

Efficient gene therapy with a combination of ultrasound-targeted microbubble destruction and PEI/DNA/NLS complexes

YI-JIA WANG, QING ZHOU, SHENG CAO, BO HU, QING DENG, NAN JIANG and JINGJING CUI

Department of Ultrasound Imaging, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. Current strategies of gene transfection are not efficient at achieving a notable therapeutic effect. The aim of the present study was to combine ultrasound-targeted microbubble destruction (UTMD) with a polyethylenimine/pEGFP-N₃ plasmid/nuclear localization sequence (PEI/DNA/NLS) complex gene delivery system, and evaluate the transfection efficiency of enhanced green fluorescent protein (EGFP) gene delivery to 293T cells using this system. The formation of PEI/DNA/NLS complexes and the protective effects of PEI/NLS were verified by gel electrophoresis. Solutions consisting of the plasmid alone, PEI/DNA complexes, PEI/DNA/NLS complexes, UTMD+DNA, UTMD+PEI/DNA complexes, and UTMD+PEI/DNA/NLS complexes were transduced into 293T cells via ultrasound irradiation. The expression of GFP was observed using an inverted microscope and transfection efficiency was detected by flow cytometry following 24 h incubation *in vitro*. Cell activity was detected using a Cell Counting kit (CCK)-8 assay. Gel electrophoresis confirmed the formation of PEI/DNA/NLS complexes and demonstrated that PEI/NLS exhibited protective effects on plasmid integrity for a limited time. Inverted microscope observations revealed that a greater GFP signal was observed with the combined action of PEI/DNA/NLS complexes with UTMD, and flow cytometry analysis demonstrated the highest level of transfection efficiency in this group. In addition, the viability of the cells detected by CCK-8 and treated with PEI/DNA/NLS complexes with UTMD was >80%. In conclusion, the combination of UTMD and PEI/DNA/NLS complexes was highly effective for the efficient transfection of 293T cells without causing excessive

cell damage. This method may provide a novel and effective gene transduction system to be applied in clinical treatments.

Introduction

In recent years, gene therapy has become a widely-used approach to treat a variety of inherited and malignant diseases. It requires safe, efficient and specific gene delivery systems. Despite the safety advantages, the efficiency of non-viral vectors is usually poor (1,2). Therefore, the development of efficient non-viral transfection systems to improve transfection efficiency is an important area of research.

In 1962, Feldherr (3) described the process by which nucleocytoplasmic transport of colloidal gold particles occurs through specialized pores in the nuclear envelope known as nuclear pore complexes (NPCs). Transport through NPCs is selective and energy dependent (4). Ions and small molecules with a diameter of <9 nm enter the nucleus passively, whereas larger molecules require nuclear localization sequences (NLSs) that are recognized by the cytoplasmic transport receptors responsible for mediating nuclear uptake (5). The most studied, and therefore best known, NLSs are virus-derived peptides, such as the Tat (transactivating) protein or Antennapedia homeodomain protein. However, arginine/lysine-rich NLSs, such as the Simian virus 40 (SV40) large T antigen (PKKKRKV), appear to be far more efficient (6-8). The immunogenicity of this peptide has been reported to be markedly lower when compared with additional NLS-peptides (9,10). In addition, this peptide binds proteins and DNA via electrostatic interactions. Therefore, conjugation of this peptide to DNA may improve nuclear import and thus increase the transfection efficiency of non-viral gene delivery systems (8,11).

Cationic lipids and polymers have been employed as non-viral gene transfer agents. These cationic substances form complexes with anionic DNA via electrostatic interactions. The subsequent cationic DNA complexes are taken up by cells via electrostatic interactions due to the negative charge of the cell surface (12). Among these complexes, cationic liposomes are widely used for almost all animal cells, as they demonstrate nonspecific ionic interactions and a low level of toxicity (13). Therefore, a number of polymeric cationic systems including polyethylenimine (PEI), cationic peptides (poly L-lysine), cationic dendrimers and chitosan have been studied. In a previous study, PEI has been proposed as a safer alternative to other non-viral vectors (14).

