Ultrasound-Mediated Gene Transfection *In vitro*: Enhanced Efficiency by Complexation of Plasmid DNA

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Ultrasound-Mediated Gene Transfection *In vitro*: Enhanced Efficiency by Complexation of Plasmid DNA

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Ultrasound-mediated gene transfection in the presence of microbubbles is a recently developed promising nonviral gene delivery method. The main obstacle towards its clinical application is its low transfection efficiency. In this work, we investigate the effect of the complexation of plasmid DNA (pDNA) into polyplex micelles on the transfection efficiency. Complexation changes the structure of pDNA and results in the condensation in size and enhanced stability. Both naked and complexed pDNAs were transfected into cultured cells using ultrasound in the presence of microbubbles. The transfection rate using complexed pDNA is considerably enhanced (from ~0.92 to ~1.67%, by ~82%) compared with the rate using naked pDNA. Our method provides an alternative for the improvement of the transfection efficiency of the ultrasound-mediated method.

1. Introduction

Gene therapy has long held the promise of treating various inherited and acquired diseases, as well as clinical difficulties such as cancer and vascular diseases owing to the radical idea that the very therapy deals with the cause rather than the symptoms.[1,2)] Decades of work have now put forward related research to finding a safe and effective vector, which can be either viral or nonviral.[3)] Generally, viral vectors are more efficient; however, more applications are obstructed by immune recognition, mutagenic integration, inflammatory toxicity, and low productivity.[4–6)] The nonviral method, with relatively simpler quantitative production, low host immunogenicity, and flexibility in the size of the targeted gene, is becoming a topic of intense interest.[7,8)] Even with lower transfection efficiency, nonviral delivery systems are still preferred, and various methods have been developed such as gene bombardment, electroporation, ultrasonography, and the use of lipid-based vectors, polymeric vectors, and dendrimer-based vectors.[9–11)] The ultrasound-mediated method is relatively new among all the nonviral delivery systems.[12)] Each nonviral method holds its own potential for clinical use, but the ultrasound-related system has its unique advantages.[13,14)] It is less invasive than the electroporation method as acoustic waves can propagate through soft tissue and reach internal organs. Besides, both spatial and temporal control of delivery can be achieved exclusively by adjusting the exposure parameters, which will make the delivery more targeted to the desired area and lessen side effects.

Ultrasound is a mechanical wave carrying energy and can induce phenomena such as heating, streaming, and cavitation when it propagates in cells or tissues.[15)] Cavitation and cavitation-induced activities can increase cell membrane permeability temporarily by creating transient holes in the phospholipid bilayer, termed sonoporation, and thus allow the transfer of large DNA molecules into the cell.[16)] Although the detailed mechanism of sonoporation remains unclear to date, successful transfection to various types of cell or tissue both *in vivo* and *in vitro* has been achieved.[17–21)] Until now, the largest barrier to a clinical setting of the ultrasound-mediated method is the low transfection efficiency. Experimental studies showed that exposure parameters such as pulse central frequency, intensity, repetition frequency, microbubble (gas bodies added artificially; in our case, commercial ultrasound contrast agent, UCA) concentration, and plasmid density can all affect the transfection efficiency as well as cell viability.[22–26)] However, a common problem with parametric studies is that a higher transfection efficiency is often accompanied by a lower cell viability. Our previous results led to similar conclusions, with a maximum transfection efficiency of less than 1%.[27,28)] To address this low efficiency, a new method is proposed in the present study: the use of polyplex micelles,[29,30)] complexed with pDNA and poly(ethylene glycol) (PEG) polycation block catiomers, instead of with naked pDNA. By packaging within the polyplex micelles, pDNA reduces its hydrodynamic size,[31)] so that facilitated pDNA permeation into the cytosol is expected after ultrasound application.[32)]

In this study, the ultrasound-mediated transfection method is employed together with the complexation of pDNA in order to improve the transfection efficiency. Both naked and complexed pDNAs are delivered to cultured cells. The improved stability is verified by electrophoresis and the enhanced transfection efficiency is validated by flow cytometry. The objective of this study is to provide a new way of enhancing the transfection efficiency without increasing ultrasound intensity, which can sometimes be clinically unacceptable owing to severe side effects.

