surface charge of gelatin derivatives-plasmid DNA complexes prepared at different mixing weight ratios of gelatin-DNA. The apparent molecular size of plasmid DNA changed with mixing of the gelatin derivative and decreased with the increased mixing ratio, irrespective of the gelatin type. Free plasmid DNA exhibited a negative zeta potential. However, mixing with the gelatin derivative changed it to positive values. The value tended to increase as the mixing weight ratio increased, although the increased extent was somewhat high for the Sm gelatin derivative compared with that of the Ed and Sd gelatin derivatives.

Transfection of Different Gelatin Derivatives-Plasmid DNA Complexes with or without US Irradiation

Figure 4 shows the effect of the gelatin-DNA mixing weight ratio on the luciferase activity of RGM-1 cells treated with gelatin derivatives-plasmid DNA complexes or other agents with or without US irradiation. Irrespective of the gelatin-DNA mixing ratio and US irradiation, the luciferase

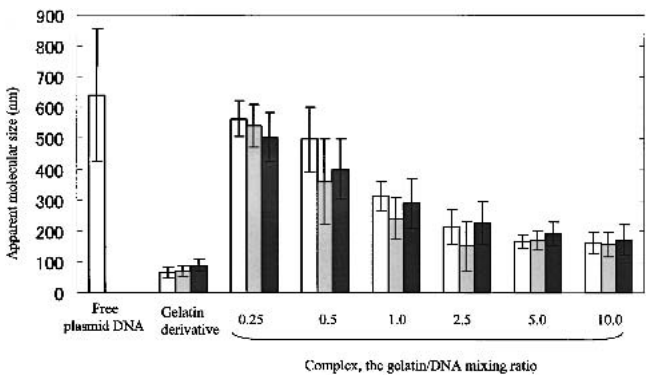

Fig. 2. Apparent molecular size of free plasmid DNA, gelatin derivatives of ethylenediamnie, spermidine, and spermine, and gelatin derivative-plasmid DNA complexes prepared at different mixing weight ratios of gelatin-DNA: Ed-50 (□), Sd-50 (▩), and Sm-50 (■). The concentration of plasmid DNA used is 2 mg/mL.

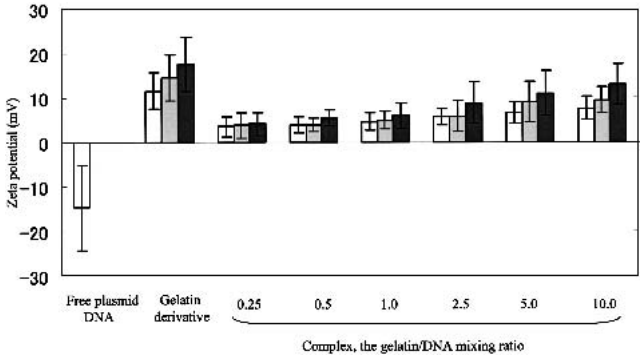

Fig. 3. Zeta potential of free plasmid DNA, gelatin derivatives of ethylenediamnie, spermidine, and spermine, and gelatin derivative-plasmid DNA complexes prepared at different mixing weight ratios of gelatin-DNA: Ed-50 (□), Sd-50 (■), and Sm-50 (■). The concentration of plasmid DNA used is 2 mg/mL.

activity was enhanced by the incubation of any gelatin derivative-plasmid DNA complex to a significantly higher extent than that of free plasmid DNA. The activity enhancement was higher for the Sm derivative complex than that of the corresponding other complexes, whereas it enhanced with an increase in the mixing ratio. US irradiation enabled both free plasmid DNA and the complexes to enhance their luciferase activity, although the extent was less for the former than the latter. US irradiation increased the luciferase activity of cells incubated with every complex, and the enhanced effect was saturated at the mixing ratio of 5.0. The gelatin derivative alone with or without US irradiation was not effective in the activity enhancement, and the level was similar to that of PBS treatment.