Correspondence to: Professor Qing Zhou, Department of Ultrasound Imaging, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, P.R. China
E-mail: qingzhou.wh.edu@hotmail.com

Abbreviations: UTMD, ultrasound-targeted microbubble destruction; PEI/DNA/NLS, polyethylenimine/pEGFP-N₃ plasmid/nuclear localization sequence

Key words: nuclear localization sequence, gene delivery, ultrasound irradiation, polyethylenimine, microbubble

Among the novel strategies under investigation, ultrasound-targeted microbubble destruction (UTMD) has been demonstrated to be particularly promising for the enhancement of gene and drug delivery (15-17). A number of studies have demonstrated that UTMD significantly increases the permeability of a cell membrane, thereby improving the efficiency of gene transfection (15-17). In addition, the combination of ultrasound (US) irradiation with PEI markedly improves transfection efficiency (18,19) and specific targeting (20). Yoo and Jeong (21) demonstrated the plasmid DNA/PEI complexes containing NLS attached to psoralen, a nucleic acid-intercalating agent, increased transfection efficiencies in COS-1 cells, indicating that this complex may be used as a potential DNA carrier for therapeutic applications. Current research is primarily focused on whether the combined effect of UTMD and PEI/DNA/NLS complexes may be more efficient than previously studied methods without increasing cell damage.

The present study investigated the use of SV40 large T-antigen as a nuclear localization signal non-covalently bound to a PEI/DNA complex, in an attempt to increase transfection efficiency using the unique physical non-viral method of US irradiation.

Although US is a simple, convenient and targeted method, the transfection efficiency achieved by US remains low (22). Therefore, the aim of the present study was to combine US with PEI/DNA/NLS complexes to produce a novel gene transfer system, and to investigate the safety of the new gene delivery system, as well as its effect on transfection efficiency.

Materials and methods

Ultrasound irradiation equipment. Ultrasound irradiation was generated by a UGT 2007 ultrasonic gene transfection apparatus (Ultrasonic Research Institute, Chongqing Medical School, Chongqing, China) with a varied pulse time of 1 to 10 sec and a probe area of 0.8 mm². The irradiation frequency ranged from 0.5 to 2 MHz with a continuous wave. The intensity of ultrasound exposure was set at 1.5 W/cm² and the duration was 30 sec, as previously described (23).

Plasmid. The 4.7-kb pEGFP-N3 plasmid, an expression vector for the enhanced green fluorescent protein (EGFP) gene, was obtained from Clontech Laboratories, Inc. (Mountainview, CA, USA). The plasmid was used to transform chemically-competent DH5 α *Escherichia coli* (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), which is performed according to the manufacturer's protocol. Cultures of transformed bacteria were grown in the presence of 20 μ g/ml kanamycin (Shine Star (Hubei) Bio Engineering Co., Ltd., Jingzhou, China). The plasmid DNA was extracted and purified using a QIAquick PCR Purification kit (Shine star (Hubei) Bio Engineering Co., Ltd.). The concentration of isolated plasmid DNA was determined by measuring the absorbance at 260 nm (A260) using UV spectrophotometry (Beckman DU-640; Beckman Coulter, Inc., Brea, CA, USA). Following determination of the concentration, the plasmid DNA was resuspended to a final concentration of 1 μ g/ μ l in elution buffer [2.5 mM Tris-HCl, (pH 8.5)]. In addition, the

A260/A280 ratio was between 1.8 and 2.0, indicating that the purified plasmid DNA was free of contaminants.

NLS peptide. The NLS peptide, PKKKRKV, originates from the large-T antigen in the SV40 virus (molecular weight: 1386; high-performance liquid chromatography analysis: 98.612%), and was obtained from Shanghai Science Peptide Biological Technology Co., Ltd. (Shanghai, China). The peptide powder was stored at -20°C, away from light sources at room temperature for 30 min, before it was centrifuged at 37°C and 400 x g for 5 min. A total of 1 mg peptide was dissolved in 1 ml phosphate-buffered saline (PBS) in a sterile environment, and was stored at -20°C away from light sources.

Preparation and characterization of PEI/DNA complexes with and without NLS. Branched PEI with an average molecular weight of 25 kDa was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). An aqueous stock solution of PEI was prepared by diluting 1 mg of the commercial solution in 1,000 ml ddH₂O, neutralizing with HCl and filtering through a 0.2- μ m filter (EMD Millipore, Billerica, MA, USA). The PEI/DNA complexes were prepared at various PEI nitrogen: DNA phosphate ratios (N:P) by adding the pEGFP-N3 plasmid solution to the PEI solution, the N:P ratios are 0:1, 0.25:1, 0.5:1, 1:1, 2:1, 4:1, 8:1 and 16:1. The mixture was gently mixed using a pipette for 3 to 5 sec to initiate complex formation, before the NLS solution was added and incubated for 30 min at room temperature.

