4aBA1. Size effect of complexed plasmid DNA to gene transfection efficiency of microbubble-mediated sonoporation

Yoichiro Matsumoto*, Yiwei Zhang, Takashi Azuma, Kiyoshi Yoshinaka, Kensuke Osada, Kazunori Kataoka and Shu Takagi

*Corresponding author's address: The University of Tokyo, Tokyo, 1138656, Tokyo, Japan, ymats@feli.t.u-tokyo.ac.jp

Ultrasound-mediated gene transfection in the presence of microbubbles is a recently developed, promising nonviral gene delivery method. Detailed dynamics of pore opening on the cell surface have not been clarified. In particular, pore size is one of the most essential parameters. In this study, we investigated the size effect of complexed plasmid DNA (pDNA) on transfection efficiency by packaging the pDNA within polyplex micelles. Both naked pDNA and complexed pDNA were transfected into cultured NIH3T3 cells by using ultrasound in the presence of a microbubble contrast agent, Sonazoid. The sizes of the hydrodynamic diameter of the naked and complexed pDNA, estimated by a dynamic light scattering measurement, were 600 and 120 nm, respectively. The transfection rates of the complexed pDNA, evaluated by counting the number of cells that exhibited green fluorescence, was 1.67%, while that of the naked pDNA was 0.92%. This efficiency enhancement, which depends on size reduction, showed that the pore sizes were distributed in the range of the pDNA diameters.
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 on 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 Even with lower transfection efficiency, nonviral delivery systems are still preferred, and various methods have been developed such as gene bombardment, electroporation, and ultrasonography and the use of lipid-based vectors, polymeric vectors, and dendrimer-based vectors.8 The ultrasound-mediated method is relatively new among all the nonviral delivery systems.9 Each nonviral method holds its own potential for clinical use, but the ultrasound-related system has its unique advantages.10 It is less invasive than is the electroporation method as acoustic waves can propagate through soft tissue and reach internal organs. In addition, 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.11 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.12 Although a 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.13 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.14 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%.15,16 To address this low efficiency, a novel method is proposed in the present study — the use of polyplex micelles17,18 complexed with pDNA and poly(ethylene glycol) (PEG) polycation block catiomers, instead of with naked pDNA. By packaging pDNA within the polyplex micelles, pDNA reduces the micelles hydrodynamic size19 so that facilitated pDNA permeation into cytosol is expected after ultrasound application.20

In this study, plasmid DNA was combined with copolymer and then delivered to cultured cell lines. The complexation of plasmid DNA was determined by using an atomic force microscope (AFM) and dynamic light scattering (DLS), and improved stability was then verified by electrophoresis. The enhanced transfection efficiency was validated by flow cytometry.21

2. MATERIAL 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% CO₂ in 55-cm² tissue culture dishes containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented 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 by using trypsin and transferred to a standard 24-well plate and then left to grow to 60 to 70% confluence before exposure.

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

![Diagram of Ultrasound Exposure Apparatus](image)

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

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 (C₄F₁₀) 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 10⁻⁸ m. The volume concentration was chosen to be 10%, which corresponded to about 1.7 × 10⁵ bubbles/ml, on the basis of a comparison among transfection experiments in which concentrations of 0, 1, 10, and 20% were used and also the fact that bubbles float owing to buoyancy.

2.4 Preparation of Polyplex Micelles

Block copolymers that contained 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 -NH₂ terminal group of methoxy-amino PEG (M₆: 12 k), followed by the removal of trifluoroacetyl groups (TFA) by using NaOH. The degree of polymerization (DP) of the PLys segments was determined to be 20 by comparing 1H-NMR integration ratios between PEG chain methylene protons (CH₂CH₂O) and lysine unit methylene protons (CH₂)₃CH₂NH₃. Gel permeation chromatography (GPC) measurements were carried out using a TOSOH HLC-8220. Molecular weight distribution (Mₙ/Mₚ) was determined to be 1.03.

The PEG-PLys block copolymer and pDNA were separately dissolved in a 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.

Atomic force microscopy (AFM) imaging was conducted by using a Nanoscope IIIa with MMAFM (Veeco, USA) in tapping mode with standard silicon probes on a highly orientated pyrolytic graphite (HOPG) substrate. The obtained images were processed by flattening them to remove the background slope of the substrate surface.

Dynamic light scattering measurement was carried out to estimate the size of the polyplex micelles by using NanoZS (ZEN 3600, Marvern, UK). DNA concentration was adjusted to 33.3 μg/ml. Data obtained at a detection angle of 173° at 37°C were analyzed by using the cumulant method to obtain hydrodynamic diameters.
FIGURE 2. Structures of complexed plasmid DNA. Polyanion (plasmid DNA) and polycation (block copolymer) couples and different structures form due to the ratio of negative charge to positive charge.

