

Bioeffects of Transient and Low-Intensity Ultrasound on Nanoparticles for a Safe and Efficient DNA Delivery

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Abstract

An important advantage of polymer-based gene delivery systems over viral transfection systems is that transient gene expression without the safety concerns can be achieved. In addition to the polymeric systems to deliver DNA, therapeutic ultrasound is potentially useful because ultrasound energy can be transmitted through the body without damaging tissues and could be applied on a restricted area where the desired DNA is to be expressed. In this study, bioeffects of ultrasound on the transfection efficiency and cytotoxicity of DNA-polymer complexes on mammalian cells (HEK-293 and COS-7 cell lines) were investigated.

Polymer-DNA ratios for optimal transfection efficiency and the size of PEI/DNA or PDMAEMA/DNA complexes were found not affected by ultrasound treatment. Also, electrophoresis results indicate that the tertiary DNA structure was not influenced by ultrasound when exposed up to 10 seconds. More importantly, cationic polymer-mediated cell transfection was significantly enhanced and reached a 150% increase by using ultrasound. Cytotoxicity of HEK-293 and COS-7 cell lines was not observed after ultrasound. Therefore, these results indicate that clinical applications of ultrasound could be used as a safe and efficient method for non-viral gene delivery.

Keywords: Transfection; Polymer-DNA complexes; Ultrasound; Cytotoxicity

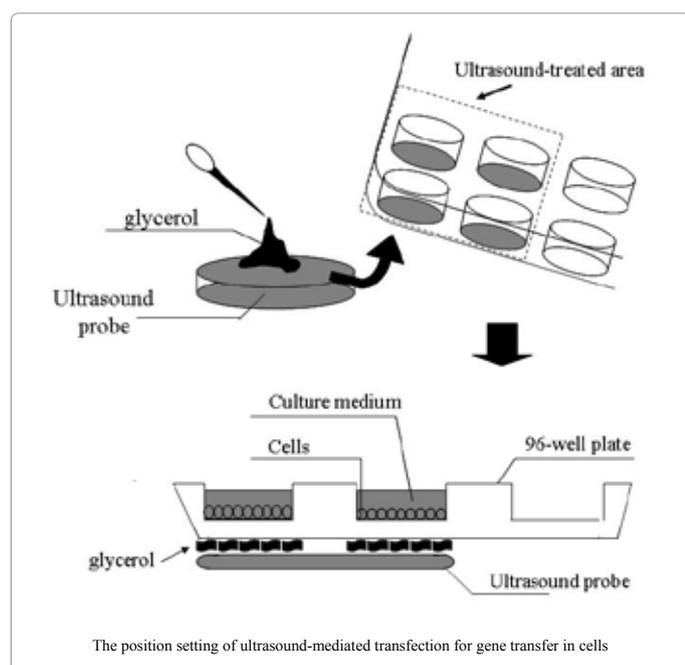
Introduction

Ultrasound has evolved from being primarily used for diagnosis into treatment of diseases (e.g. cancer) by delivery of drugs, proteins (interleukins, antibodies) or nucleic acids (DNA, siRNA) to the site of diseases [1-6]. Rapid and efficient transfer of therapeutic agents is a critical first step in therapy and mild or no cytotoxicity is a favorable step after treatment. In gene transfection, non-viral/physical *in-vitro* techniques are commonly used to mediate gene transfer and transgene expression with a varying degree of efficiency including liposome/

polymer-mediated gene transfer [7,8]. The use of ultrasound in a combination with these techniques improves transfection efficiency and site specificity [9]. Ultrasound can also increase the permeability of cell membrane to macromolecules such as plasmid DNA. Enhancement of gene expression was then observed by irradiating ultrasonic wave to the tissue after injection of DNA [10]. However, reduced cell viability *in vitro* and *in vivo* was reported because of possible acoustic pressure or ultrasound-caused cavitation [11]. Low-intensity pulsed ultrasound, transmitting as an acoustic pressure wave and applying mechanical stress indirectly to the tissues, has been reported to promote osteogenesis and protein synthesis, calcium uptake, and DNA synthesis in different cells and this ultrasound application is flexible and safe for use in gene delivery applications [12].

It was reported that a combination of microbubbles with ultrasound could further increase the gene expression level in tumor tissues or tendons [13,14]. This is because the microbubbles (could be a few μm in size) with a thin shell such as albumin are more stable compared to air bubbles (conventional sonoporation) [15]. Although microbubbles demonstrated an ultrasound-targeted capability [16], a high power pulse is required to destroy the microbubbles so concerns over cytotoxicity or irreversible damage remain [15,17].

In this study, we use a transient and low-intensity ultrasound to enhance the delivery of complexes of DNA with two polymers (PEI and PDMAEMA). The exposure time of ultrasound is 10 seconds with



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9 cycles (separated by an interval of 230 seconds). We hypothesize that the procedure would minimize cytotoxicity without compromising the increase of transfection efficiency. Also, these polymer-DNA complexes are stable with sizes smaller than 0.2 μm . Comparing to microbubbles commonly present with a size around 1-8 μm [15,18], their stability and vascular damage are major determinants of *in vivo* efficacy [19,20].

Experimental

Chemicals

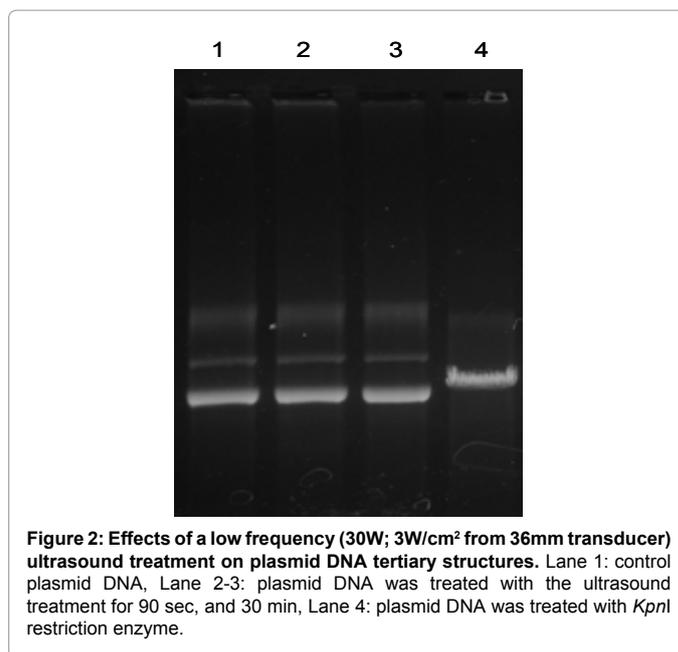
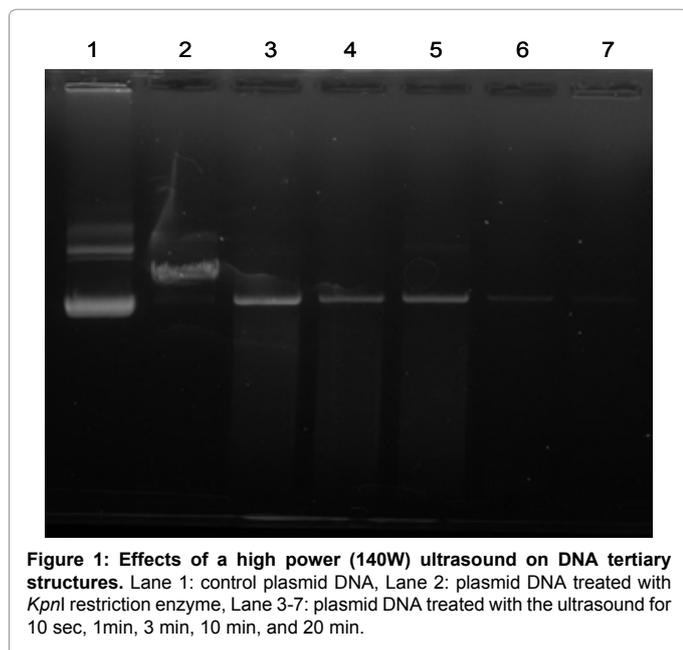
Luria broth (LB) and o-Nitrophenyl- β -D-Galactopyranoside (ONPG) were purchased from Sigma. 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT assay kit) was obtained from Roche. Agarose and restriction enzyme KpnI (10U/ μl) were purchased from Invitrogen. Polyethyleneimine (PEI, Mw=25 kDa) was from Aldrich and Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA, Mw=130 kDa) was synthesized and purified as described previously [21]. Dulbecco's modified Eagle medium (DMEM) supplemented with 10000U/ml Penicillin and 10mg/ml streptomycin for HEK-293 cell line (CCRC 60019) and COS-7 (CCRC 60094) which were obtained from Food Industry Research & Development Institute (Taiwan).

Plasmid DNA

Plasmid (pCMV *lacZ*), which encodes the *lacZ* gene for β -galactosidase, was driven by a SV40 promoter to gene expression. Plasmid DNA was amplified in *E. coli* (grown in LB medium with 50 $\mu\text{g}/\text{ml}$ of ampicillin) and purified by column chromatography (QIAGEN-Mega kit). The purity of plasmid DNA was determined by a UV Spectrometer at 260/280 nm with a ratio higher than 1.80. Agarose (0.7%) gel electrophoresis analysis using restriction enzyme (*Kpn* I) showed that plasmid DNA was mainly in a supercoiled form.

Preparation of polymer-based DNA delivery systems

Plasmid DNA (with a final concentration of 5 $\mu\text{g}/\text{ml}$) was prepared in plain DMEM. PEI and PDMAEMA were prepared in plain DMEM and various ratios of polymer/DNA (0.5/1, 1/1, 2/1, 3/1, 5/1) were



reached by adding the polymer into DNA. DNA/polymer complexes were then formed after 30 minutes prior to transfection.

Ultrasound treatment

Low-intensity pulsed ultrasound (1.5~2.2 MHz, 3W/cm², Neo-Tec N101, NeoAsia Enterprise, Taiwan) was used. The ultrasound was delivered in a form of square waves and transmitted by a 36mm flat-top probe. The 96-well plates were divided into 24 areas to apply ultrasound treatment. In order to minimize the effect of raising temperature from ultrasound, each area was treated for 10 sec before moving to the next area. Each area was treated with an upward-direction ultrasound for a total of 9 cycles (interval = 230 seconds/cycle) from bottom of culture plates (see diaphragm 1). The tertiary structure of plasmid DNA was examined after ultrasound treatment. Plasmid DNA was either treated with a ultrasonic cleaner with high power (40 KHz, 140W, WUC-D06H, Double eagle enterprise, Taiwan) for 10 sec, 1min, 3 min, 10 min, and 20 min or lower power (3W/cm²; 30W in total from a 36 mm probe) for 90 sec or 30 min prior to electrophoresis analysis.

Cell cultures

HEK-293 and COS-7 cell lines were used to evaluate the effectiveness of ultrasound on transfection efficiency. The HEK-293 cell line was cultured in completed DMEM culture medium (from Sigma), containing 10% inactivated horse serum (from HyClone), 1mM sodium pyruvate (from Sigma), and 100U/ml penicillin-100 $\mu\text{g}/\text{ml}$ streptomycin (from Invitrogen). The COS-7 cell line was cultured in completed DMEM culture medium, containing 5% inactivated FBS (from HyClone), 1mM sodium pyruvate, 25mM HEPES (from Sigma), and 100U/ml penicillin-100U/ml streptomycin-100 $\mu\text{g}/\text{ml}$ amphotericin (from Invitrogen). Both cells were cultured in an incubator (Sanyo MCO-17AIC) at 37°C and 5% CO₂.

In vitro transfection

Cells were seeded into a 96-well plate at a density of 1x10⁴ cells/well and grown overnight (with 70-80% confluence) at 37°C and 5% CO₂. Prior to transfection, cells were rinsed with plain DMEM to avoid FBS interference. 200 μl of polymer/DNA complexes was added to each

well and incubated for 1 hour. Ultrasound treatment was carried out with a low intensity ($3\text{W}/\text{cm}^2$, 90 sec in total divided in 9 cycles; that is 10 sec/cycle with an interval of 230 sec) and then incubated at 37°C and 5% CO_2 for further 30 minutes. After incubation, the polymer/DNA complex was removed by aspiration and the each well was refilled with 100 μl of completed DMEM culture medium. After 48 hours, transfection efficiency was determined by β -galactosidase gene expression. Briefly, cells were washed once with PBS prior to adding 20 μl lysis buffer (pH 8.0, contains 0.5mM tris base, 1.5mM NaCl and 1% triton X) per well and incubating at 4°C for 20 min. After that, 180 μl of ONPG staining solution (0.09M sodium phosphate, pH 7.5, 0.9mM MgCl_2 , 0.67 mg/ml ONPG) was added into each well and incubated at 37°C and 5% CO_2 for 60 minutes. The absorbance was determined at 405/630nm using an ELISA plate reader (Labsystems Multiskan MS).

Cell viability of transfected cells

To determine the influence of ultrasound on cell viability, the number of viable cells was measured using an XTT colorimetric assay according to the manufacture protocol from Roche, Taiwan. Briefly, mixing 5ml of XTT stock solution and 100 μl of electron-coupling reagent prior to the assay. 50 μl of the XTT mixture was added to each well and incubated for 60 min at 37°C and 5% CO_2 . The absorbance was determined at 490/690nm using an ELISA plate reader (Labsystems Multiskan MS).

Statistical analysis

Data of replicates from each group of six samples were combined from at least three different experiments. A two-tailed student's unpaired test was used to compare the mean values of two populations with respect to a significant difference in transfection efficiency and cytotoxicity (GraphPad Prism 3.0 software).

Results

Effects of ultrasound on plasmid DNA tertiary structures and sizes of polymer-DNA complexes

Plasmid DNA was initially in supercoiled topology (Figure 1, lane 1). After 10 sec exposure of a high power (140W) ultrasound, the amount of DNA was reduced (lane 3). The reduced DNA was not converted to a linear form which should be visible as shown in lane 2 but was degraded into small fragments (shown as a smear pattern, lane 3-5). The DNA was degraded more under 140W ultrasound as the treatment time was increased (from 1 min, to 20 min; lane 4 and lane 7, respectively). Conversely, a low power (30W; $3\text{W}/\text{cm}^2$ from 36-mm probe) ultrasound appeared not to affect the integrity of DNA in either 90 sec or 30 min treatment group (Figure 2; lane 2 and lane 3, respectively). The sizes of PEI-DNA and PDMAEMA-DNA complexes were not affected either by a low power (30W) or a high power (140W) ultrasound (data not shown).

Bioeffects of ultrasound on COS-7 cells

PEI/DNA complexes: In transfection efficiency studies with PEI and COS-7 cells, the optimal transfection ratio for PEI-DNA complexes without ultrasound treatment (control) was 1/1 (w/w) (Figure 3A). As we have shown previously [21], a lower ratio (e.g. PEI-DNA ratio =0.5/1) increased the particle size of complexes over $0.3\ \mu\text{m}$ and a high ratio (e.g. PEI-DNA ratio >2/1) also increased their cytotoxicity. Both circumstances would result in decreased transfection efficiency.