Preparation of SonoVue/DNA, SonoVue/PEI/DNA and SonoVue/PEI/DNA/NLS complexes. The SonoVueTM microbubble suspension (Bracco Suisse SA, Manno, TI, Switzerland) was reconstituted immediately prior to use by injecting 5 ml 0.9% saline solution. The mean diameter of the microbubbles was 2.5 μ m, and the concentration was 2x10⁸ to 5x10⁸ microbubbles/ml. Prior to the experiments, 400 μ l SonoVue was mixed with the pEGFP-N₃ plasmid, PEI/DNA complexes or PEI/DNA/NLS complexes. The complex formation was confirmed by gel electrophoresis. All complexes were prepared by incubation for 30 min at room temperature.

Gel electrophoresis assays. In the first set of experiments, the aim was to evaluate whether PEI can be combined with the pEGFP-N₃ plasmid using static electricity adsorption. Therefore, the pEGFP-N₃ plasmid (1/1 μ l) was incubated with PEI at N:P ratios ranging from 0:1 to 16:1. In the second set of experiments, NLS (120 μ g) was added to each well (5). Agarose gels were prepared with a 0.7% agarose solution in Tris-acetate buffer containing ethidium bromide (0.5 μ g/ml). Electrophoresis was performed for 60 min at 90 V.

Effect of NLS peptide and PEI on DNA integrity using a deoxyribonuclease (DNase) protection assay. To determine whether the PEI/NLS complexes protect the pEGFP-N₃ plasmid from DNase degradation reference earlier works (24) in the present study, the pEGFP-N₃ plasmid was incubated with the PEI at N:P ratios of 6:1 (19) with or without 120 μ g NLS (24) for 1 h at room temperature. DNase endonuclease (0.25 U; Thermo Fisher Scientific, Inc.) was then added to pEGFP-N3 plasmid, PEI/DNA and PEI/DNA/NLS solutions

for 5, 10 or 15 min at 37°C. Following incubation at 80°C for 10 min using a thermo cycler to terminate enzyme digestion, the samples were transferred to wells at the top of a 1.0% agarose gel. Electrophoresis was performed at 100 V for 1 h.

Cell culture and transfection groups. A total of 2×10^6 293T cells (China Center for Type Culture Collection, Wuhan, China) were placed into 6-well culture plates in 90% Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.). The cell cultures were maintained at 37°C and 5% CO₂ for 24 h until the cell adherence rate was ~95%. The different transfection conditions were grouped as follows: Group 1 (control group), 10 µg pEGFP-N3 plasmid was added to a single well, and the plate was incubated in 5% CO₂ at 37°C for 2 h; group 2, pEGFP-N3 plasmid+PEI (PEI/DNA), whereby 10 µg pEGFP-N3 plasmid and 7.8 µg PEI (N:P, 6:1) were added to a single well, and the plate was incubated in 5% CO₂ at 37°C for 24 h; group 3, pEGFP-N3 plasmid+PEI+NLS peptide (PEI/DNA/NLS), whereby 10 µg pEGFP-N3 plasmid, 7.8 µg PEI (N:P, 6:1) and 120 µg NLS peptide were added to a single well, and the plate was incubated in 5% CO₂ at 37°C for 24 h; group 4, UTMD+pEGFP-N₃ plasmid (UTMD+DNA), whereby 400 µl SonoVue (20% of total volume) and 10 µg pEGFP-N₃ plasmid were added into one well before the plate was exposed to US generated by the UGT 2007 ultrasonic gene transfection apparatus. The probe was placed under the 6-well plate at a distance of 3 to 5 mm, and the intensity of the ultrasound exposure was set at 1.5 W/cm² for 30 sec. The plate was then incubated in 5% CO₂ at 37°C for 24 h; group 5: UTMD+pEGFP-N₃ plasmid+PEI (UTMD+PEI/DNA), whereby 400 µl SonoVue (20% of total volume), 10 µg pEGFP-N₃ plasmid and 7.8 µg PEI (N:P, 6:1) were added to a single well and the plate was exposed to US generated by a UGT 2007 ultrasonic gene transfection apparatus using the same conditions described for the UTMD+DNA group. The plate was then incubated in 5% CO₂ at 37°C for 24 h; group 6, UTMD+pEGFP-N₃ plasmid+PEI+NLS peptide (UTMD+PEI/DNA/NLS), whereby 400 µl SonoVue (20% of total volume), 10 µg pEGFP-N₃ plasmid, 7.8 µg PEI (N:P, 6:1) and 120 µg NLS peptide were placed into a single well and the plate was exposed to the US generated by a UGT 2007 ultrasonic gene transfection apparatus using the aforementioned conditions. The plate was then incubated in 5% CO₂ at 37°C for 24 h. Following 24 h of incubation, the expression of GFP were observed using an inverted microscope (Olympus IX51; Olympus Corporation, Tokyo, Japan).