Figure 5 shows the luciferase activity after US irradiation at different time intervals after application of the gelatin derivatives-plasmid DNA complexes. The enhanced activity was achieved when the time interval was prolonged $\geq$ 10 min, although the longer time period did not contribute to the activity enhancement.

Viability of Cells Incubated with Gelatin Derivatives-Plasmid DNA Complexes with or without US Irradiation

Figure 6 shows the viability of cells incubated with gelatin derivatives-plasmid DNA complexes with or without US irradiation. Irrespective of the gelatin type, the cell viability decreased through treatment with the gelatin derivative-plasmid DNA complex, whereas US irradiation did not affect the cell viability.

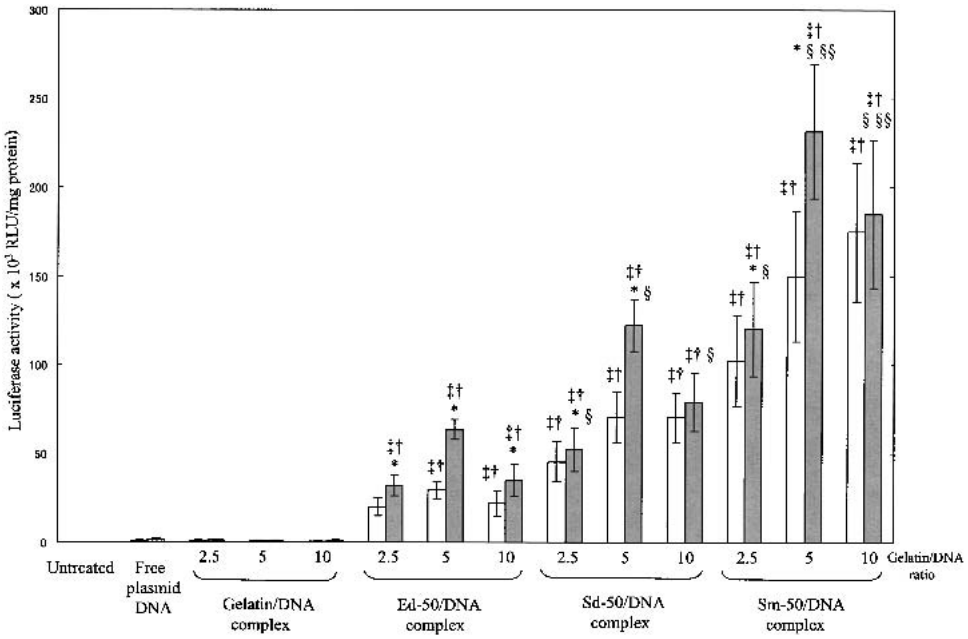

Fig. 4. Luciferase activity of RGM-1 cells 48 h after treatment of free plasmid DNA, gelatin-plasmid DNA complex, and gelatin derivative-plasmid DNA complexes with (■) or without US irradiation (□). The dose of plasmid DNA is 0.1 mg/mL, and the mixing weight ratios of gelatin-DNA is 2.5, 5.0, and 10. The gelatin derivatives used are Ed-50, Sd-50, and Sm-50. The US irradiation was performed 10 min after Sm-50 gelatin-plasmid DNA complex, 30 min after Sd-50 gelatin-plasmid DNA complex, and 60 min after Ed-50 gelatin-plasmid DNA complex application (irradiation time periods = 60 s, intensity = 1 W/cm^2, frequency = 3 MHz, and duty cycle = 10%). *$p < 0.05$; significant against the activity of RGM-1 cells after treatment of the corresponding gelatin derivative-plasmid DNA complex without US irradiation. †$p < 0.05$; significant against the activity of RGM-1 cells after treatment of free plasmid DNA without US irradiation. ‡$p < 0.05$; significant against the activity of RGM-1 cells after treatment of free plasmid DNA with US irradiation. $^{§}p < 0.05$; significant against the activity of RGM-1 cells after treatment of the Ed-50 gelatin-plasmid DNA complex at the corresponding gelatin/DNA ratio and US irradiation. $^{§§}p < 0.05$; significant against the activity of RGM-1 cells after treatment of the Sd-50 gelatin-plasmid DNA complex at the corresponding gelatin-DNA ratio and US irradiation.

