Retraction Note to “In vitro transfection of plasmid DNA by different-cationized gelatin with or without ultrasound irradiation”


Mr. Hossein Hosseinkhani, the first author of this paper, published 18 papers with me when he was in my laboratory. The editors or I recently found that at least 8 of his papers carry figures that are apparently duplicated or modified. These papers have been retracted. I have found similar apparent duplications and modifications in Figure 1 of the above-mentioned paper published in Proc. Jpn. Acad. Ser. B, and am uncertain about the scientific integrity of the paper.

Despite many attempts, I cannot reach Mr. Hossein Hosseinkhani via E-mail. All other authors agree with this retraction.

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In vitro transfection of plasmid DNA by different-cationized gelatin with or without ultrasound irradiation

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Abstract: In vitro transfection of a plasmid DNA encoding luciferase by cationized gelatin was investigated with or without ultrasound (US) irradiation. Cationized gelatins with various introduction percentages of amino residues could be prepared by changing the introduction condition of ethylenediamine (ED) to the carboxyl groups of gelatin. The zeta potential of cationized gelatin became larger as the percent introduced increased, whereas a big change in the apparent molecular size of gelatin was not observed. An electrophoresis experiment revealed that the cationized gelatin was mixed with the plasmid DNA with the increased gelatin/DNA ratio to form cationized gelatin-plasmid DNA complexes. When the amount of amine residues introduced was 47.8% or higher, the complex obtained was about 200 nm in diameter with a positive charge. The rat gastric mucosal (RGM-1) cells incubated with the cationized gelatin-plasmid DNA complex exhibited a luciferase activity and the activity was further enhanced by US irradiation. The amount of plasmid DNA internalized was increased not only by the complexation but also by an increase in the cationization of gelatin. The viability of cells decreased by their incubation with complexes, irrespective of the gelatin type and US irradiation. We conclude that it is necessary to use US-enhanced gene expression to use cationized gelatin with the percent aminization of 47.8% or higher as the vector of plasmid DNA.

Key words: Plasmid DNA; cationized gelatin; amination; ultrasound; gene expression.

Introduction. Several viral and non-viral vectors have been explored aiming at enhanced efficiency of gene transfection.1-3 Although the former vector possess the inherently high efficiency of gene transfer, there are some points to be improved from clinical trials, such as immune and/or toxic reactions toward the vectors themselves. To tackle these problems, several genetic modulations have been attempted for genes. On the other hand, non-viral vectors have some advantages over the viral vectors although the transfection efficiency is much lower than that of the viral system. Their induction nature of toxicity and immune responses is low compared with that of viral vectors and possibility of the integration into genome does not need to be considered. In addition, the non-viral vector system is applicable for any molecular size of gene to be introduced. It should be noted that it is practically easy for non-viral vectors to produce and chemically modify on a large scale. Many macromolecules and liposomes of positive charges have been designed to demonstrate their feasibility as the non-viral vector in enhancing gene expression.4-6 As one of the former trials to enhance the transfection efficiency of DNA, it is practically promising to take advantage of external physical stimuli, such as electronic and ultrasound (US) for enhanced internalization of plasmid DNA into cells. Indeed it has been demonstrated that the transfection efficiency of plasmid DNA in vitro and in vivo was enhanced by either of them.5-6

The present study was undertaken to examine the effect of the cationization of gelatin vectors on the US-enhanced gene expression by cationized gelatin-plasmid DNA complexes. Introduction of ED to gelatin was performed under different reaction conditions to prepare cationized gelatin with varied introduction percentages of amino residues. Following incubation with the cationized gelatin-plasmid DNA complexes with or without the subsequent exposure to US, the DNA expression of cells treated was measured to evaluate the effect of the cationization of vector gelatin on the expression extent. We examine the amount of plasmid...
DNA internalized into cells as well as the cell viability with or without US irradiation.

Materials and methods. Materials. Gelatin was prepared through an acid process of pig skin, type I collagen and kindly supplied from Nitta Gelatin Co. (Osaka, Japan). Protein assay kit (Lot. No. LB900) were purchased from Nakalai Tesque (Kyoto, Japan), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC) and DNA MW Standard Marker (1 kb DNA Ladder) were obtained from Dojindo Laboratories (Kumamoto, Japan) and Takara Shuzo Co., Ltd. (Shiga, Japan), respectively. Rodamine B isothiocyanate (RTIC) was obtained from Sigma-Aldrich (St Louis, MO, USA) and cationized gelatin-plasmid DNA complexes.