Flow cytometry analysis. Gene transfection efficiency was examined by flow cytometry analysis (FACS Calibur™; BD Biosciences, Franklin Lakes, NJ, USA). Both the control and treated 293T cells were collected to measure the expression of the plasmid-encoded EGFP gene. Flow cytometry with the excitation setting at 488 nm was used to analyze the transfection efficiency. The 293T cells were digested with 0.25% trypsin and washed three times in 1 ml pre-cooled PBS. At least 10,000 cells were acquired for each test measurement. All experiments were performed in triplicate. The data was analyzed by FlowJo software (version 10; FlowJo, LLC., Ashland, OR, USA).

Evaluation of cytotoxicity using the cell counting kit (CCK)-8 assay. 293T cells were seeded in a 96-well plate at a density of 5×10^3 cells/cm² in 100 µl of growth medium, and incubated for 24 h in 5% CO₂ at 37°C to ensure that the cell adherence rate was ~90%. Prior to transfection, the medium was removed, and the cells were washed with PBS. The cells were then divided into the aforementioned groups and treated accordingly in 100 µl of mixed medium. Experiments performed on each sample were repeated three times. The cells were then incubated with 10 µl CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and 100 µl growth medium for 4 h. Relative cell viability was calculated by measuring the A450 values using a microplate reader (Victor3; PerkinElmer, Inc., Waltham, MA, USA). The viability of untreated control cells was arbitrarily defined as 100%.

Statistical analysis. Variables were normally distributed and presented as the mean ± standard deviation. Differences between the data prior to and following gene transfection were analyzed using the Student's t-test. Comparisons among multiple stages were made using one-way analysis of variance with post hoc analysis by Student-Newman-Keuls test. Statistical analyses were performed using SPSS software (version, 19.0; IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of DNA/PEI complexes and DNA/PEI/NLS complex interactions. Gel retardation experiments were used to measure the DNA binding ability of PEI (Fig. 1). The polymers could neutralize the negative charge of DNA and prevent the DNA from moving to the anode (12). However, plasmid (p)-DNA with complexes were retained in the gel loading well at an N:P ratio >1, indicating that PEI/DNA complexes were formed (Fig. 1). In contrast to the preparation of the PEI/DNA complexes containing 120 µg NLS peptides, even though the PEI/DNA complexes possessed a N:P ratio <2, the PEI/DNA complexes were retained in the gel loading wells. This indicated that the PEI/DNA/NLS complexes were formed, and the NLS peptide exhibited a positive charge.

Effect of PEI and NLS on protection of pDNA from DNase I. To evaluate the ability of the PEI and NLS to protect pDNA from cytosolic nucleases, DNase I was added to the pEGFP-N3 plasmid, DNA/PEI and PEI/DNA/NLS solutions for different durations. The pEGFP-N3 plasmid was digested into small fragments following 5 min of incubation with DNase I, as demonstrated by the smeared bands at the bottom of the gel (lane 2; Fig. 2). By contrast, the PEI/DNA complex was not degraded until 10 min (lane 6), and the PEI/DNA/NLS complex was not degraded until 15 min (lane 10; Fig. 2). This indicated PEI and NLS may have a protective effect on pDNA against degradation by lysosomes and degeneration enzymes in the cytoplasm; however, this function appeared to be time-dependent.

Expression of GFP under an inverted fluorescence microscope. pEGFP-N₃ is an expression vector for the EGFP gene; the presence of which can be observed by detecting GFP expression in 293T cells following transfection under an inverted

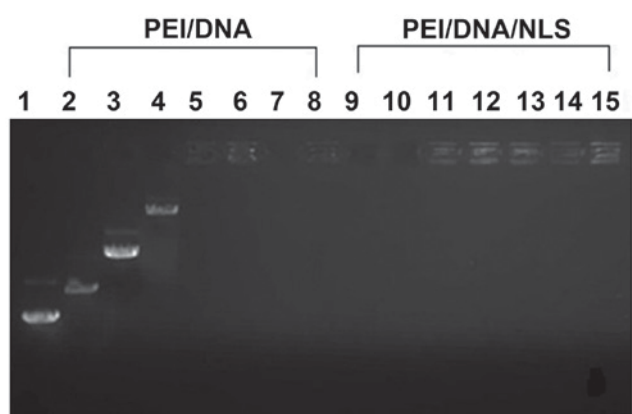


Figure 1. Gel retardation analysis of PEI/DNA complexes generated in the presence or absence of NLS. Lane 1, pEGFP-N₃ plasmid; lanes 2 to 8, PEI/DNA complexes at N:P ratios 0.25:1, 0.5:1, 1:1, 2:1, 4:1, 8:1 and 16:1, respectively; lanes 9 to 15, PEI/DNA complexes with 120 μ g of NLS at weight ratios of at N:P ratios 0.25:1, 0.5:1, 1:1, 2:1, 4:1, 8:1 and 16:1, respectively. PEI, polyethylenimine; NLS, nuclear localization sequence; PEI/DNA, pEGFP-N₃ plasmid plus PEI.

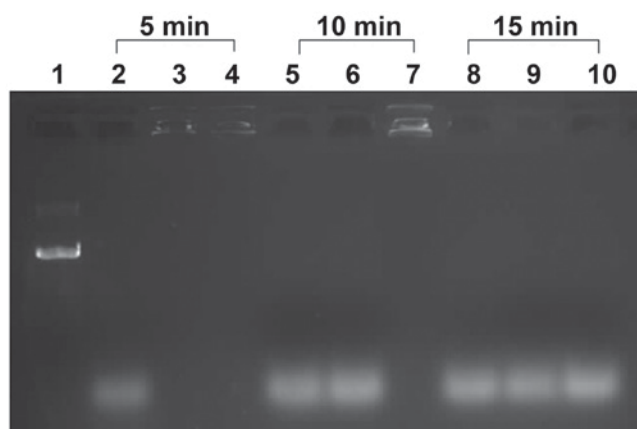


Figure 2. The effect of PEI and NLS on the protection of pDNA from DNase digestion as determined by gel electrophoresis. The pEGFP-N₃ plasmid was incubated with the PEI at N:P ratios of 6:1 and in the presence or absence of 120 μ g NLS. Lane 1, pEGFP-N₃ plasmid; lanes 2 to 4, the pEGFP-N₃ plasmid, PEI/DNA and PEI/DNA/NLS, respectively, following exposure to DNase for 5 min; lanes 5 to 7, the pEGFP-N₃ plasmid, PEI/DNA and PEI/DNA/NLS, respectively, following exposure to DNase for 10 min; lanes 8 to 10, the pEGFP-N₃ plasmid, PEI/DNA and PEI/DNA/NLS, respectively following exposure to DNase for 15 min. PEI, polyethylenimine; NLS, nuclear localization sequence; pDNA, plasmid DNA; DNase, deoxyribonuclease; N:P ratio, PEI nitrogen: DNA phosphate ratio; PEI/DNA, pEGFP-N₃ plasmid plus PEI; PEI/DNA/NLS, pEGFP-N₃ plasmid plus PEI and NLS.

fluorescence microscope. Fig. 3 reveals almost no expression of GFP in the pEGFP-N₃ plasmid-only group (Fig. 3A). By contrast, group 3 exhibited a higher level of green fluorescence when compared with group 2 (Fig. 3B and C), demonstrating that the non-covalent binding of pDNA to NLS may improve the efficiency of transfection. In addition, the highest level of GFP expression was observed in group 6 (Fig. 3). This indicated that UTMD combined with PEI/DNA/NLS complexes may improve the transfection efficiency when compared with PEI/DNA/NLS and UTMD+PEI/DNA.