Exposure to ultrasound (10 sec, 9 cycles with 230 sec interval) increased transfection efficiency (Figure 3A, $p<0.01$) for PEI/DNA

complexes at 1/1 ratio. For other ratios of PEI/DNA, no significant increase in transfection efficiency was observed before and after the ultrasound exposure. In Figure 3B, there is a slight decrease in cell viability of PEI/DNA complexes on COS-7 cells at higher polymer-DNA ratios (from 2/1 to 5/1). This decrease was due to the effect of cationic polymers upon cells which is well documented [22]. Importantly, ultrasound treatment did not cause any cytotoxicity under our test conditions when compared to controls.

PDMAEMA/DNA complexes: The optimal ratio of PDMAEMA/DNA complexes for transfection was determined to be at 5/1 (w/w) (Figure 4A). Further higher polymer/DNA ratios would result in significant cytotoxicity. However, for lower ratios (from 0.5/1 to 2/1), particle sizes of complexes were observed over $0.3\ \mu\text{m}$ and were the cause of lower transfection efficiency [21] compared to the size of complexes at 3/1 or 5/1 was $0.2\ \mu\text{m}$ and $0.15\ \mu\text{m}$, respectively. After ultrasound treatments, a 150 % increase in transfection efficiency was found in the complexes at ratios of 3/1 and 5/1 with small sizes ($<0.2\ \mu\text{m}$). In contrast, the transfection efficiency of complexes at 2/1 with a larger size ($0.3\ \mu\text{m}$) did not show a beneficial effect on increasing gene transfer. Again, the ultrasound treatment did not induce more cytotoxicity in comparison with controls (without ultrasound) (Figure 3B).

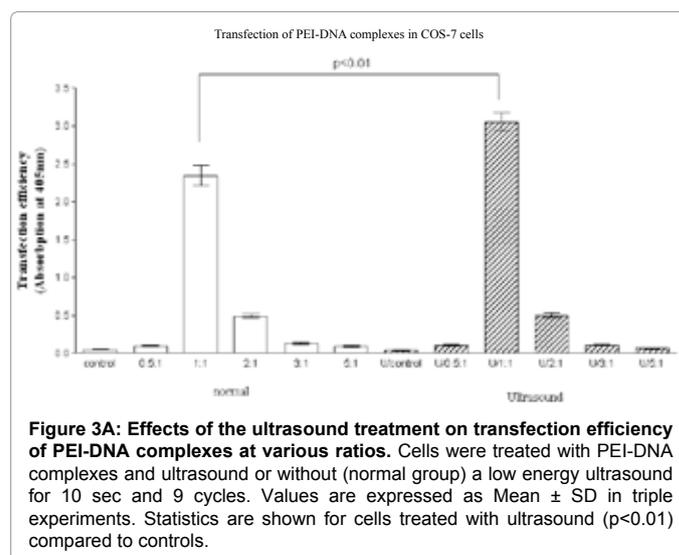


Figure 3A: Effects of the ultrasound treatment on transfection efficiency of PEI-DNA complexes at various ratios. Cells were treated with PEI-DNA complexes and ultrasound or without (normal group) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD in triple experiments. Statistics are shown for cells treated with ultrasound ($p<0.01$) compared to controls.

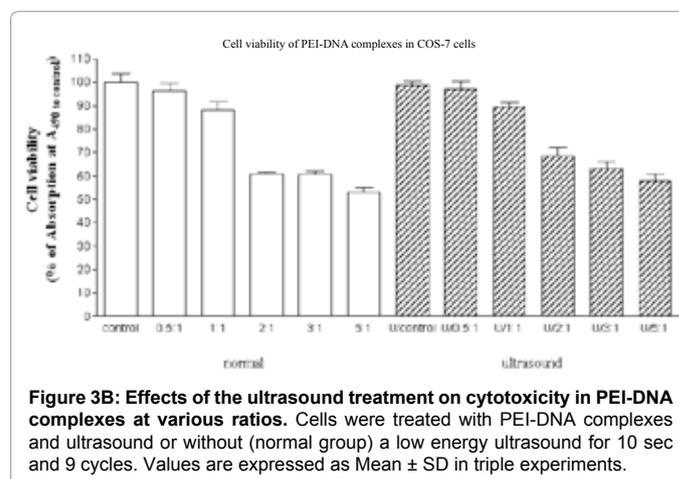


Figure 3B: Effects of the ultrasound treatment on cytotoxicity in PEI-DNA complexes at various ratios. Cells were treated with PEI-DNA complexes and ultrasound or without (normal group) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD in triple experiments.

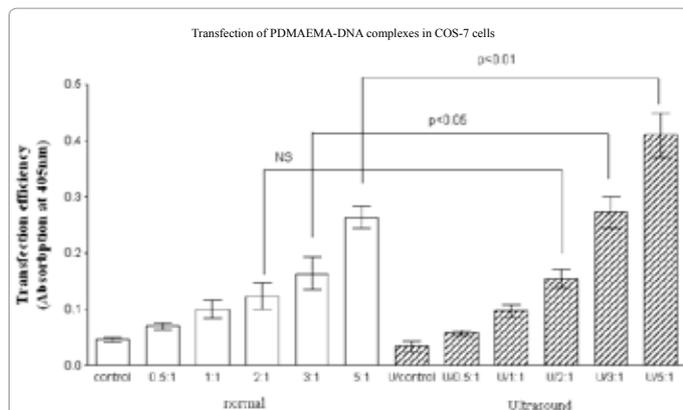


Figure 4A: Effects of the ultrasound treatment on transfection efficiency of PDMAEMA-DNA complexes at various ratios. Cells were treated with PDMAEMA-DNA complexes and ultrasound or without (normal group) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD in triple experiments. Statistics are shown for cells treated with ultrasound compared to untreated cells at ratios of 5/1 ($p < 0.01$), 3/1 ($p < 0.05$) and 2/1 (NS; not statistically different).

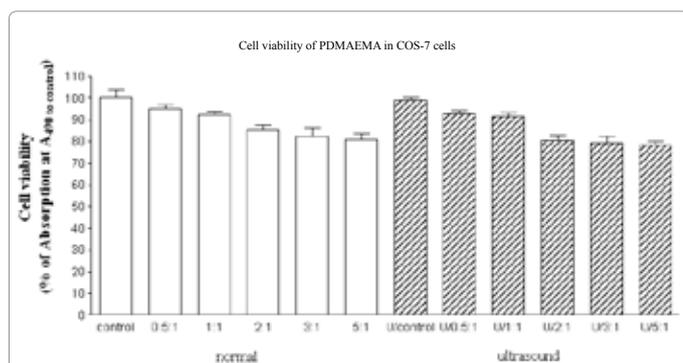


Figure 4B: Effects of the ultrasound treatment on cytotoxicity of PDMAEMA-DNA complexes at various ratios. Cells were treated with PDMAEMA-DNA complexes and ultrasound or without (normal group) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD in triple experiments.

Bioeffects of ultrasound on 293 cells

PEI/DNA complexes: Similar transfection results were obtained in the use of PEI-DNA complexes on 293 cells compared to COS-7 cells. As in the experiment with COS cells, a significant increase in transfection efficiency at a 1/1 ratio of PEI-DNA complexes was found (Figure 5A, $p < 0.05$) after the ultrasound treatment. Again, no additional cytotoxicity was observed from our test conditions with ultrasound (Figure 5B).

PDMAEMA/DNA complexes: In agreement with the findings of PDMAEMA-DNA complexes on COS cells, PDMAEMA-DNA complexes were optimally transfected at a ratio of 5/1 followed by ratios of 3/1, 2/1, 1/1, and 0.5/1 (Figure 6A). After ultrasound treatment, a 140% increase in transfection efficiency was observed for the 3/1 and 5/1 ratios (Figure 6A, $p < 0.05$). When larger PDMAEMA-DNA complexes were used (ex. at 2/1 ratio), ultrasound did not improve transfection efficiency (no statistical difference from control). Moreover, cell viability of 293 cells treated with PDMAEMA/DNA complexes was not influenced by ultrasound treatment (Figure 6B) which suggests that this is a safe and effective method to improve gene transfer.