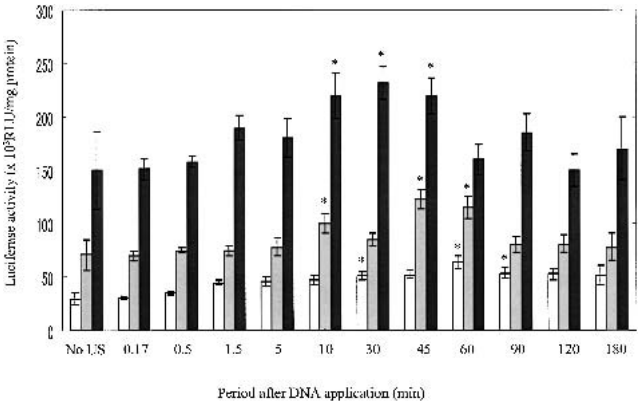

Fig. 5. Luciferase activity of RGM-1 cells 48 h after treatment of gelatin derivative-plasmid DNA complexes: Ed-50/DNA complex (□), Sd-50/DNA complex (■), and Sm-50/DNA complex (▩). The dose of plasmid DNA is 0.1 mg/mL, and the mixing weight ratio of gelatin-DNA is 5.0. The US irradiation was performed at different time periods after DNA application (irradiation time period = 60 s, intensity = 1 W/cm^2, frequency = 3 MHz, and duty cycle = 10%). *$p < 0.05$; significant against the activity of RGM-1 cells after treatment of the corresponding gelatin derivative-plasmid DNA complex without US irradiation.

Cell Uptake of Plasmid DNA

Figure 7 shows the percent internalized of gelatin derivatives-plasmid DNA complexes or other agents into RGM-1 cells with or without US irradiation. The internalization of plasmid DNA into cells was significantly enhanced by the complexation with every gelatin derivative, irrespective of US irradiation. The internalization of the complexes was not further increased by US irradiation. The internalization level of original gelatin-plasmid DNA with or without US irradiation was similar to that of free plasmid DNA.

Buffering Capacity of Gelatin Derivatives

Figure 8 shows the buffering capacity of different gelatin derivatives and PEI. The shape of titration curve for Ed and Sd gelatin derivatives was similar to that of original gelatin without any aminization. On the contrary, the Sm gelatin derivative exhibited the titration curve similar to that of PEI, and the buffering pH range was wider than that of other derivatives.

DISCUSSION

The present study undoubtedly shows that US irradiation significantly enhanced the gene expression of cells *in vitro* treated with different gelatin derivatives-plasmid DNA complexes. The expression enhancement depended on the type of gelatin derivatives used and was higher for the Sm gelatin derivative than Ed and Sd derivatives. All the gelatin derivatives were electrostatically interacted with the plasmid DNA (Fig. 1). Retarded or no electrophoretic migration of plasmid DNA complexed with the gelatin derivative is ascribed to the neutralization of DNA negative charge and the positive charge. No electrophoretic migration of Sm gelatin derivative-plasmid DNA complex at the lower gelatin-DNA ratio can be explained by the higher zeta potential of the derivative than other derivatives. Aminization gave gelatin positive charges, irrespective of the type of amine compounds used (Fig. 1). Because the Sm has more amino groups in one molecule than the Ed and Sd, the introduction enables gelatin to increase the positive charge on comparing at the same extent of aminization. It is possible that this results in larger molecu-