Effect of UTMD combined with PEI/DNA/NLS complexes on transfection efficiency. Fig. 4 depicts the transfection

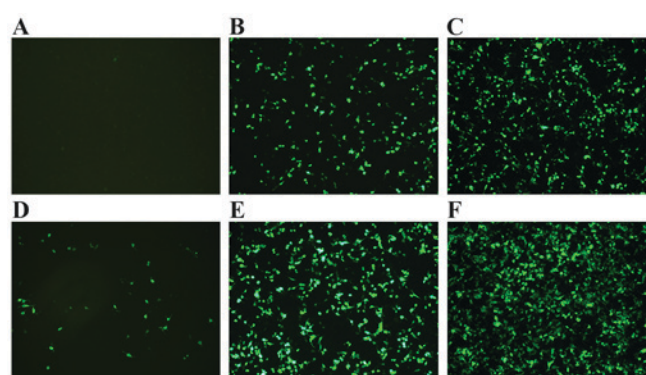


Figure 3. Expression of green fluorescent protein was observed under an inverted fluorescence microscope (magnification, x400). (A) pEGFP-N₃ plasmid, (B) pEGFP-N₃ plasmid with PEI, (C) pEGFP-N₃ plasmid with PEI and NLS, (D) pEGFP-N₃ plasmid treated by UTMD, (E) pEGFP-N₃ plasmid with PEI treated by UTMD, (F) pEGFP-N₃ plasmid with PEI and NLS treated by UTMD. PEI, polyethylenimine; NLS, nuclear localization sequence, UTMD, ultrasound-targeted microbubble destruction.

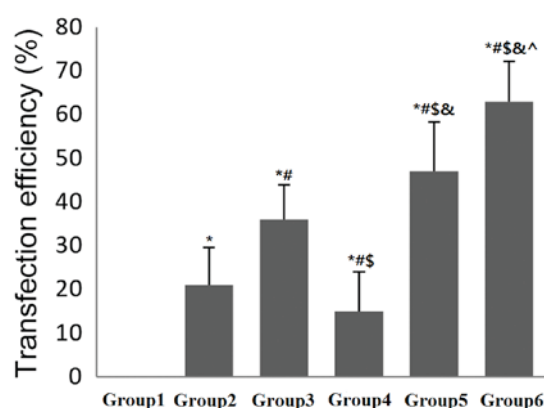


Figure 4. Effect of NLS on PEI/DNA complexes following US-mediated transfection of 293T cells. US was applied at 1.5 W/cm² for 30 sec. Transfection efficiency was detected by flow cytometry following 24 h. *P<0.05 vs. DNA; ^aP<0.05 vs. PEI/DNA; ^bP<0.05 vs. PEI/DNA/NLS; ^cP<0.05 vs. UTMD+DNA; ^dP<0.05 vs. UTMD+PEI/DNA. NLS, nuclear localization sequence; PEI, polyethylenimine; US, ultrasound; UTMD, ultrasound-targeted microbubble destruction; group 1, DNA, pEGFP-N₃ plasmid only; group 2, PEI/DNA, pEGFP-N₃ plasmid with PEI; group 3, PEI/DNA/NLS, pEGFP-N₃ plasmid with PEI and NLS; group 4, UTMD+DNA, pEGFP-N₃ plasmid treated by UTMD; group 5, UTMD+PEI/DNA, pEGFP-N₃ plasmid with PEI treated by UTMD; group 6, UTMD+PEI/DNA/NLS, pEGFP-N₃ plasmid with PEI and NLS treated by UTMD.

efficiency in each group by flow cytometry analysis. All groups displayed a significantly higher level of transfection efficiency when compared with the DNA group (P<0.05). In addition, the transfection efficiency in group 6 was significantly higher when compared with groups 3 and 5, respectively indicating that UTMD combined with PEI/DNA/NLS complexes may express an increased quantity of GFP compared with PEI/DNA/NLS or UTMD+PEI/DNA. These results implied that UTMD combined with PEI/DNA/NLS may be an effective method to increase the expression of the target gene (both P<0.05).