Preparation of cationized gelatin and complexation with plasmid DNA. Cationization of gelatin was carried out by introduction of ED into the carboxyl groups of gelatin. Briefly, ED and EDC were added into 2,200 ml of double-distilled water (DDW) containing 44 mg of gelatin at different molar ratios to the carboxyl groups of gelatin. The reaction mixture was agitated at pH 5.0 at 37 °C for various time periods and then dialyzed against DDW for 48 hr at 25 °C. The dialyzed solution was freeze-dried to obtain powdered cationized gelatins. The percentage of amino groups introduced into gelatin, so-called the cationization of gelatin, was determined by the trinitrobenzene sulfonate method based on the calibration curve prepared by using gelatins.

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 cationized gelatin with plasmid DNA was performed by simple mixing the two materials at various 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 cationized gelatin 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 cationized gelatin-plasmid DNA complexes.

Electrophoresis of cationized gelatin-plasmid DNA complexes. After prepared at various mixing weight ratios of cationized gelatin to plasmid DNA at the DNA amount of 0.1 µg, the complex samples were applied to 0.75 wt% of agarose gel and electrophoresed for 40 min at 100 V. The electrophoresis solution used was 45 mM Tris-Borate and 1 mM EDTA buffer (pH 8.0). The gel was stained with 0.5 mg/ml etidium bromide solution for 30 min to visualize the location of DNA with a Gel Doc 2000 (Bio-Rad, Tokyo, Japan).

Measurement of dynamic light scattering (DLS) and electrophoretic light scattering (ELS). For complex preparation, 2.5 ml of PBS containing 25 mg cationized gelatin was mixed with 2.5 ml of PBS containing 5 mg of plasmid DNA by the similar procedure described above. DLS measurement was carried out using 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, can be calculated from Einstein-Stokes’ equation: \( R = kT/3\pi \eta D \), where k is the Boltzmann constant, T is the absolute temperature, \( \eta \) is the solution viscosity, and D is translational diffusion coefficient. ELS measurement was carried out on an ELS-7000AS instrument (Otsuka Electronic Co., Ltd., Osaka, Japan) at 37 °C and an electric field strength of 100 V/cm and performed three times for every sample. The zeta potential (\( \zeta \)) was automatically calculated using the Smoluchowski equation based on the electrophoretic mobility measured as: \( \zeta = 4\pi \eta \epsilon / e \) where \( \eta \) and \( \epsilon \) are the viscosity and the dielectric constant of the solvent, respectively.

Transfection experiment by cationized gelatin-plasmid DNA complexes. Rat Gastric Mucosal (RMG)-1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Lot. No. 1073750, Gibco BRL, Life Technology, NY, U.S.A.) supplemented with 10 wt% fetal calf serum, 0.12 wt% sodium bicarbonate, and 100 units/ml mixed penicilline-streptomycin solution. The cell suspension (1 × 10³ cells/ml) was plated into each well of 6 well multi-well culture plates (Code 3800-6100, Iwaki brand, Scitech Div. Asahi Techno Glass, Chiba, Japan) and cultured at 37 °C in a 95% air-5% CO₂ atmosphere. When the cell confluency reached almost 70% after 1 day incubation, the cationized gelatin-plasmid DNA complexes were added to each well. After incubation for 1 hr, the cells were exposed to US for 60 sec or not. US irradiation was performed by an US machine