Discussion

In this study, we used a transient, low intensity ultrasonic system to safely deliver genes *in vitro*. The resonant frequency around 2 MHz (1.5~2.2 MHz in our use) was chosen because it is approved for clinical applications with advantages of good penetration through soft tissues and no influence on DNA integrity [23-25]. Also, the acoustic wave with a frequency around 2 MHz helps in-depth transmission through the bottom of 96-well plates. Lower frequency (e.g. 500 kHz) would cause significant damage to DNA and a negative effect on cell viability [26]. Up to date, the exposure time and setting of ultrasound treatment (i.e. directions of irradiation, sequences in using ultrasound and cells to be transfected) were different. Our methodology has brought the use of ultrasound closer to the clinical setting. The ultrasound irradiation for only 10 seconds is rare and has only been used for transfection of naked DNA [27]. Meanwhile, very rare research is available on the subject of using ultrasound with polymer or liposome/DNA complexes [28,29].

Mechanisms of ultrasound to increase transfection efficiency have been shown in many studies. Acoustic cavitation effect either from air bubbles or added microbubbles is commonly accepted [30-32].

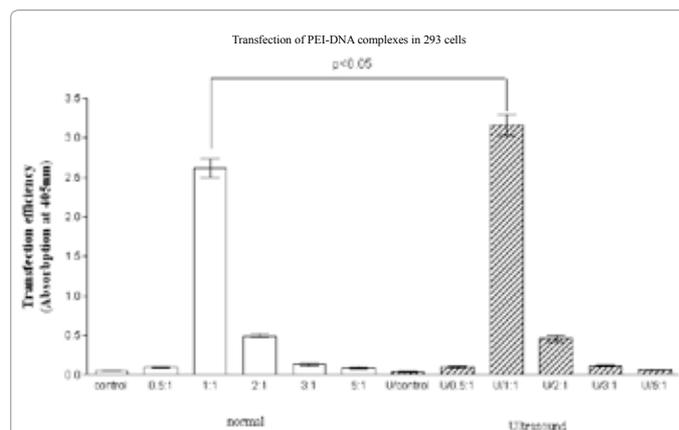


Figure 5A: Effects of the ultrasound treatment on transfection efficiency of PEI-DNA complexes at various ratios. Cells were treated with PEI-DNA complexes and ultrasound or without (normal group) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD in triple experiments. Statistics are shown for cells treated with ultrasound ($p < 0.05$) compared to controls.

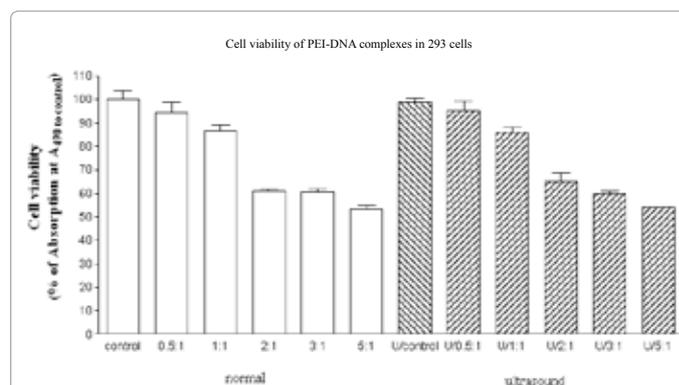


Figure 5B: Effects of ultrasound treatment on the cytotoxicity of PEI-DNA complexes at various ratios. Cells were treated with PEI-DNA complexes and employ ultrasound or without (normal) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD. Triple experiments were performed.