Effect of combining UTMD with PEI/DNA/NLS complexes on cell viability. One of the major requirements for cationic

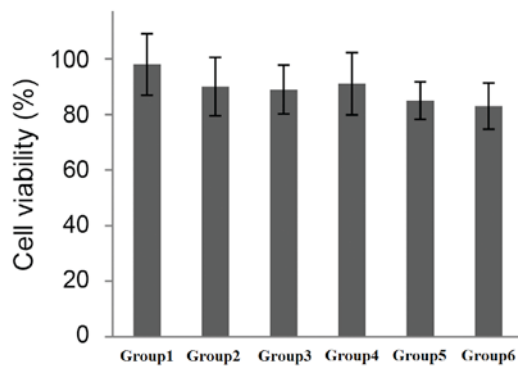


Figure 5. Cytotoxic effect of NLS peptide interaction with PEI/DNA complexes following US mediated transfection of 293T cells. Cell viability was measured using the Cell Counting Kit-8 assay. The viability of the treated cells were expressed as a percentage of untreated controls (100%). NLS, nuclear localization sequence; PEI, polyethylenimine; US, ultrasound; UTMD, ultrasound-targeted microbubble destruction; group 1, DNA, pEGFP-N₃ plasmid only; group 2, PEI/DNA, pEGFP-N₃ plasmid with PEI; group 3, PEI/DNA/NLS, pEGFP-N₃ plasmid with PEI and NLS; group 4, UTMD+DNA, pEGFP-N₃ plasmid treated by UTMD; group 5, UTMD+PEI/DNA, pEGFP-N₃ plasmid with PEI treated by UTMD; group 6, UTMD+PEI/DNA/NLS, pEGFP-N₃ plasmid with PEI and NLS treated by UTMD.

polymer vectors in gene delivery is low cytotoxicity. In the present study, cells in the different groups were incubated for 4 h following treatment, and cell viability was assessed using a CCK-8 assay and a microplate reader. As shown in Fig. 5, cell viability decreased as a result of US irradiation and PEI. However, as the differences were not statistically significant and cell viability was >80% among all groups, the level of cell toxicity was considered to be acceptable (Fig. 5).

Discussion

Successful gene therapy requires the development of technologies capable of transferring exogenous genes into a wide variety of cells and tissues safely and effectively. To enhance exogenous DNA delivery, the therapy must demonstrate the ability to affect membrane permeability, to bind and condense DNA in a reversible manner and to promote nuclear delivery of DNA through non-viral gene delivery methods (25). When considering the above requirements, UTMD presents a number of advantages for gene therapy, as it is capable of reversibly opening the cell membrane in a safe manner, and demonstrates a high targeting ability and visibility (26). Therefore, the present study employed US as the chosen method of transfection. Previous studies have demonstrated that UTMD improves cell membrane permeability (26,27); however, additional studies have suggested that UTMD only facilitates gene entry to the cytoplasm, and not to the nucleus (28,29). These discrepancies may be due to differences in US irradiation frequency, the radiation pattern and experiment selections. However, a previous study confirmed that US irradiation facilitates access of DNA to the nucleus (5). Duvshani-Eshet *et al* (15,16) demonstrated that US induces exogenous genes to enter the nucleus of three different cell types, including baby hamster kidney (BHK) cells, human prostate carcinoma PC-3 cells and basal cell epithelioma (BCE) cells; this is achieved only when the frequency of US irradiation and total radiation energy is

sufficiently high. When compared with the control group, this increased target gene expression to 1,200-fold higher levels, increased the transfection efficiency to 28% and reduced the cell mortality rate to <20%. In addition, several studies have demonstrated that, regardless of whether it is combined with microbubble technology, long-pulse ultrasonic irradiation leads to entry of DNA to the cytoplasm and the nucleus, while maintaining a cell survival rate of >80% (15,16). In the present study, ultrasonic irradiation conditions were set according to the previously described methods (30). PEI was used as a non-viral gene transfer agent, as PEI is cationic and has demonstrated the capacity to produce strong DNA compaction, thus providing effective DNA protection (31). The negatively-charged plasmid DNA may be protected from degradation by PEI during mechanical processes (32). PEI serves an important role in this process of transfection.

