

In vitro gene transfer to mammalian cells by the use of laser-induced stress waves: effects of stress wave parameters, ambient temperature, and cell type

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1 Introduction

Recently, various types of nonviral gene transfections using plasmid DNA, which is an easier method for mass production and quality control than that using viral vectors, have been developed.¹⁻⁴ Nonviral vectors are highly attractive since they can overcome serious problems associated with viral vectors such as mutagenesis and unexpected immune responses. Although the efficiency for plasmid DNA to enter cells is limited, a number of methods using chemical carriers and physical energy have been reported to improve transfection efficiency. Laser-mediated gene transfection has received much attention as a new method for targeted gene transfection because of the high spatial controllability of laser energy. In addition, since laser energy can be transmitted through an optical fiber, catheter-based gene transfer may come into prac-

Abstract. Laser-mediated gene transfection has received much attention as a new method for targeted gene therapy because of the high spatial controllability of laser energy. We previously demonstrated both *in vivo* and *in vitro* that plasmid DNA can be transfected by applying nanosecond pulsed laser-induced stress waves (LISWs). In the present study, we investigated the dependence of transfection efficiency on the laser irradiation conditions and hence stress wave conditions *in vitro*. We measured characteristics of LISWs used for gene transfection. For NIH 3T3 cells, transfection efficiency was evaluated as functions of laser fluence and number of pulses. The effect of ambient temperature was also investigated, and it was found that change in ambient temperature in a specific range resulted in drastic change in transfection efficiency for NIH 3T3 cells. Gene transfection of different types of cell lines were also demonstrated, where cellular heating increased transfection efficiency for nonmalignant cells, while heating decreased transfection efficiency for malignant cells. © 2006 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2160407]

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tical use. In 1984, Tsukakoshi et al. first demonstrated that exogenous DNA can be transferred into cells by irradiation with a focused UV pulsed laser beam.⁵ A tightly focused laser beam was used to make a small pore on the plasma membrane, allowing foreign DNA to enter the cells. The method has been used to deliver DNAs into mammalian cells⁵⁻⁸ and plant cells.^{9,10} Recently, Tirlapur and König improved this method by using focused femtosecond laser pulses, where two-photon absorption was used to create a ultrafine hole in the cell membrane.¹¹ As another approach, Palumbo et al. made the cell membrane permeable for gene transfection by irradiating a solution of a light-absorbing dye, phenol-red, with a focused laser beam, by which local phase transition might occur in the cell membrane.¹² These methods were single-cell-basis transfections and useful for analysis of cell regulations. However, higher throughput is required for clinical gene therapy.

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We previously demonstrated both *in vivo* and *in vitro* that plasmid DNA can be transfected by the use of stress waves generated by irradiating a solid target with nanosecond laser pulses (laser-induced stress waves, LISWs).^{13,14} For *in vitro* experiments, an LISW was generated by irradiating a black rubber disk with a single nanosecond laser pulse from the second harmonics of a Q-switched Nd:YAG laser at a fluence of 1.7 J/cm². Plasmid DNA coding for EGFP has been successfully delivered to NIH 3T3 cells. The transfection efficiency increased by a factor of 2.5 by applying transient cellular heating. By using this method, a large number of cells can be treated simultaneously. In addition, this method enables treatment of deeper-located tissue because stress waves can be propagated more efficiently in tissue than laser light can. However, the mechanism of LISW-induced gene permeation through the plasma membrane is not clear. Investigation of the dependence of transfection efficiency on the laser and hence stress wave conditions may help to reveal the mechanism.

The purpose of the present study was to investigate the mechanism of LISW-mediated gene transfer. We measured characteristics of LISWs used for gene transfection. Transfection efficiency was evaluated as functions of laser fluence and number of pulses. Temperature dependence was also investigated in detail. We also investigated the applicability of LISW-mediated gene transfection to various cell lines.

2 Materials and Methods

2.1 Cell Culture

Five types of mammalian cells were used in this study. NIH 3T3 cells, HeLa cells, Chinese hamster ovary (CHO) cells, and human glioma cells were obtained from RIKEN (Tsukuba, Japan). Human fibroblasts were obtained from American Type Culture Collection (Manassas, VA). All types of cells were cultured as a monolayer in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (11995-065, Invitrogen, Carlsbad, CA, USA) with 10% calf serum (CS) and antibiotics (penicillin/streptomycin). HeLa cells were cultured in Minimum essential medium (11095-080, Invitrogen) with 10% CS and the same antibiotics. CHO cells were cultured in HAM's/F-12 (SH30026.01, HyClone, South Logan, UT) with 10% fetal bovine serum (FBS) and the same antibiotics. Human glioma cells and human fibroblasts were cultured in DMEM with 10% FBS and the same antibiotics. After reaching 70 to 90% confluence, cells were harvested, seeded in glass-bottom culture dishes (P35Gcol-0-14-C, MatTek, Ashland, MA), and incubated at 37°C for 24 h.

2.2 Plasmid DNA

Plasmid DNA coding for enhanced green fluorescent protein (EGFP) was obtained from Clontech (pEGFP-C1, Palo Alto, CA). EGFP expression vector was driven by the cytomegalovirus promoter. *Escherichia coli* competent cells were transformed and amplified in media supplied with kanamycin by standard procedures, and then plasmid DNA was purified on a Qiagen column (Qiagen Inc., Chatsworth, CA, USA).¹⁵

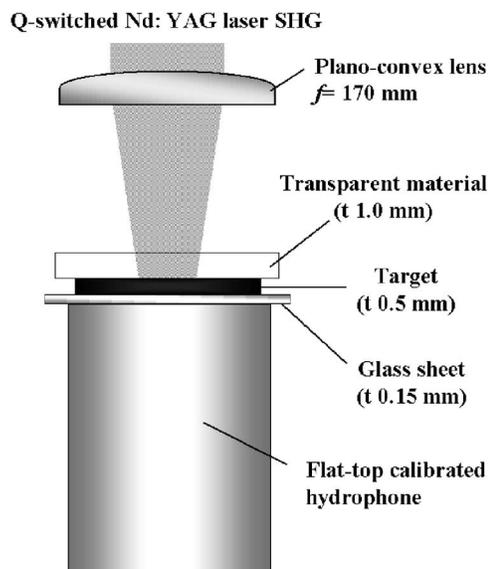


Fig. 1 The configuration for measurement of LISWs.

2.3 Generation and Measurements of LISWs

The experimental setup for measurement of LISWs is shown in Fig. 1. A black rubber disk of 0.5 mm in thickness was used as a laser target; to the top surface of the target, a 1-mm-thick transparent polymer (polyethylene terephthalate) sheet was bonded to confine laser-induced plasma. Laser-induced stress waves were generated by irradiating the target with nanosecond laser pulses from the second harmonics (532 nm) of a Q-switched Nd:YAG laser (Surelite I-10, Hoya-Continuum). The pulsed light was focused with a plano-convex lens to a spot of 3 mm in diameter on the target. To generate multipulse LISWs, the laser was operated at 10 Hz, using a single target. Pressures of LISWs were measured with a flat-top calibrated PVDF/TrFE (Polyvinylidene fluoride and trifluoroethylene copolymer) hydrophone. A glass sheet of 0.1 mm in thickness was placed under the laser target to simulate the same conditions as those used for gene transfer experiments. Silicone grease (FS silicone grease, Dow Corning) was used to match the acoustic impedances of black rubber, glass sheet, and surface material of the hydrophone. Measured data were recorded by a digital oscilloscope (Tektronics, TDS4874D).

2.4 Gene Transfer

Figure 2 shows the experimental configuration for gene transfection by the use of LISWs. Cells adhered to the bottom of a well and formed a monolayer. Experiments were performed in a thermostatic water bath for cells with 80% to 90% confluence. Immediately before laser irradiation, the cells were washed with phosphate-buffered saline (PBS) and covered with a suspension including plasmid DNA (pEGFP-C1, Clontech) coding for EGFP (0.1 mg/ml). A laser target attached on a glass sheet was placed above the cells. Because the black rubber was deformed at the moment of plasma formation, a glass sheet was attached under the laser target to prevent direct contact of the black rubber with the cells. The distance between the glass sheet and the cells was 0.7 mm. Pulsed

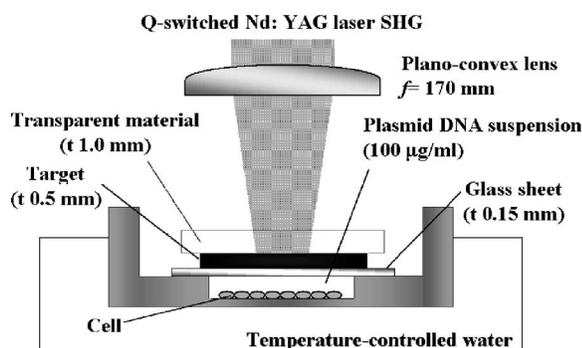


Fig. 2 The configuration for gene transfection of cultured cells by use of LISWs.

laser light was focused to a spot of 3 mm in diameter on the target. Cells that were exposed to LISWs were detached from the bottom of the well. Two minutes after irradiation, culture medium was added to the well. Detached cells in the culture medium were collected from a well by using a Pasteur pipette and moved to a new dish, which was kept in a 37°C incubator for 24 h. In our previous study, we used a different configuration, in which the laser target was attached to the backside of the well bottom.¹⁴ In the present study, the target was placed above the cells to investigate the effect of temperature under steady conditions.

2.5 Transfection Efficiency and Viability Measurement

Expression of EGFP in the cells was observed by using a fluorescence microscope (Eclipse E600, Nikon). The cell samples were excited at 460–500 nm, and the emitted fluorescence was observed through a 510–550 nm band-pass filter. Neutral density filters were used to attenuate autofluorescence, by which only EGFP fluorescence could be observed. Transfection efficiency was defined as the ratio of the number of cells emitting EGFP fluorescence to the total number of cells. Cells emitting EGFP fluorescence were counted for an area of 14 mm in diameter under a microscope; the number of cells emitting EGFP fluorescence in each well was not larger than 350. The total number of cells was estimated, based on the cell densities for 640×480 µm images that were randomly captured for each sample. Cell viability was evaluated by the trypan blue dye exclusion test (0.4%, Sigma, Saint Louis, MO) 24 h after application of LISWs.

2.6 Statistical Analysis

Gene transfer experiments were performed five times in each experimental condition ($n=5$). Statistical analysis based on the nonparametric Mann-Whitney test was performed on the data of transfection efficiency. A value of $p < 0.05$ was considered statistically significant. All values are expressed as means \pm standard error.

3 Results

3.1 Characteristics of LISWs

Figure 3 shows a typical temporal profile of an LISW at a laser fluence of 1.3 J/cm². Strong positive pressure was recorded, while negative pressure, i.e., the tensile wave, was

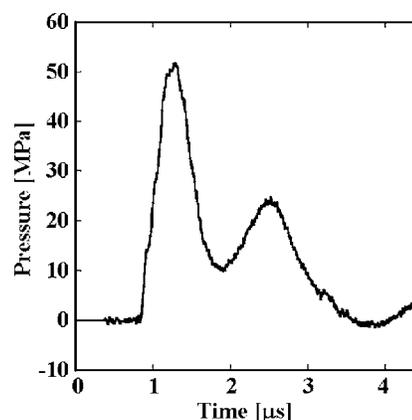


Fig. 3 Typical temporal profile of an LISW at a laser fluence of 1.3 J/cm². Note the positive pressure and the less negative peak of the stress wave.

limited. The pressure rose to a peak and fell to about 10 MPa within 1 µs. The second peak appeared at about 1 µs after the first peak; the formation of the second peak is attributable to the reflection of the stress wave at the surface of the black rubber target. Figure 4 shows the maximum positive pressure and the maximum negative pressure as a function of laser fluence. The maximum positive pressure increased with increasing laser fluence and gradually became saturated at higher fluence. The maximum negative pressure decreased with increasing laser fluence. Figure 5 shows the dependence of stress gradient, defined as peak stress divided by rise time and impulse (i.e., pressure integrated over time), on laser fluence. Rise time of the stress waves was approximately 160±8 ns, regardless of laser fluence, and the stress gradient therefore increased with increasing peak pressure. Impulse increased with increasing laser fluence.

3.2 Gene Transfer

Figure 6 shows the dependence of transfection efficiency on laser fluence for NIH 3T3 cells to which a single-pulse LISW was applied. No gene expression was observed without application of an LISW. Increase in laser fluence increased transfection efficiency. Transfection efficiency was drastically in-

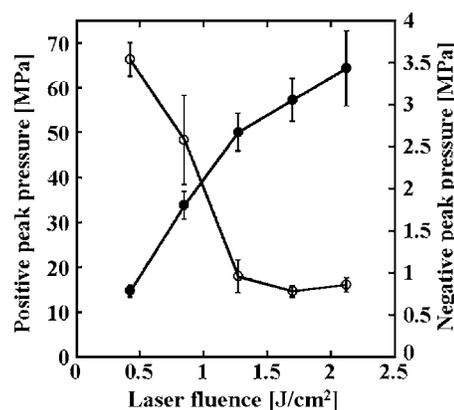


Fig. 4 Dependence of positive (solid circles) and negative peak pressures (open circles) of generated LISWs on laser fluence.

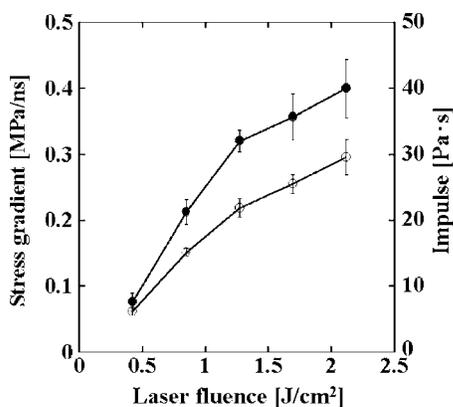


Fig. 5 Stress gradient (solid circles) and impulse (open circles) of an LISW as a function of laser fluence.

creased at fluences higher than 1.3 J/cm^2 ; a four-fold increase in transfection efficiency was obtained when compared with the efficiency at 1.3 J/cm^2 . However, reproducibility of transfection efficiency was low at a fluence of 2.1 J/cm^2 .

Figure 7 shows the dependence of transfection efficiency on number of laser pulses at fluences of 0.4 , 0.9 , 1.3 , and 1.7 J/cm^2 . Transfection efficiency increased monotonously with increase in number of pulses at a fluence of 0.4 J/cm^2 , while at fluences of 0.9 and 1.7 J/cm^2 , transfection efficiency increased with increase in number of pulses up to 20 pulses and was saturated over 20 pulses. At a fluence of 1.7 J/cm^2 , no increase in transfection efficiency was observed with increase in pulse number. After multi-pulse irradiation, the transparent material was detached from the black rubber target, creating a space at the boundary (Fig. 8), and thermal denaturation was observed in the transparent material. We performed temperature measurements for the suspension by using a thermocouple. The maximum steady-state increase in temperature was less than 0.3°C under the conditions used for the present gene transfection experiments (data not shown).

The dependence of transfection efficiency on temperature is shown in Fig. 9. The experiments were performed under

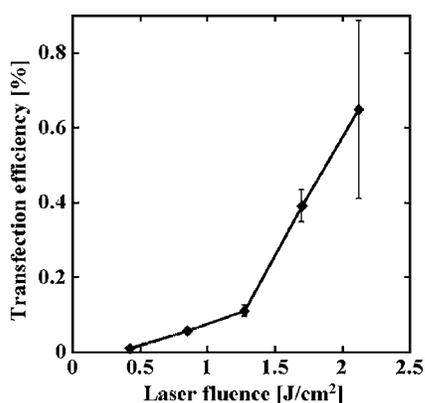


Fig. 6 Effect of laser fluence on transfection efficiency for NIH 3T3 cells exposed to a single-pulse LISW. The temperature was 37°C . Transfection efficiency was evaluated 24 h after LISW application.

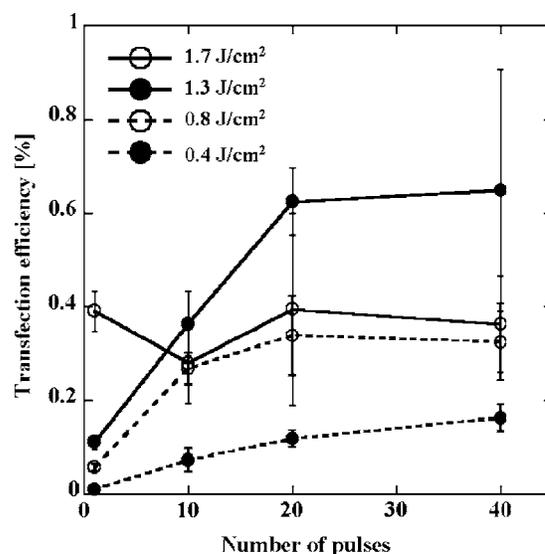


Fig. 7 Effect of pulse number on transfection efficiency for NIH 3T3 cells. The temperature was 37°C . Transfection efficiency was evaluated 24 h after LISW application.

three different conditions: (1) 0.4 J/cm^2 , 20 pulses, (2) 1.3 J/cm^2 , 20 pulses, and (3) 1.7 J/cm^2 , single pulse. Increasing temperature from 43 to 45°C drastically improved transfection efficiency.

Figure 10 shows the temperature dependence of viability of NIH 3T3 cells after application of LISWs (1.3 J/cm^2 , 20 pulses). Survival rate was slightly decreased by increasing temperature, but it was higher than 80% even at the highest temperature of 45°C . Survival rate of cells treated at 45°C with LISWs was not significantly different from that at 43°C . Without application of LISWs, survival rate was $99.4 \pm 0.1\%$ at a temperature of 37°C .

Transfection efficiencies for five cell lines to which LISWs were applied at temperatures of 37 and 45°C are shown in Fig. 11. The gene could be transfected to all cell lines. Cellular heating to 45°C increased transfection efficiency for NIH 3T3 cells, CHO cells, and human fibroblasts, while heating decreased transfection efficiency for HeLa cells and human glioma cells. No gene expression was observed in any of the cell lines without application of an LISW.

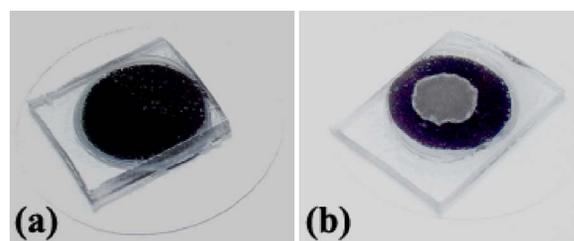


Fig. 8 Photographs of a target before irradiation (a) and a target after irradiation with multiple pulses (b). In (b), a space created at the boundary is observed.

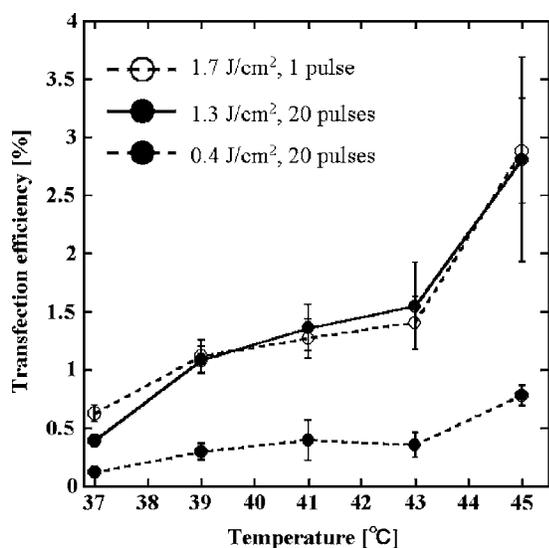


Fig. 9 Effect of ambient temperature on transfection efficiency for NIH 3T3 cells under three different laser irradiation conditions. Transfection efficiency was evaluated 24 h after LISW application.

4 Discussion

We previously showed that plasmid DNA can be transfected by the use of nanosecond pulsed laser-induced stress waves.^{13,14} In the present study, we investigated the characteristics of LISWs and the effects of physical parameters on transfection efficiency for NIH 3T3 cells. The transfection efficiency depended on laser fluence, pulse number and ambient temperature for the cells. The optimum conditions for transfection of NIH 3T3 cells were found to be 1.3 J/cm² (laser fluence), 20 pulses, and 45 °C (ambient temperature). We also investigated the applicability of LISW-mediated gene transfer to various cell lines.

Time resolution of a hydrophone, t , is given by d/c , where d is the thickness of a piezoelectric film and c is the velocity of a stress wave in the film.¹⁶ The average thickness of a

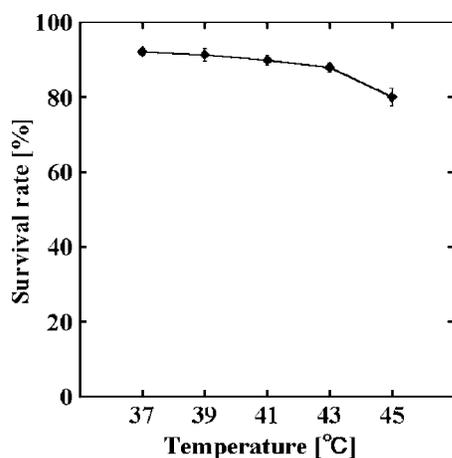


Fig. 10 Effect of ambient temperature on viability of NIH 3T3 cells exposed to LISW at the laser fluence of 1.3 J/cm² for 20 pulses. Viability was evaluated by the trypan blue dye exclusion test 24 h after irradiation.

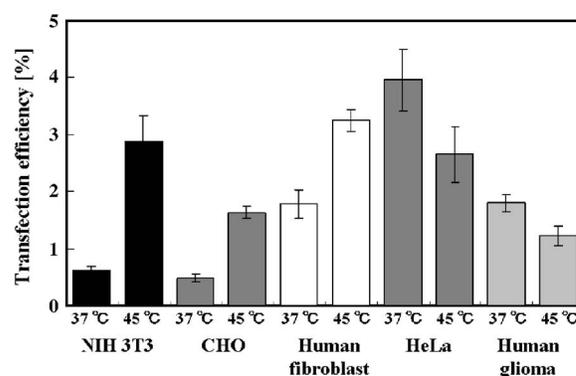


Fig. 11 Comparison of transfection efficiencies for five cell lines at temperatures of 37 and 45 °C. NIH 3T3 cells, Chinese hamster ovary cells (CHO), human fibroblasts, HeLa cells, and human glioma cells were exposed to LISWs at a laser fluence of 1.3 J/cm² for 20 pulses.

PVDF/TrFE film used in this study was 20 μ m and velocity of the stress wave was 2400 m/s, giving a time resolution of 8.3 ns. The rise time of LISWs generated was 160 ± 8 ns, regardless of the laser fluence. Thus, time resolution of the hydrophone was sufficiently high for measurement of stress gradients.