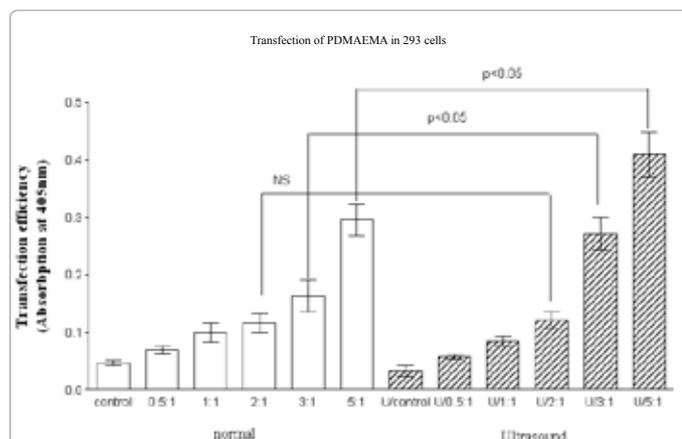


Figure 6A: Effects of the ultrasound treatment on transfection efficiency of PDMAEMA-DNA complexes at various ratios. Cells were treated with PDMAEMA-DNA complexes and ultrasound or without (normal) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD in triple experiments. Statistics are shown for cells treated with ultrasound compared to untreated cells at ratio of 5/1 ($p < 0.05$), 3/1 ($p < 0.05$) and 2/1 (NS).

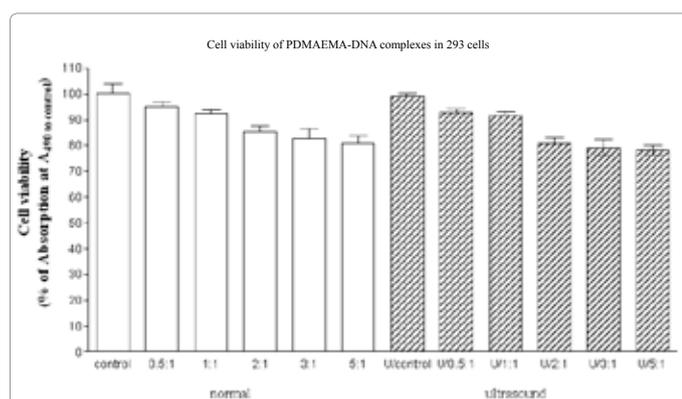


Figure 6B: Effects of the ultrasound treatment on cytotoxicity of PDMAEMA-DNA complexes at various ratios. Cells were treated with PDMAEMA-DNA complexes and ultrasound or without (normal group) a low energy ultrasound for 10 sec and 9 cycles. Values are expressed as Mean \pm SD in triple experiments.

However, this effect also induce toxicity or apoptosis in cells due to disturbing the cell membranes [33-35] especially with high power of ultrasound (e.g. 130W) [36] or a combination of high power and low frequency (e.g. 20 kHz) [37] as we shown in Figure 1. Nevertheless, a cycle burst ultrasound treatment which we used only up to 10 seconds bypasses the problem of cytotoxicity without compromising the transfection efficiency. Besides the improvement in mechanical bioeffect from ultrasound, our methodology minimized the thermal bioeffect which might also contribute to cell toxicity [38].

Further key findings of our studies were: (1) the ratios for optimal transfection of polymer-DNA complexes are not affected before/after ultrasound treatment (Figure 3A, 4A, 5A, 6A). This indicates that the ultrasound effect is evenly affecting complexes prepared at all polymer-DNA ratios; (2) the sizes of polymer-DNA complexes before/after ultrasound treatment were also not affected. Therefore, the reason of ultrasound in increasing transfection efficiency before/after was not due to particle sizes nor cytotoxicity (Figure 3B, 4B, 5B, 6B); (3) for each polymer-DNA ratio, the extent of transfection efficiency enhancement by ultrasound is dependent to the particle size

of polymer-DNA complexes, so smaller complex sizes lead to higher transfection efficiency (Figure 4A and Figure 6A).

Conclusions

A transient, low intensity ultrasound was found to be non-cytotoxic and exhibited an ability to overcome the major limitations of polymer-DNA complexes such as low transfection efficiency. This new transfection method was shown to be a safe, simple and efficient approach with clinical applications for gene transfer.

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