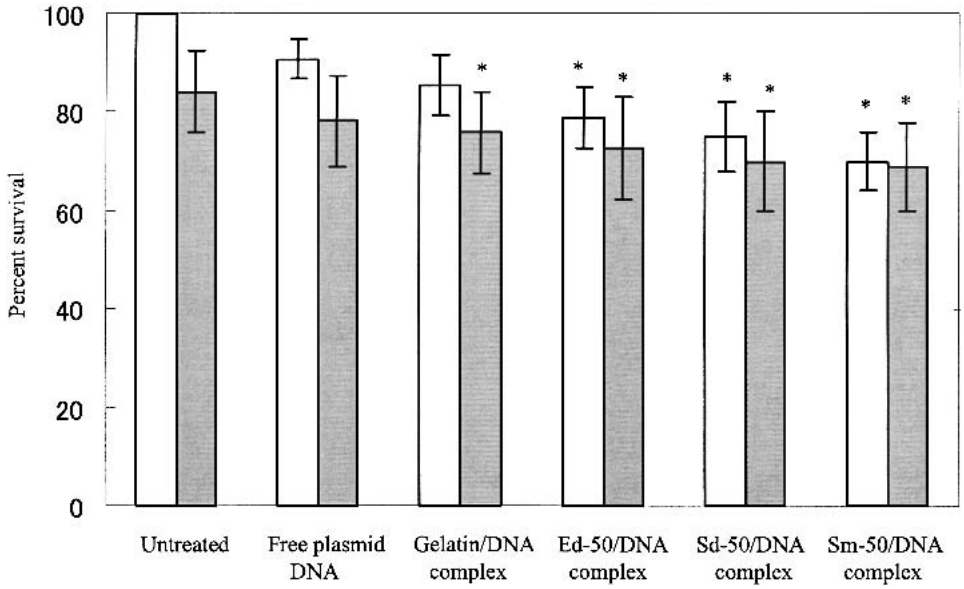

Fig. 6. Survival of RGM-1 cells 48 h after treatment of free plasmid DNA, gelatin-plasmid DNA complex, and gelatin derivative-plasmid DNA complexes with (▩) or without US irradiation (□). The gelatin derivatives used are Ed-50, Sd-50, and Sm-50 (irradiation time period = 60 s, intensity = 1 W/cm^2, frequency = 3 MHz, and duty cycle = 10%). The dose of plasmid DNA is 0.1 mg/mL, and the mixing weight ratio of gelatin-DNA is 5.0. The percent survival is defined as 100% when RGM-1 cells are incubated in the absence of free plasmid DNA and the complex without US irradiation. *$p < 0.05$; significant against the survival percentage of untreated RGM-1 cells without US irradiation.

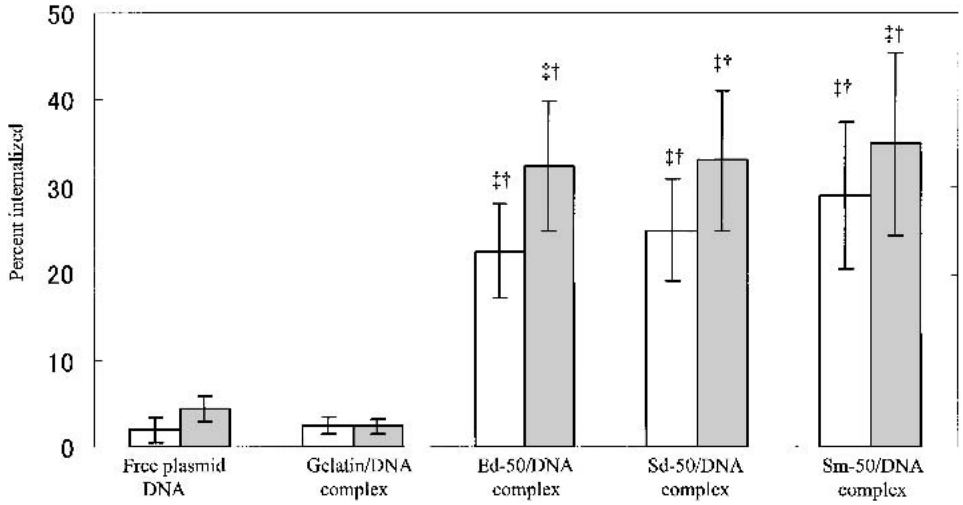

Fig. 7. Internalization of free plasmid DNA, gelatin-plasmid DNA complex, and gelatin derivative-plasmid DNA complexes with (■) or without US irradiation (□). The dose of plasmid DNA is 0.1 mg/mL, and the mixing weight ratio of gelatin-DNA is 5.0. The gelatin derivatives used are Ed-50, Sd-50, and Sm-50. The US irradiation was performed 60 min after DNA application (irradiation time period = 60 s, intensity = 1 W/cm^2, frequency = 3 MHz, and duty cycle = 10%). †$p < 0.05$; significant against the internalization percentage of free plasmid DNA without US irradiation. ‡$p < 0.05$; significant against the internalization percentage of free plasmid DNA with US irradiation.

lar size of the Sm gelatin derivative, because of the stronger electric repulsion. It is likely that electrostatic interaction with the positively charged gelatin enabled plasmid DNA of a negative charge to electrically shield the intramolecular repulsion, resulting in formation of condensed complex between the two molecules (Fig. 2). However, the condensation effect on the plasmid DNA was similar among the gelatin derivatives. This is because the molecular size of amine compounds is not large enough to affect that of complexes. The higher zeta potential of Sm gelatin derivative complexes is due to the higher positive charge (Fig. 3).

Complexation enabled the plasmid DNA to significantly enhance the gene expression. The enhanced effect depended on the gelatin-DNA mixing ratio and was the highest for the Sm gelatin derivative (Fig. 4). This is explained from the viewpoint of the molecular size and charge of complexes and their buffering capacity. It is no doubt that the electrostatic interaction between the cell membrane of negative charge and the

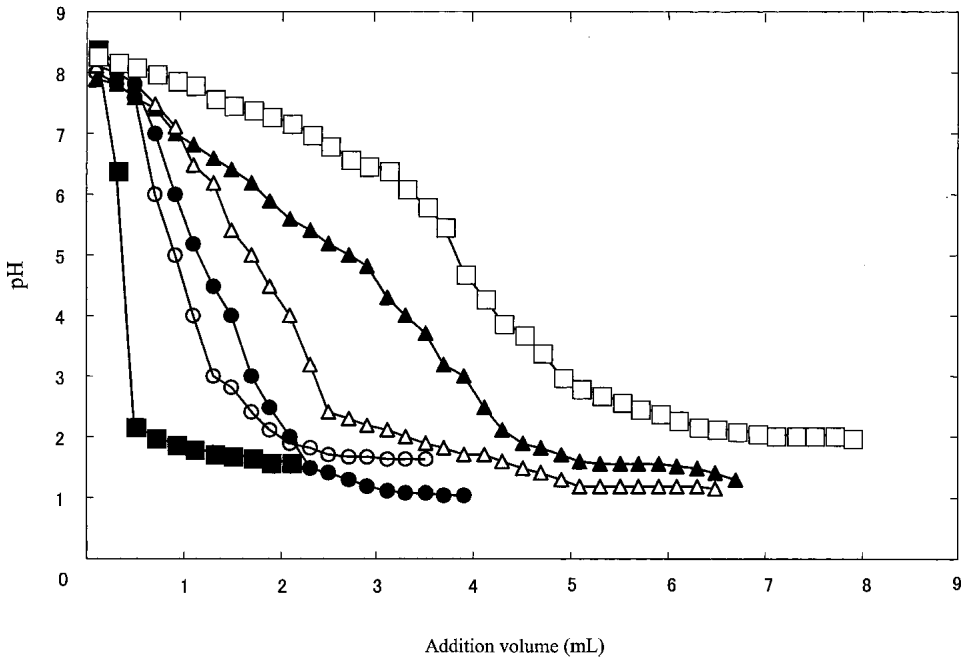

Fig. 8. Buffering capacity of gelatin and gelatin derivatives of ethylenediamnie, spermidine, and spermine in DDW at 25°C: original gelatin (○), Ed-50 (●), Sd-50 (△, Sm-50 (▲), PEI (□), and no substance (■).