To promote nuclear delivery of target DNA in the present study, the SV40 large T antigen (PKKKRKV) was applied as a type of NLS. NLS sequences bind to receptors on the nuclear membrane, thus mediating the delivery of DNA into the nucleus. Previous studies have demonstrated that NLS effectively promote DNA delivery into the nucleus, thus improving exogenous gene expression (33,34); however, a number of studies have provided evidence to suggest that NLS-coupled DNA does not promote the transport of DNA to the nucleus (8). It is possible that the non-covalent coupling method does not effectively combine NLS with DNA, or that the covalent coupling method does not expose the NLS epitope. Although previous studies have confirmed that NLS promotes DNA entry to the nucleus, it may impede gene expression following covalent coupling (8,35). Fixed-point coupling technologies, including peptide nuclear acids (36), triple helix formation oligonucleotides (37) and restriction enzyme recognition domain structures (38), combine the NLS to the outside of the target gene expression cassette and promote DNA delivery to the nucleus without affecting gene expression. However, due to a number of limitations with these techniques, they are unable to be widely used for gene therapy. These limitations include the tedious preparation of the complex, the difficult generation of a connection with exogenous genes and the challenging detection of successful complex construction. Taking the cost into consideration, the present study used NLS peptides combined with PEI/DNA produced by electrostatic adsorption to form the complex, as electrostatic adsorption does not affect the properties of microbubbles, NLS, PEI and DNA. The results indicated that PEI and NLS may demonstrate protective effects on DNA against degradation by lysosomes and degenerative enzyme in the cytoplasm.

The present study applied UTMD as the method of transfection due to the cavitation effect, which reversibly increases the permeability of the cell membrane (15-17). In addition, the surface of the SonoVue microbubbles, cell membranes and DNA are all negatively charged, whereas the PEI and NLS peptide were positively charged. The present study therefore combined the NLS peptide with the PEI/DNA complex by electrostatic interactions, which reduced the cost of peptide synthesis and simplified the procedure. US irradiation is considered to be an alternative approach for non-viral gene delivery, as it increases the opportunity for interactions between non-viral vectors and target cells, thereby facilitating the targeted release of PEI/DNA/NLS

complexes. The destruction of microbubbles stimulates binding of PEI/DNA/NLS complexes to the cell membrane and promotes endocytosis, thus enhancing gene transfection.

The results of the current study indicated that PEI/DNA complexes and PEI/DNA/NLS complexes were formed successfully. In addition, PEI and NLS may demonstrate protective effects on DNA integrity. Although the protective effect was time-dependent, it induced the delivery of more exogenous genes into the nucleus, and this result is consistent with the study by Collins *et al* (39). The results indicated that combining UTMD with the PEI/DNA/NLS complexes may effectively increase the rate of exogenous gene transfection and expression. Even though cell viability was decreased through the mechanical damage of the ultrasonic irradiation and the low level of cytotoxicity of PEI, no significant difference in cell viability in all groups was observed when compared with group 1. In addition, the cell survival rate was >80%. Therefore, the combination of UTMD and PEI/DNA/NLS complexes as a method of gene therapy may be worthy of further investigation in future studies.

The results of the present study indicated that combining UTMD with PEI/DNA/NLS complexes promotes the delivery of target genes and improves the rate of transfection and gene expression effectively. However, further studies are required to clarify the mechanism by which the complex facilitated gene delivery. In addition, as the PEI/DNA/NLS complexes are coated in microbubbles instead of connected to the surface of the microbubbles, further studies are required to determine whether additional genes could be delivered to the cells. In addition, as the present study was conducted *in vitro*, the effect of UTMD combined with PEI/DNA/NLS complexes requires further verification through *in vivo* studies. Finally, the use of liposomes with low or no toxicity requires further investigation.

In conclusion, the results of the current study suggest that UTMD is a simple, effective, non-invasive targeting technology, which has been demonstrated to be a feasible approach to enhance the efficiency of non-viral transfection. The combination of UTMD and PEI/DNA/NLS complexes was developed as a gene delivery method; however, it requires improvement prior to its use in clinical applications. It is a promising technique for gene therapy and may serve an important role in future treatments. The present study successfully produced a UTMD-PEI/DNA/NLS complex gene delivery system, and the results demonstrated that the transfection efficiency of the system was superior to that of UTMD+PEI or PEI/DNA/NLS complexes alone, without subsequently increasing the rate of cell injury. Therefore, it may be an effective method for the clinical application of gene transfection.

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