The decrease in negative peak pressure with increasing laser fluence (Fig. 4) may be due to the fact that stress waves generated by ablation of target material dominated over the thermoelastic stress waves.^{17,18} At fluences higher than 1.0 J/cm², negative peak pressure did not increase. In ultrasound-mediated gene transfection, application of repetitive tensile waves results in the formation of cavitation bubbles, which can disrupt cell membranes and, as a result, plasmid DNA can be delivered into the cells.¹⁹ Our results showed that transfection efficiency markedly increased with increasing laser fluence, while negative peak pressure did not increase at higher fluences (Fig. 6). These findings suggest that cavitation is not the dominant mechanism of LISW-mediated gene transfection.

In the present study, the maximal stress gradient of LISWs generated was 0.4 MPa/ns. Impulses of LISWs ranged from 6 to 30 Pa's (Fig. 5); under these conditions, gene expressions were observed (Fig. 6). Mulholland et al. delivered 4.4-kDa dextran-fluorescein isothiocyanate (FITC-dextran) to human red blood cells by applying LISWs with different stress gradients.²⁰ They reported that permeabilization of the cell membrane was dependent on the stress gradient and that an LISW with a stress gradient 1.5 MPa/ns did not cause any measurable increase in the uptake of FITC-dextran. Lee et al. suggested that denaturation or expansion of membrane proteins such as aquaporin is the mechanism of cell membrane permeabilization by LISWs.²¹ It should be noted that stress gradients in the present study were much smaller than those in their experiments. On the other hand, Kodama et al. investigated the delivery of 71.6-kDa FITC-dextran into human promyelocytic leukemia cells using shock waves generated by a shock tube, and they showed that impulse of the shock wave was a dominant factor determining permeabilization of the cell membrane.²² They suggested that acceleration of exogenous molecules or transient disruption of the plasma membrane by shear force is the possible mechanism of LISW-

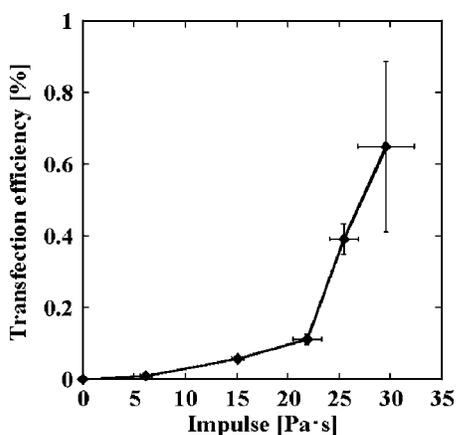


Fig. 12 Relationship between transfection efficiency and impulse of the LISW.

mediated delivery of molecules. Figure 12 shows the dependence of transfection efficiency on impulses of LISWs derived from the results shown in Fig. 5 and Fig. 6. Transfection efficiency increased with increasing impulse of LISWs, and the slope of transfection efficiency drastically increased for impulses higher than 25 Pa·s. It seems that transfection efficiency depends on impulse of an LISW rather than on stress gradient. Therefore, we speculate that the mechanism of LISW-mediated permeabilization depends on the size of exogenous molecules. Denaturation and expansion of membrane proteins by application of LISWs might be the mechanism for delivery of low-molecular-weight drugs, while there seems to be other mechanisms such as transient membrane disruption by shear force for delivery of macromolecules including plasmid DNA.

In our experiments, a scheme for plasma confinement was adopted to increase peak pressures and pulse widths of generated stress waves. As described above, after irradiating with multiple pulses, the transparent material was detached from the black rubber target, creating a space at the boundary. Under such conditions, LISWs could not be generated efficiently. This may be the reason why transfection efficiency was saturated for more than 20 pulses at laser fluences of 0.8 and 1.3 J/cm² (Fig. 7). At 1.7 J/cm², LISWs might be strong enough to create a space with the first pulse, and succeeding pulses could therefore not contribute to efficient LISW generation. Thermal denaturation was also observed in the transparent material after multi-pulse irradiation, by which transmittance for laser pulses might be decreased, causing loss of laser energy.

We previously demonstrated for NIH 3T3 cells that transient cellular heating was effective in improving transfection efficiency.¹⁴ It is known that fluidity of the plasma membrane, which works as a barrier to entry of exogenous molecules, increases with increasing temperature; a phase transition occurs in the membrane at a specific range of temperatures, typically 40 to 45°C.^{23,24} Therefore, we assumed that cellular heating contributed to enhancement of transfection efficiency. Figure 9 shows that an increase in temperature from 43 to 45°C drastically improved transfection efficiency. This result supports our hypothesis that phase transition of the plasma membrane induced by heating accelerates gene transfection

by LISWs. It is known that heating decreases cell viability and that cell viability largely depends on the time of heating.²⁵ However, our heating time was only a few minutes. This might be one of the reasons for achieving enhancement of transfection efficiency at a survival rate of NIH 3T3 cells higher than 80% even at a temperature of 45°C (Fig. 10).

The gene could be transfected to all of the five cell lines by applying LISWs (Fig. 11). Interestingly, we found that cellular heating decreased transfection efficiency for malignant cells (HeLa cells and human glioma cells), while heating increased transfection efficiency for nonmalignant cells (NIH 3T3 cells, CHO cells, and human fibroblasts). It is not clear why cellular heating decreased transfection efficiency for malignant cell lines, but there might be a different cellular response to heating for malignant cells. Since we determined transfection efficiency based on the ratio of number of cells emitting EGFP fluorescence, transfection efficiency depends not only on the permeabilization of the cell membrane but also on the process of gene expression. Since it is known that malignant cells are generally more sensitive to heating than are nonmalignant cells, it is possible that heating decreased the efficiency of transcription and translation processes of EGFP expression for malignant cells. Recently, gene transfer by the use of a femtosecond laser has been reported both *in vitro* and *in vivo*. In an *in vitro* study, individual cells were transfected by direct irradiation of their membranes with a focused laser beam.¹¹ In an *in vivo* study, DNA-injected muscle in mice was directly irradiated with focused laser pulses, and gene expression was observed in the irradiated tissue.²⁶ The use of a femtosecond laser has the advantage of enabling treatment of an extremely small volume of cells or tissue. However, higher throughput is required for clinical gene therapy. The present method using LISWs enables treatment of a large number of cells or a large area of tissue in a relatively short time period. In addition, nanosecond pulsed lasers are more compact and cost-effective compared with femtosecond lasers. Furthermore, nanosecond laser pulses can be delivered through an optical fiber, and catheter-based gene transfer may therefore be possible. At present, the highest transfection efficiency obtained was 4.0% (HeLa cells, 37°C), but transfection efficiency may be improved by optimizing pulse interval, frequency of LISWs, heating time, plasmid DNA concentration, and pH of the suspension.

5 Conclusions

In the LISW-mediated gene transfection, transfection efficiency was increased by increasing laser fluence, while negative peak pressure was decreased at higher fluences. Therefore, it seems that the cavitation effect is not the dominant mechanism of LISW-mediated gene transfection; transient disruption by shear force in the cell membrane is the most probable mechanism. For NIH 3T3 cells, the highest transfection efficiency was achieved by irradiation with twenty laser pulses with a fluence of 1.3 J/cm² at a temperature of 45°C; under these conditions, cellular viability was higher than 80%. Increase in ambient temperature from 43 to 45°C resulted in a drastic increase in transfection efficiency for NIH 3T3 cells. These findings suggest that phase transition of the plasma membrane induced by heating accelerates gene transfection by LISWs. We also demonstrated that all cell lines tested in

this study (NIH 3T3 cells, CHO cells, human fibroblasts, HeLa cells, and human glioma cells) were successfully transfected by the use of LISWs. However, nonmalignant cells and malignant cells showed different temperature dependence of transfection efficiency.

Acknowledgments

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