2. Materials and Methods

2.1 Cell line

Mouse embryonic fibroblast cells (NIH3T3) were incubated as a monolayer at 37 °C under a humidified atmosphere of 5% CO_2 in 55 cm^2 tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemen-

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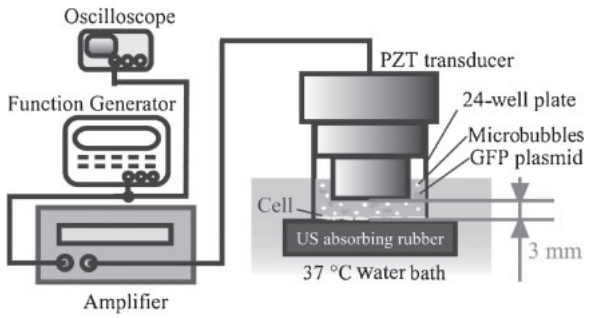

Fig. 1. Ultrasound exposure system. A 2 MHz plane transducer irradiates sound wave to cells attached to the bottom of a 24-well plate.

ted with 10% v/v fetal bovine serum (FBS, not used in one case, as will be pointed out later) and 1% v/v antibiotics (penicillin–streptomycin and L-glutamine). All the reagents were purchased from Invitrogen. The cells were detached using trypsin and transferred to a standard 24-well plate and then left to grow to 60 to 70% confluence before exposure.

The plasmid encoding green fluorescent protein (GFP) was derived from a coliform bacterium. In transfection experiments, the concentration of plasmid was kept at 15 μg/ml.

2.2 Ultrasound exposure apparatus

A schematic diagram of the ultrasound exposure apparatus is shown in Fig. 1. The system comprised an arbitrary waveform generator (NF WF1944A), a 50 dB gain radio frequency amplifier (E&I 2100L), an oscilloscope, and a custom-designed, single piezoceramic element plane transducer (13.5 mm diameter) with a central frequency of 2 MHz. During the experiments, the transducer was inserted into the wells of a 24-well plate (BD Falcon), which was placed on a sound-absorbing material in a 37 °C water bath. All the manipulations of cells were performed in a clean bench.

2.3 Ultrasound contrast agent

The ultrasound contrast agent used in all the experiments was Sonazoid® (Daiichi Sankyo). Sonazoid® is a kind of perfluorobutane microbubble (C_4F_{10}) with a resonance frequency of 2 MHz (according to the size distribution data supplied by the manufacturer), which is the exact value that was referred to when designing the transducers. The bubble sample was prepared right before the irradiation experiments by injecting 2 ml of pure distilled water into each bottle of powder and then shaking the mixture for 1 min. According to the manufacturer, the microbubble diameters range from 1 to 10 μm. On the basis of a comparison among transfection experiments using 0, 1, 10, and 20% concentrations and also the fact that bubbles float owing to buoyancy, the volume concentration was chosen to be 10%, which corresponded to about 1.7×10^5 bubbles/ml.

2.4 Preparation of polyplex micelles

Block copolymers poly(ethylene glycol)-*b*-poly(L-lysine) (PEG–PLys) were synthesized via the ring-opening polymerization of *N*-trifluoroacetyl-L-lysine *N*-carboxyanhydride initiated by the $-NH_2$ terminal group of methoxy-amino PEG (M_w 12 k), followed by the removal of trifluoroacetyl groups (TFA) using NaOH. The degree of polymerization (DP) of the PLys segments was determined to be 20 by comparing ^{1}H-NMR integration ratios between PEG chain methylene protons (CH_2CH_2O) and lysine unit methylene protons ($(CH_2)_3CH_2NH_3$. Gel permeation chromatography (GPC) measurements were carried out using a TOSOH HLC-8220. Molecular weight distribution (M_w/M_n) was determined to be 1.03.

The PEG–PLys block copolymer and pDNA were separately dissolved in 10 mM Tris–HCl buffer adjusted to pH 7.4. Polyion complexes between PEG–PLys and pDNA (polyplex micelles) were obtained by simply mixing both solutions at a charge ratio of lysine units in PEG–PLys to nucleotide units in pDNA of 1.5 when the complexed pDNA presented long rod and toroid structures. The complexed pDNA represents a core–shell structure with DNA covered and surrounded by PEG, and a structure sketch is shown in ref. 31.