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(Intertron R 6100 US, Nihon Medix, Ultrasound Williams Health Care Systems, Kyoto, Japan) at the frequency of 3 MHz, the intensity of 1 W/cm², and the duty cycle of 10%. During sonication, the temperature of the transducer head ranged from 26 to 30 °C. Following incubation for 4 hr, the culture medium was exchanged to exclude cationized gelatin, free plasmid DNA, and cationized gelatin-plasmid DNA complexes added. Each experiment was carried out for three wells. As controls, US irradiation was or not performed to cells incubated in the cultured medium alone or that containing free plasmid DNA. Then, cells were incubated for additional 48 hr, washed twice with 1 ml of PBS, and lysed by 100 µl of a lysis buffer (Luciferase Assay System, Cat# E 1500, Promega, U.S.A.). The cell lysate was centrifuged at 12,000 rpm for 5 sec at 4 °C and the supernatant sample (16 µl) was mixed with 80 µl of reconstituted luciferase assay solution (Luciferase Assay System, Cat# E 1500, Promega, U.S.A.) and the relative light unit (RLU) of the solution mixture was determined by a luminometer (Lumat Lb 9507-Berthold, Germany). The protein concentration of the lysate was also assayed by the Lowry kit (Nacalai Tesque, Lot. No. L8900, Japan).

**Internalization evaluation of cationized gelatin-plasmid-DNA complexes into cells.** For fluorescent labeling of plasmid DNA, the pCMV-Luciferase and RITC were mixed in 0.2 M sodium cacodylate-buffered solution (pH 9.7) at 4 °C for 12 hr at both the concentrations of 1 mg/ml. The reaction mixture was applied to gel filtration with a PD 10 column (Amersham Pharmacia Biotech V.K., Tokyo, Japan) to separate the RITC-labeled pCMV-luciferase from the uncoupled RITC reagent, followed by ethanol precipitation to obtain a RITC-labeled pCMV-luciferase. The RITC-labeled pCMV-luciferase were mixed with the cationized gelatin at the gelatin/DNA weight ratio of 5 in PBS to prepare various complexes. The complex prepared was added to each well where RGM-1 cells were grown at the 70% confluence. After incubation for 1 hr, the cells were exposed to US under the same condition previously described. As controls, after incubated in the cultured medium alone or that containing RITC-labeled-plasmid DNA, the cells underwent US irradiation or not. The cells incubated for 48 hr were washed carefully three times with 1 ml of PBS, and lysed by 500 µl of a lysis buffer (Luciferase Assay System, Cat# E 1500, Promega, U.S.A.). The fluorescent intensity of cell lysates was measured by a fluorescent spectrophotometer (Ex 570 nm/Em 595 nm) and divided by that of RITC-labeled plasmid DNA initially added to obtain the percent internalized. Each experiment was carried out for six wells.

**Evaluation of cell survival.** The cationic gelatin-plasmid DNA complexes were added to RGM-1 cells at the confluence of 70% and the cells were exposed 1 hr later to US similarly, followed by further incubation for 48 hr. The number of alive cells was counted by use of a hemocytometer to calculate the number percentage to cells grown without the complex treatment nor US exposure (percent survival). Each experiment was carried out for three wells. As controls, cells were incubated in the medium alone or that containing free plasmid DNA with or without US irradiation.

**Statistical analysis.** All the data were statistically analyzed to express the mean±standard deviation (SD) of the mean. Student’s t test was performed and p < 0.05 was accepted to be significance.

**Results.** Preparation and characterization of cationized gelatin. The gelatin cationization was controllable by changing the reaction time, the molar ratio of ED and EDC added to the carboxyl groups of gelatin. The amount of amino residues introduced and the subsequent zeta potential of cationized gelatin increased with an increase in the ED amount (Table I). The apparent molecular size of cationized gelatin was hardly influenced by the cationization of gelatin.

**Characterization of cationized gelatin-plasmid DNA complexes.** Migration of plasmid DNA was retarded with an increase in the cationized gelatin/plasmid DNA ratio and was not observed anymore at the ratio higher than a certain value (Fig. 1). The value became smaller as the cationization of gelatin was larger. Neither the original gelatin without any cationization nor cationized gelatin with the smallest cationization induced the electrophoretic migration of plasmid DNA. The apparent molecular size of plasmid DNA decreased by mixing with the cationized gelatin and attained to around 200 nm when E-50 and E-100 gelatins were used (Fig. 2). After mixing with the cationized gelatin, the negative charge of plasmid DNA converted to positive, irrespective of the gelatin type. The zeta potential of complexes tended to increase with an increase in the cationization of gelatin.

**Transfection of cationized gelatin-plasmid DNA complexes with or without US irradiation.** Irrespective of US irradiation, the luciferase activity was enhanced by treatment of plasmid DNA complexes with E-25, E-50, and E-100 gelatins to a significantly higher extent than that of free plasmid DNA (Fig. 3). US irradiation enhanced the luciferase activity of free plasm-
mid DNA and the complexes although the extent was less for the former than the latter. Cationized gelatin alone with or without US irradiation was not effective in the activity enhancement and the level was similar to that of PBS treatment.

**Cell uptake of plasmid DNA and cell viability.** (Fig. 4). However, the enhancement was not observed for other types of cationized gelatin. In addition, US irradiation enhanced the cell internalization of the complexes more strongly than free plasmid DNA. The cell viability decreased through treatment with the cationized gelatin-plasmid DNA complex, irrespective of the complex type. On the other hand, for any treatment, US irradiation decreased the cell viability (Fig. 5).

**Discussion.** The present study undoubtedly demonstrates that US irradiation significantly enhanced the gene expression of cells in vitro treated with cationized gelatin-plasmid DNA complexes. The expression enhancement was significant when the E-25, E-50, and E-100 gelatins were used, whereas other
cationized gelatins were not. The cationized gelatin prepared was electrostatically interacted with the plasmid DNA (Fig. 1). The cationization of gelatin by ED introduction is experimentally supported by the ELS data (Table I). It is likely that the electrostatic interaction with cationized gelatin of positive charge enabled plasmid DNA of negative charge to electrically shield the intramolecular repulsion, resulting in formation of condensed complex between the two molecules (Fig. 2). Probably, such a condensed structure of complex with positive charges enabled the plasmid to efficiently transfect to the cells, resulting in enhanced gene expression. This enhancement can be further promoted by US irradiation.

US irradiation was effective in enhancing the in vitro gene transfection of plasmid DNA for both mammalian cells[15,16] and plant cells[17-19]. Cationization of biological cells in vitro may result in heat production, cavitation phenomena, and direct mechanical forces, leading to changes in the function of cell plasma membrane. The use of low intensity US reduces both tissue heating and the occurrence of cavitation[16]. It is possible that US enables the cell membrane to enhance the permeability.
for the cell uptake of DNA through diffusion. The main mechanism contributing to the US-induced enhancement of membrane permeability is considered to be cavitation.\textsuperscript{10,15,16} Cavitation is the phenomenon that microbubbles are formed or destructed in a liquid exposed to an acoustic field. 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,\textsuperscript{17} which hypothetically increases the permeability of cell membrane to some extents to allow the uptake of foreign DNA.\textsuperscript{10,16} In addition, US can be focussed and transmitted into the body even until to the deep site. US-induced gene transfection will be also a promising way to enhance the \textit{in vivo} efficacy of gene therapy.

The complexes with the cationized gelatin/plasmid DNA mixing ratio of 5.0 showed maximum enhancement of gene expression (Fig. 3). This is explained in terms of the molecular size and positive charge of complex. It is highly conceivable that the electrostatic interaction between the cell membrane of negative charge and the cationized gelatin-plasmid DNA complex with a positive charge is a driving force to attach the complex to cells. However, the complex with lower mixing ratios would be large enough not to be taken up by cells. The complex formation and a net positive charge of cationized polymer-plasmid DNA complexes have been reported to be key for gene transfection.\textsuperscript{18} This two key properties could be achieved only by mixing with the cationized gelatin of an appropriate amionicization. The reason for less gene expression at the higher gelatin/DNA mixing ratio is not clear at present.

The amount of plasmid DNA internalized increased with the cationization of gelatin used for complexation, while it was further enhanced by US irradiation (Fig. 4). The enhancement well correlated with that of gene expression. It is highly possible that the amount of plasmid DNA internalized results in increasing the level of gene expression. The decrement of cell viability was not influenced by the cationization of gelatin for complexation (Fig. 5). As the increasing cationization of gelatin hardly increased the zeta potential of the complex (Fig. 2), it is conceivable that the similar level of positive charge induce that of cell viability. Decreased cell viability by US irradiation may be ascribed to change in the permeability of cell membrane. It is possible that such a permeability change increased the amount of plasmid DNA internalized into cells, resulting in enhanced gene expression. It has been well recognized that an appropriate US exposure brings about reversible change of cell membrane permeability.\textsuperscript{19,20}

**References**