gelatin derivative-plasmid DNA complex of positive charge is a driving force for the complex attachment to cells, which is an important initial step for gene transfection. At the lower mixing ratio, the complex size would not be small enough to be taken up by cells, although the zeta potential of complex is positive. With the increased mixing ratio, the complex size decreased, which facilitates the cell uptake of complexes in addition to their attachment to the cells due to the positive charge. The cell uptake of plasmid DNA was enhanced by the complexation (Fig. 7). Both the condensed formation and a net positive charge of cationized polymer-plasmid DNA complexes have been reported to be key for gene transfection (9). On the other hand, it has been experimentally demonstrated that the buffering capacity of plasmid DNA vectors plays an important role in the gene transfection (22–24). It is well known that PEI is a representative nonviral vector with a buffering effect (22). The Sm gelatin derivative possessed higher buffering than other gelatin derivatives, and the effect was comparable with that of PEI (Fig. 8). The similarity in the molecular structure between the Sm and PEI may be one of the reasons why their buffering capacity is comparable.

For the compounds, there are many residues that can be protonable at a physiologic pH. It is likely that the protonable residues act as an endosomal buffering system, resulting in protecting of plasmid DNA from degradation of lysosomal enzymes. The lysosomal enzymes are probably not activated in digestion activity because the pH inside the phagolysosome does not decrease to an acidic level. It is conceivable that no enzyme activation enables the plasmid DNA to transfer into the nucleus more readily and, consequently, enhance the gene transfection.

The interval time period between DNA application and US exposure for 5 min or shorter did not contribute to any enhancement of gene expression for all the gelatin derivative-plasmid DNA complexes (Fig. 5). It will probably need some time periods to interact the complex with the cell membrane. It is conceivable that US irradiation does not result in enhanced gene expression if the complex-cells interaction is not completed.

The enhancement effect of plasmid DNA internalization into cells did not depend on the type of gelatin derivatives (Fig. 7). This finding is mainly due to the similarity of gelatin derivatives in the apparent molecular size and zeta potential. Basically, interaction of any cationized substance often causes the damage of cell membranes, which is responsible for the decreased percent survival of cells. It is apparent from Fig. 6 that the incubation of every complex decreased the cell viability. It is well recognized that *in vitro* insonation may give cells at least three types of potentially damaging interactions: heat production, cavitation phenomena, and direct mechanical forces (25). The interactions alter the functions of cell plasma membrane. It has been shown that US irradiation enables the cell membrane to enhance the permeability for cell uptake of DNA through diffusion. The main mechanism contributing to the US-induced enhancement of membrane permeability is considered to be cavitation (12,26,27). Cavitation is the phenomenon in which microbubbles are formed or destroyed in a liquid exposed to an acoustic field. It is known that cavitation begins as propagating pressure waves strike bubbles that are formed by the low-pressure portion of acoustic wave as the wave passes through media rich in dissolved gases. As the bubbles grow, they quickly reach resonant diameter and then are destroyed. This destruction can concentrate the intensity of the acoustic field up to 11 orders of magnitude in very small and localized volumes (28), which hypothetically increases the permeability of cell membrane to some extents to allow the uptake of foreign DNA (12,27). It seems that the cell uptake of complexes was enhanced by US irradiation, irrespective of the gelatin type, which is ascribed to the enhanced diffusion of plasmid DNA into cells by US irradiation. The diffusion enhancement does not depend on the type of complexes to permeate. As a result, there was no big difference in the US-enhanced internalization among the three gelatin derivatives.

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