2.5 Gel electrophoresis

For both naked and complexed pDNAs, four samples were prepared containing 6 μl of plasmid solutions (with a density of 100 μg/ml) and 194 μl of FBS containing a medium at 0 °C to prevent any enzyme activities, followed by incubation for 0, 15, 30, and 60 min at 37 °C. Ethylenediaminetetraacetate (EDTA) was then added to stop the enzyme, and dextran sulfate (M_w 25,000) was added to dissociate the polyplex micelles. Fluorescent images for all four incubation time cases with both naked and complexed pDNAs were obtained after electrophoresis and 1 h dyeing. The gel electrophoresis was carried out with an FAS-201 unit (TOYOBO).

2.6 Flow cytometry

The transfection of GFP was measured by flow cytometry (BD Biosciences International, BD LSRII). After ultrasound irradiation, the plates were incubated for 48 h and then rinsed with phosphate-buffered saline (PBS). Dead cells floating in the medium were not considered. Cells were then detached using trypsin and then analyzed in a cytometer (at least 10,000 cells were analyzed for each well). The gene transfection rate was taken as the number of cells that exhibited green fluorescence divided by the number of total cells after two days of incubation.

2.7 Statistical analysis

All the data are averaged from three independent replicates (12 samples) and presented as the mean ± standard deviation (SD). Test of significance between different conditions (naked/complexed; serum free/containing) was performed using single-factor analysis of variance (ANOVA) in Excel. Significance between groups was determined using two-way ANOVA with replication. Alpha level is set at 0.05 for both tests.

3. Results

3.1 Acoustic field

Considering that the plastic bottom of plates can cause the absorption and reflection of incident waves and thus result in a standing wave, the acoustic field variance from the plate

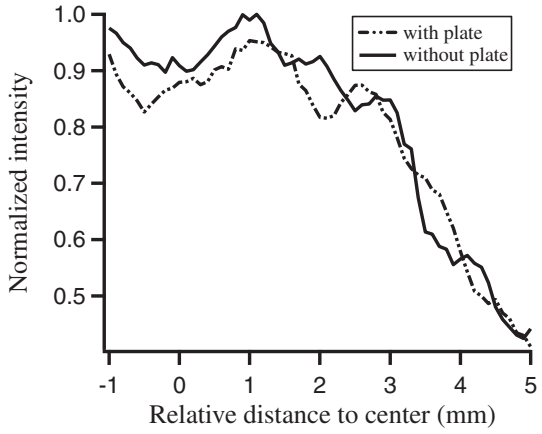

Fig. 2. Peak-to-peak acoustic pressure field measured with/without 24-well plate by needle hydrophone. Dotted line: 24-well plate positions between the hydrophone and the transducer; solid line: no 24-well plate between the hydrophone and the transducer.

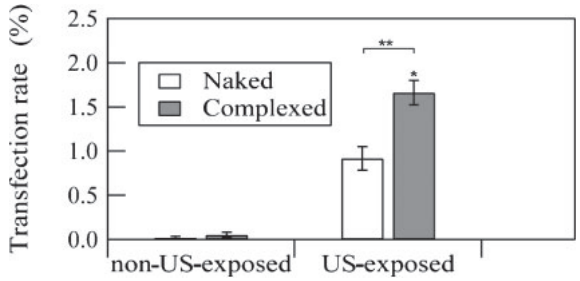

Fig. 3. Transfection rates of naked and complexed pDNAs. Plasmid DNA concentration: 15 μg/ml; microbubble concentration: 10 v/v (about 10^5/mm^3); intensity: 5.08 W/cm^2; duty ratio: 10%; pulse repetition frequency: 5 kHz; irradiation time: 60 s. Data are averaged from three independent replicates (12 samples) and presented as the mean ± standard deviation (SD). Asterisks indicate $p < 0.05$. ∗ indicates the significance between naked and complexed cases with ultrasound exposure, and ∗∗ indicates the significance between the group with exposure and the group without exposure.

was measured before the transfection experiment. A needle hydrophone was positioned 3 mm down away from the plate bottom (6 mm away from the transducer surface under a 24-well plate, as shown in Fig. 1), and the pressure field was recorded with/without the plate, as shown in Fig. 2. The diameter of the piezoelement was 12.3 mm, and taking symmetry into consideration, the acoustic pressure distribution along the axial direction was given. The acoustic pressure variance was measured to be very small (no significant difference found), so neither attenuation nor reflection was considered.

3.2 Transfection efficiency

Both naked and complexed pDNAs were transfected to NIH3T3 cells by ultrasound in the presence of microbubbles. The medium containing 10% v/v microbubbles and 15 μg/ml pDNA was prepared, and the cells were then rinsed once with phosphate buffer. A 0.8 ml mixture of microbubbles and the culture medium plus 100 μl of pDNA solution was injected into each well, and the cells inside the well were then irradiated. Eight out of the 24 wells in a plate were used for one independent experimental replicate. Taking the time when pDNA was mixed together with the FBS-containing medium as the beginning and the time when the exposure was finished as the end, the coexistence time of pDNA and serum was between 10 and 15 min, differing by manipulation. The plates were placed back into the incubator without rinsing immediately after irradiation.

The DNA concentration (15 μg/ml), microbubble concentration (10 v/v, about 10^5/mm^3), and exposure parameters (intensity: 5.08 W/cm^2; duty ratio: 10%; pulse repetition frequency: 5 kHz; irradiation time: 60 s) are identical and the transfection rates (at least 10,000 cells were analyzed for each well) are shown in Fig. 3. In the cases without exposure (intensity equals 0), no transfection was observed, which agreed with the results in the literature.[21,25] With exposure, transfection was confirmed for both types of DNA (the difference between with exposure and without exposure is statistically significant). The rate was sharply enhanced owing to the complexation of pDNA: ∼0.92 to ∼1.67%, which is an ∼82% increase (the difference between the naked and complexed plasmids is statistically significant). The efficiency increase here proved that complexation is effective and provides an alternative choice for solving the low-efficiency issue faced by the ultrasound-mediated delivery method.

4. Discussion

The enhanced transfection efficiency by complexation that we obtained is attributed to two factors: size reduction and stability against nucleases. Viability is also an important issue.

4.1 Size reduction

It is clear that the reduced size of pDNA would benefit the internalization process. The size reduction can be attributed to the structure change due to complexation, also termed as DNA condensation.[33] DNA condensation was achieved and characterized as the large volume transition from a coil to a globule state. In other words, the naked pDNA molecules present a supercoiled close-circular structure; however, for complexed molecules, the block copolymer regulates the supercoil to toroid and rod structure. Quantitatively, the hydrodynamic diameter of pDNA is dependent on the base pair (bp) number. The pDNA encoding GFP applied in this study has 6 kbp, corresponding to a hydrodynamic radius of approximately 600 nm. For naked pDNA, the expanded conformation due to electrostatic repulsion within a DNA chain makes it difficult to internaliz. Using dynamic light scattering measurement (Marvern ZEN 3600), the hydrodynamic diameters of complexed pGL3–Luc (5256 bp) and Cag–Luc (6400 bp) pDNA are measured to be 110 and 118 nm, respectively.[34,35] With the same copolymer and N/P ratio applied in this study, the size of the complexed pDNA encoding GFP should fall between the interval of 110 to 118 nm. The size reduction is then apparent since the hydrodynamic diameter of complexed pDNA is less than one-fifth of the naked pDNA value and will surely benefit gene delivery.

4.2 Stability

The importance of the stability of pDNA is reflected by two

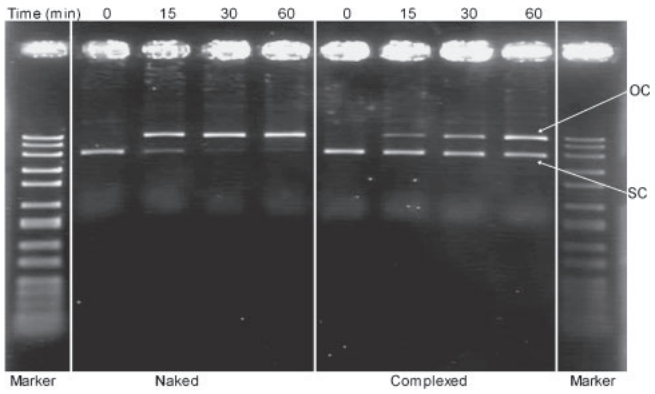

Fig. 4. Gel electrophoresis result of naked and complexed pDNAs added to serum-containing medium. OC means open circular and SC means supercoil. Naked DNAs are mostly degraded to open circular structures, while complexed DNAs partly remain as supercoiled structures.

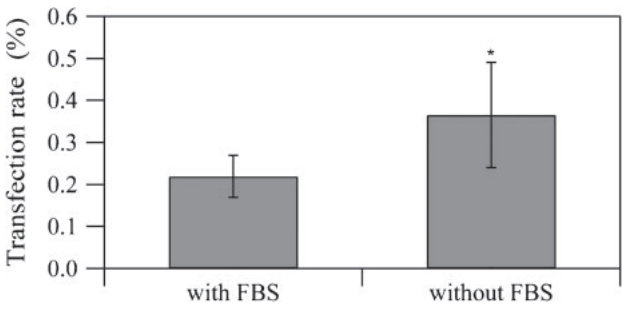

Fig. 5. Transfection rates of naked pDNA in both serum-free medium and serum containing medium. All the parameters (concentration of naked DNA, microbubble concentration, intensity, duty ratio, pulse repetition frequency and irradiation time) are identical for two cases. Data are averaged from three independent replicates (12 samples) and presented as the mean ± standard deviation (SD). Asterisks indicate $p < 0.05$.

factors: size and efficacy for transcription. Nucleases causing degradation are present in the serum and also in the cytoplasm of cells.

As mentioned in the irradiation protocols, pDNA coexists with the serum for about 15 min. pDNA molecules presenting a supercoiled structure, as mentioned before, may be subject to degradation by certain nucleases in FBS, resulting in a morphology change to an open circular topology and also to an increased size.

With the described protocol, the gel electrophoresis result is shown in Fig. 4. The original supercoiled topology of naked pDNA was degraded to an open circular structure within 30 min of incubation together in the FBS-containing medium. However, for complexed pDNA, the supercoiled structure was a major component after 15 min of incubation and coexisted with an open circular structure after 30 min of incubation. After 60 min of incubation, the open circular structure was the major component in both pDNA cases, but only in a complexed case was the supercoiled structure found. The electrophoresis result shows that by packaging pDNA into a PEG base, the stability is improved against nuclease in the serum. Since the open circular structure is larger than the supercoiled structure, complexation helps pDNA maintain its compact size, which is beneficial to the delivery process. This point was further proved by a transfection experiment. Naked pDNA was transfected in both serum-free medium and serum-containing medium. The cells were rinsed once after irradiation and the medium was replaced with FBS-containing medium to facilitate proliferation. All the parameters (concentration of naked DNA, microbubble concentration, intensity, duty ratio, pulse repetition frequency, and irradiation time) are identical, and the transfection rates (at least 30,000 cells were analyzed for each well) under the two conditions are shown in Fig. 5. The transfection rate achieved in the serum-free medium is higher (the difference is statistically significant), showing that the complexation protects DNA from degradation by nucleases contained in the serum and thus benefits the delivery.

The situation inside the cell is more complicated since various nucleases inside the cell can degrade DNA. Once internalized, pDNA should be transcribed. The complexed pDNA used in this work showed comparable transcription efficiency to naked pDNAs.[32] However, complexed pDNA has nuclease stability thus it remains intact in the cytoplasm, while naked pDNA is readily digested.[36]

4.3 Viability

Our previous study[27] showed that cell viability after irradiation is dependent on exposure parameters, more specifically, the energy level of the ultrasound applied. For the transfection experiments in this study, the ultrasound exposure parameters applied to naked pDNA and complexed pDNA are identical, so no cell viability difference would be induced by the ultrasound aspect. For the results shown in Fig. 3, the only difference between the two groups was the type of pDNA. Only 12 μg of pDNA was added to each well, so it could be expected that no cavitational activities, which is critical to the delivery, are affected. Besides, when the N/P ratio equals 1.5 neither naked nor complexed pDNA contained toxicity.[31] Thus, the outcome of the cell viability would be identical. For the results shown in Fig. 5, the cells irradiated without FBS experienced lack of nutrition for less than 15 min. However, the doubling time of NIH3T3 is approximately 20 h; the effect of 15 min can be neglected. Thus, here again, the same cell viability can be expected under the two conditions.

5. Conclusions

Plasmid DNA was successfully transfected to NIH3T3 cells in this study. A method to improve the transfection efficiency has been proposed: DNA complexation. The transfection efficiency with a plasmid after complexation is obviously higher than the efficiency without complexation, because complexed pDNA is smaller and has higher stability. This study presents a new angle for improving the efficiency of the ultrasound-mediated gene transfection method and will accelerate its adaptation to a clinical setting.

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