2.5 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 by 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.

3. RESULTS

3.1 Complexation of Plasmid DNA

The AFM observation of plasmid DNA before and after complexation is shown in Fig. 3. In this figure, size difference was clearly observed. Quantitatively, the hydrodynamic diameter of pDNA is dependent on the base pair (bp) number. The pDNA encoding GFP applied in this study had 6 kbp, corresponding to a hydrodynamic radius of approximately 600 nm. For naked pDNA, the expanded conformation caused by electrostatic repulsion within a DNA chain makes it difficult to be internalized. Using dynamic light scattering measurement, the hydrodynamic diameters of complexed pGL3–Luc (5256 bp) and Cag–Luc (6400 bp) pDNA were measured to be 110 and 118 nm, respectively. With the same copolymer and N/P ratio applied in this study, the size of the complexed pDNA encoding GFP should have fallen between an interval of 110 to 118 nm. The size reduction was then apparent since the hydrodynamic diameter of complexed pDNA was less than one-fifth of the naked pDNA value, which will surely benefit gene delivery.

FIGURE 3. AFM observation of plasmid DNA before (a) and after complexation (b). The DNA molecular presents a supercoiled structure before complexation; however, block copolymer regulates supercoil to a toroidal structure after complexation.
3.2 Transfection Efficiency

Before transfection experimentation, the acoustic field was measured with a needle hydrophone, and the intensity value was calculated from the measurement. Considering that the plastic bottom of the plates could possibly cause incident waves to be absorbed and reflected and may thus result in standing waves, we also measured the acoustic field with 24-well plates that was placed between the transducer and hydrophone. The result is that both absorption and reflection could be neglected.

Before an experiment of complexed plasmid, a microbubbles enhancement to the transfection of naked DNA was confirmed. When bubble number of density was larger than $10^4$ count/mm$^3$, the transfection of naked DNA was enhanced as shown in Fig.4. Both naked and complexed plasmid to DNA were transfected to NIH3T3 cells by using ultrasound with the presence of microbubbles. The concentration of DNA (15 µg/ml), microbubble concentration (10% v/v, about $10^7$/mm$^3$), and exposure parameters (intensity: 5.08 W/cm$^2$; duty ratio: 10%; pulse repetition frequency: 5 kHz; irradiation time: 60 seconds) were identical, and the transfection ratio (a count of at least 10,000 cells was analyzed for each well) for two types of DNA is shown in Fig. 5. For cases without exposure (US intensity equals 0), no transfection was found in literature. Therefore, the ultra-low transfection (0.02% for naked DNA and 0.05% for complexed DNA) here is believed to be a measuring error. With exposure, transfection was confirmed for both types of DNA (the difference between with exposure group and without exposure group is statistically significant). The efficiency was sharply enhanced due to the complexation of plasmid DNA (the difference between naked and complexed plasmid is statistically significant), i.e. ~0.92% to ~1.67%, a ~82% increase.

**FIGURE 4.** Transfection ratio of naked plasmid DNA depending bubble concentration. Plasmid DNA concentration: 15 µg/ml; microbubble concentration: from 0 to $10^7$/mm$^3$; intensity: 5.08 W/cm$^2$; duty ratio: 10%; pulse repetition frequency: 5 kHz; irradiation time: 60 seconds.

**FIGURE 5.** Transfection ratio of naked and complexed plasmid DNA. Plasmid DNA concentration: 15 µg/ml; microbubble concentration: 10% v/v (about $10^7$/mm$^3$); intensity: 5.08 W/cm$^2$; duty ratio: 10%; pulse repetition frequency: 5 kHz; irradiation time: 60 seconds. Data were averaged from three independent replicates (12 samples) and presented as the mean ± standard deviation (SD). Asterisks indicate $p < 0.05$. 
4. DISCUSSION

Complexation changes the structure of pDNA in size and stability, and the stability is thought to be also important factor to affect the transfection efficiency. The importance of the stability of pDNA is reflected by two factors: size and efficacy for transcription. Nucleases causing degradation were 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. The gel electrophoresis result is shown in Fig. 4 with the described protocol. 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. To evaluate both effects of the structure of pDNA in size and stability independently, more detailed study will be discussed in the presentation.

5. CONCLUSION

Plasmid DNA was successfully transfected to NIH3T3 cell lines in this study. A method to improve efficiency has been proposed: DNA complexation. Complexed plasmid DNA has a smaller size and higher stability. The transfection efficiency with plasmid after complexation was obviously higher than that without complexation. The study presents a new angle on improving the efficiency of the ultrasound-mediated gene transfection method and will accelerate the process to a clinical setting.

ACKNOWLEDGMENTS

This work was partially supported by the Translational Systems Biology and Medicine Initiative (TBSBMI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES