DNA uptake, intracellular trafficking and gene transfection after ultrasound exposure

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Abstract

Ultrasound has been studied as a promising tool for intracellular gene delivery. In this work, we studied gene transfection of a human prostate cancer cell line exposed to megahertz pulsed ultrasound in the presence of contrast agent and assessed the efficiency of fluorescently labelled DNA delivery into cell nuclei, which is necessary for gene transfection. At the sonication conditions studied, ~30% of cells showed DNA uptake 30 min after sonication, but that fraction decreased over time to ~10% of cells after 24 h. Most cells containing DNA had DNA in their nuclei, but the amount varied significantly. Transfection efficiency peaked at ~10% at 8 h post sonication. Among those cells containing DNA, ~30% of DNA was localized in the cell nuclei, ~30% was in autophagosomes/autophagolysosomes and the remainder was “free” in the cytoplasm 30 min after sonication. At later times up to 24 h, ~30% of DNA continued to be found in the nuclei and most or all of the rest of the DNA was in autophagosomes/autophagolysosomes. These results demonstrate that ultrasound can deliver DNA into cell nuclei shortly after sonication and that the rest of the DNA can be cleared by autophagosomes/autophagolysosomes.

Keywords: acoustic cavitation; DNA uptake; gene transfection; intracellular trafficking; sonoporation; ultrasound sonication.

1. Introduction

Gene transfection of cells requires overcoming both extracellular and intracellular barriers to DNA transport into the cell nucleus. These barriers include the plasma membrane, which is the barrier of intracellular DNA uptake; the cytoskeletal meshwork in the cytoplasm, which hinders DNA trafficking in the cytoplasm; and the nuclear envelope, which limits DNA entry into the nucleus. Therefore, a gene delivery system should facilitate DNA transport across these barriers and into the nucleus to enable transcription. This process needs to be fast, because nucleases in the cytoplasm start to degrade exogenous DNA in minutes [1, 2]. However, passive diffusion of plasmid DNA in the cytoplasm is generally slow, especially for large DNA molecules (e.g., >1000 base pairs), because the multiple cytoskeletal elements (e.g., microfilaments, microtubules and intermediated filaments) in the cytoplasm form a complex and crowded latticework that significantly impedes the diffusion of large molecules [3]. Therefore, DNA trafficking in the cytoplasm after delivery into cells is of interest because it is believed to be a limiting step for non-viral gene delivery methods [4].

Many studies of intracellular DNA trafficking have focused on chemical-based gene delivery systems that employ lipids, polymers and other chemical components [5]. In these methods, multiple trafficking steps are typically required, such as cell attachment, endocytosis and entrapment into endocytic vesicles, maturation of endosomes into lysosomes, escape from vesicular compartments, migration toward the nucleus periphery, dissociation between carriers and exogenous DNA, and finally entry of DNA into the nucleus. There are fewer reports of DNA trafficking studies using physical delivery systems such as electroporation [6], microinjection [7, 8] and ultrasound [9], which involve significantly different pathways because they cause uptake through breaches in the plasma membrane and generally do not rely on endocytosis, although some studies have reported endocytic pathways playing a role [10, 11]. This study seeks to understand the non-endocytic pathways of pDNA trafficking in cells following ultrasound exposure.

Ultrasound (US) is a promising tool for gene therapy that has been shown to facilitate DNA transfection of cells. US-mediated delivery is of interest due to its expected low toxicity, low immunogenicity, potential for repeated application, organ specificity and broad applicability to acoustically accessible organs [12-14]. Various studies have examined gene transfection in different types of cells *in vitro* [15-18] and with various organs and tissues *in vivo*, including skeletal muscle [19-22], brain [23-25], heart [26-28], liver [29] and kidney [30-33]. However, US-mediated gene transfection is still in preclinical development and has the major challenge of relatively low transfection efficiency compared to viral vectors and optimal chemical formulations [34].

Previous studies have shown that gene delivery using viral vectors can be very efficient and has been evaluated in numerous clinical trials, but may be limited by safety concerns and adverse immune responses [35-37]. Lipid and polymer-based formulations have also been used for intracellular gene delivery. While some systems appear to be best suited to *in vitro* studies, a number of such chemical formulations have shown success for delivery *in vivo* [38-40]. Often, these formulations are optimized to increase gene delivery while minimizing cytotoxicity.

US-mediated delivery of DNA into cells offers advantages and limitations compared to other intracellular delivery methods. An advantage is that only acoustic energy is introduced into the cellular environment, which avoids possible safety concerns associated with chemical, viral or other materials introduced and left behind by other methods. Another advantage is that US-mediated delivery has been seen in many different cells types and therefore may be broadly applicable, in contrast to other methods that often require reformulation for specific cell types. A

limitation of the US-based approach is that transfection efficiency is often lower than by other leading methods. This study seeks to address this limitation by examining the pathways of DNA trafficking in cells, which may provide insight into strategies to improve transfection.

Unlike chemical delivery systems, US-mediated DNA uptake is often reported to be non-endocytotic. Acoustic cavitation is believed to play a major role in the cell membrane permeabilization that facilitates DNA uptake [41, 42]. US can possibly deliver plasmid DNA (pDNA) to the periphery of the cell nucleus and facilitate rapid transfection by altering the cytoskeletal network [43, 44]. There is, however, a need to better understand the DNA trafficking pathways in cells after sonication in order to identify the rate-limiting steps that should be addressed to improve transfection efficiency.

In this study, we use confocal microscopy and flow cytometry to analyze the location of pDNA during its intracellular trafficking after US exposure both qualitatively and quantitatively. We consider pDNA localization in the nucleus, in endosomes/lysosomes, in autophagosomes/autophagolysosomes and “free” in the cytoplasm. This study seeks to test the hypothesis that pDNA introduced into the cytoplasm during sonication is either transported rapidly into the nucleus (i.e., within 30 min) or removed by autophagosomes/autophagolysosomes. DNA that reaches and remains in the nucleus can be transcribed and translated to produce its expression product.

2. Materials and methods

2.1. Cell sample preparation

DU145 human prostate cancer cells (American Type Culture Collection, Manassas, VA, item no. HTB-81) in RPMI-1640 medium (Cellgro, Mediatech, Herndon, VA) were cultured as monolayers in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Cellgro).

DU145 cells were harvested by trypsin/EDTA (Cellgro) digestion and re-suspended in RPMI-1640 medium at a final concentration of 1×10^7 cells/mL before sonication. Cell concentration was determined by a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). The pDNA gWiz-GFP (Aldevron, Fargo, ND) encoding green fluorescent protein (GFP) and the gWiz control vector, gWiz Blank (Aldevron) were used for gene delivery and transfection studies. The pDNA was added to the cell suspension at a concentration of 20 µg/mL before sonication. Definity US contrast agent (Bristol-Myers Squibb Medical Imaging, North Billerica, MA) was added to cell samples at a concentration of 1 vol% to serve as cavitation nucleation sites.

2.2. Ultrasound apparatus

Sonication was carried out using an immersible, focused, piezoceramic US transducer (Sonic Concepts, Woodinville, WA, USA, model no. H-101) supplied with matching resistance networks allowing production of sound at 1MHz. The transducer had a diameter of 70 mm, a 52 mm focal length and a 1.5 mm focal width at half-amplitude (-6 dB). A sinewave was provided by two programmable waveform generators (Stanford Research Instruments, Sunnyvale, CA, model no. DS345 and Agilent, Austin, TX, model no. 33120A) and amplified by an RF broadband power amplifier (Electronic Navigation Industries, Rochester, NY, model no. 3100LA).

The transducer was submerged in deionized and partially degassed water at 37 °C placed in a polycarbonate tank (30.5 x 29 x 37 cm) to sonicate a 375 µL sample held within a disposable micropipette (Samco, San Fernando, CA). A 5 cm thick acoustic absorber (SC-501 Acoustic Rubber, Sonic Concepts) was placed opposite the transducer in the tank to minimize standing-

wave formation. A three-axis positioning system (10 μm resolution, Velmex, Bloomfield, NY) was mounted on top of the tank to position samples and a hydrophone at desired locations in the tank.

The US transducer was calibrated versus the peak-to-peak voltage of the signal by a PVDF membrane hydrophone (NTR Systems, Seattle, WA, model no. HMA-0200) at a distance of 1 cm from the transducer. Sonication was carried out at a peak positive amplitude pressure of 0.78 MPa and total treatment time of 1 min with a burst length of 0.25 ms and a duty cycle of 25%. The corresponding acoustic energy fluence was therefore 306 J/cm². Additional characterization of the US apparatus has been reported previously [45].

2.3. *Fluorescent probes*

To measure pDNA uptake efficiency and track the localization of pDNA in the cytoplasm, MirusLabelIT Tracker Cy3 kit and Cy5 kit (Mirus, Madison, WI) were used to label pDNA before sonication. The labeling reaction was conducted according to the procedure recommended by the manufacturer.

Hoechst 33342 (trihydrochloride, Invitrogen, Carlsbad, CA) was added to cell samples at a final concentration of 10 $\mu\text{g/mL}$ for 10 min at room temperature to stain cell nuclei.

LysoTracker Green DND-26 (Molecular Probes, Invitrogen) was used to label acidic organelles in live cells. The reagent was added to cells in growth medium at a final concentration of 50 nM and incubated for 5 min at growth conditions. Cells were then washed three times before analysis.

EEA-1 (final dilution ratio 1:250, BD Bioscience, San Jose, CA), CD63 (final dilution ratio 1:10, Developmental Studies Hybridoma Bank, Iowa City, IA) and LAMP-1 (final dilution ratio 1:5, Developmental Studies Hybridoma Bank) were used as primary antibodies to label early endosomes, late endosomes and lysosomes, respectively. Alexa fluor 488 Donkey anti-Mouse A-21202 (Invitrogen) was used as the secondary antibody at a final concentration of 2 $\mu\text{g/mL}$. Cells were plated on a coverslip (VWR International, West Chester, PA), fixed in 2% formaldehyde for 10 min at room temperature and washed twice with phosphate-buffered saline (PBS). Cells were treated with 10% FBS and 0.02%(w/v) sodium azide in PBS for 5min at room temperature and washed twice with PBS. Cells were then incubated with the primary antibody diluted in PBS with 10% FBS and 0.2% saponin for 1 h in darkness at room temperature, and washed twice in PBS with 10% FBS. After that, cells were incubated with the secondary antibody diluted in PBS with 10% FBS and 0.2% saponin for 1h in darkness at room temperature, and washed twice in PBS with 10% FBS.

2.4. *Flow cytometry*

In this study, we used flow cytometry as a means to acquire data from large populations of cells, but without spatial resolution. We coupled that with data from fluorescence microscopy to obtain data with spatial resolution, but only from small populations of cells. Uptake of pDNA, transfection efficiency and cell viability were determined by a BD LSR benchtop flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed by FCS Express V3 (De Novo Software, Los Angeles, CA). Typical analyses sampled approximately 10,000 cells. Samples were excited with a 488 nm laser to measure GFP expression (i.e., GFP fluorescence) with a 530/30 nm bandpass filter for emission. A 633 nm laser was used to measure uptake of pDNA labeled with Cy5 (i.e., Cy5 fluorescence) using a 660/20 nm bandpass filter for emission. Cell populations were first elucidated by gating (e.g., relative to “sham” exposure controls that

received the same experimental protocols, but ultrasound was not applied, i.e., the negative control), and histogram data were then analyzed to determine % population.

We considered the use of two different cell populations as the negative control. One choice was untreated cells (i.e., no exposure to DNA or other dyes, no exposure to ultrasound). The other was sham-sonicated cells (i.e., exposure to fluorescent dyes, no exposure to ultrasound). We selected sham-sonicated cells as the most appropriate negative control, because this population has fluorescence associated with surface binding of fluorescent molecules, cell autofluorescence and any other signals not associated with the US exposure. Indeed, if one compares the fluorescence of sham-sonicated cells to untreated cells, the sham-sonicated cells have greater fluorescence (data not shown), indicating the importance of accounting for this non-US-associated fluorescence.

The viability of DU145 cells after sonication was determined by comparing the cell concentration in sonicated samples to that in the sham-exposure controls. Briefly, viable cells were counted in each sample, normalized based on the fluid volume analyzed by the flow cytometer and then normalized to the viability of sham-sonicated control samples. The analysis time of a sample in the flow cytometer was used as a measure of the sample volume analyzed, since the flow cytometer operated at a constant flow rate. Transfection efficiency and pDNA uptake were measured by determining the percentage of cells with the corresponding fluorescence greater than a threshold value based on sham-sonicated control cells. Flow cytometry methods have been described with additional detail previously [45].

2.5. Confocal microscopy

A Zeiss LSM 510 confocal laser-scanning microscope or Zeiss LSM META/NLO 510 multiphoton microscope (Zeiss, Thornwood, NY) was used to image pDNA uptake and GFP expression. The lasers for blue, green and red fluorescence were ultraviolet (wavelength 364 nm), argon (wavelength 488 nm) and helium-neon (wavelength 543 nm). Images were captured through a 60x magnification oil objective. Cell samples were viewed on a 25 mm round glass cover slip in an Attotfluor Cell Chamber (Molecular Probes, Invitrogen). Zen software (Zeiss) and Volocity 3DM software (Improvision, Lexington, MA) were used for 3D image analysis to identify and quantify co-localization of signals.

2.6. Statistical analysis

A minimum of three replicates was performed for all samples. Replicates were used to calculate experimental means and standard deviations (SD). Data points acquired by microscopy represent the average $\pm$ SD of three biological samples with at least five images for each. In total, more than 100 cells were examined by microscopy at each time point. Analysis of variance (ANOVA, $p < 0.05$) was used to determine statistical significance of the data.

3. Results

3.1. Heterogeneous bioeffects caused by sonication

We first looked at the heterogeneity of cellular bioeffects by imaging cells using confocal microscopy 8 h after sonication. We assume that most of these cells were viable, because nonviable cells had lifted off the glass coverslip during incubation. Three conditions were observed (Figure 1): cells with pDNA uptake (cells with red fluorescence), cells with GFP transfection (cells with green fluorescence) and cells with neither of these bioeffects (e.g., like sham-sonicated controls).

Figure 1. Confocal micrograph showing Cy3-labeled pDNA uptake (red) and GFP expression (green) in DU145 prostate cancer cells. Cells were harvested after incubation for 8 h after sonication and stained with a nuclear counterstain, Hoechst 33342 (blue).

3.2. *Co-localization of pDNA with nuclei after sonication*

Transfection requires pDNA entry into the nucleus. We therefore distinguished between pDNA outside the nucleus and pDNA co-localized within the nucleus, which is believed to represent pDNA within the nucleus or possibly on its surface. When imaged by confocal microscopy 30 min after sonication, pDNA was seen in many cells, largely in the cytosol and to some degree co-localized with the nucleus (Figure 2a). This rapid delivery of pDNA apparently into the nucleus is notably different from chemical-based transfection methods relying on endocytic pathways, which generally require hours of pDNA to reach the nucleus [46].

Figure 2. Representative microscopy images (two images at each time point) of Cy3-labeled pDNA (red) and cell nuclei (blue) 30 min (a), 8 h (b), 16 h (c) and 24 h (d) after sonication. The yellow pseudo-color indicates co-localization of pDNA and nuclei.

Initially, the pDNA appeared as many small red dots under the confocal microscope (Figure 2a). Over time, the red dots became fewer in number, larger in size and often located in the perinuclear region (Figure 2b, c), suggesting pDNA segregation and accumulation, possibly in organelles. Eventually, very little pDNA was evident in the cells (Figure 2d), presumably indicating pDNA loss due to degradation or cell division.

3.3. Quantitative analysis of cells containing pDNA

Using both confocal microscopy and flow cytometry, we quantified the distribution of cells over time among cells containing DNA (Figure 3a), cells with DNA in their nuclei (Figure 3b) and cells with GFP expression (Figure 3c). Thirty minutes after sonication, $32.2 \pm 4.9\%$ (mean $\pm$ SD) of cells contained pDNA when measured by flow cytometry (black triangles, Figure 3a) and $15.0 \pm 3.0\%$ of cells contained pDNA when measured by confocal microscopy (white triangles, Figure 3a). This difference was significant (two-way ANOVA, $p < 0.05$) and can probably be explained by the greater sensitivity of flow cytometry. We previously noted that the red dots indicating pDNA were small in size and weak in light intensity when observed 30 min after sonication, which made it difficult to see with the naked eye (Figure 2a). We therefore believe that the flow cytometry measurement is more accurate.

Figure 3. Extent and kinetics of pDNA uptake (a) into cells, (b) into the nucleus and (c) resulting in GFP expression after sonication. (a) Fraction of cells containing Cy5-labeled pDNA after sonication determined by flow cytometry (black triangles, $n \geq 4$ replicates, $\sim 10,000$ events per sample) and confocal microscopy (white triangles). (b) Fraction of cells containing Cy3-labeled pDNA co-localized with the nucleus after sonication determined by confocal microscopy. (c) Fraction of cells expressing GFP after sonication due to transfection with Cy5-labeled pDNA (black rectangles) and unlabeled pDNA (white rectangles) determined by flow cytometry ($n \geq 4$ replicates, $\sim 10,000$ events per sample). In (a) and (b), data points from confocal microscopy image analysis were each obtained from 3 biological samples with ≥ 5 fields of cells imaged from each sample and a total of >100 cells analyzed at each time point. All data points represent the average $\pm$ SD.

Over time, the fraction of cells containing pDNA decreased to $\sim 10\%$ after 24 h (one-way ANOVA, $p < 0.01$, Figure 3a) possibly due to pDNA degradation and cell division. At these later time points, the flow cytometry and confocal microscopy data were no longer significantly different (two-way ANOVA, $p > 0.05$). This may be explained by the appearance of larger, brighter red dots indicating pDNA at later time points (Figure 2b, c), which made their detection by confocal microscopy more reliable.

We used confocal microscopy and 3D image analysis to count cells containing pDNA co-localized with nuclei and interpreted this as cells containing pDNA in their nuclei. Because determination of co-localization required spatial information, this analysis could only be done by

microscopy and not by flow cytometry. Previous work suggested that cells only need three plasmid copies per nucleus to express detectable transgene product [47], which means that detection of any pDNA in the nucleus could be significant.

Four hours after sonication, $14.0 \pm 8.9\%$ of cells had pDNA in the nuclei (Figure 3b). This value is lower than the $17.7 \pm 5.6\%$ of cells that had pDNA anywhere in the cell (Figure 3a, measured by microscopy), but this difference is not significant (Student's t-test, $p > 0.05$). Over time up to 16 h, there was no significant change in the fraction of cells containing pDNA in the nucleus (one-way ANOVA, $p > 0.05$), but after 24 h this value dropped to $4.6 \pm 0.1\%$ of cells.

It is notable that the fraction of cells containing pDNA decreased, but the fraction of cells containing pDNA in the nucleus remained constant over the first 16 h. At the 16 h time point, the fraction of cells containing pDNA ($15.3 \pm 1.1\%$, Figure 3a) and those containing pDNA in the nucleus ($11.2 \pm 10.7\%$, Figure 3b) were almost the same (Student's t-test, $p > 0.05$). This suggests that cells containing pDNA only in the cytoplasm lost that pDNA within 16 h, but cells containing pDNA in the nucleus retained a significant amount of pDNA. This observation could be explained by removal of pDNA from the cytoplasm, perhaps by active processes that are not operative in the nucleus [48].

Gene transfection was observed as early as 4 h after sonication (Figure 3c). Transfection efficiency peaked at 8 h ($12.4 \pm 2.2\%$ of cells) and then decreased over time (one-way ANOVA, $p < 0.01$). These kinetics are in agreement with results found in previous studies of US-mediated transfection [49, 50]. It is notable that the fraction of cells with pDNA in the nucleus and the fraction of cells expression GFP are not significantly different (two-way ANOVA, $p > 0.05$), suggesting that cells with pDNA delivered to their nuclei expressed the reporter protein. The kinetics of reporter protein expression within 3.5 h after pDNA entry into the nucleus (i.e., pDNA uptake seen at 30 min and reporter protein expression seen at 4 h) is consistent with previous findings [49].

The transfection data discussed immediately above is for unlabeled pDNA delivered to cells (white squares, Figure 3c). When pDNA labeled with Cy5 was used (black squares, Figure 3c), the transfection efficiency was significantly lower (two-way ANOVA, $p < 0.01$), probably because the Cy5 labeling interfered with the transfection process, which has been reported before [51].

3.4. Quantitative analysis of pDNA distribution in cells

We more closely examined the distribution of pDNA within cells by quantifying the fraction of pDNA located in the nucleus, in endosomes/lysosomes, in autophagosomes/autophagolysosomes and “free” in the cytoplasm. Additional analysis of the confocal microscopy images (Figure 2) allowed us to determine what fraction of the pDNA in each cell was co-localized with nuclei. After sonication, ~30% of intracellular DNA was co-localized with the nucleus, and this value did not significantly change over the course of the study (Figure 4, one-way ANOVA, $p > 0.05$). This indicates that while the majority of intracellular pDNA remained in the cytoplasm, a large fraction was transported into the nucleus within 30 min after sonication.

Figure 4. Fraction of Cy3-labeled pDNA that co-localized with cell nuclei as a function of time after sonication. Data points represent the average $\pm$ SD of 3 biological samples with ≥ 5 fields of cells imaged from each sample and a total of >100 cells analyzed at each time point.

To better understand the heterogeneity of nuclear co-localization of pDNA on a cell-by-cell basis, we randomly picked individual cells with pDNA uptake at various time points, measured the fraction of pDNA in the nucleus and displayed the results as histograms (Figure 5). The resulting distributions are highly non-Gaussian and different at different time points. Even though we collected data from only a limited number of cells, it is clear that there is not a single distribution about a common mean value. As a comparison between this single-cell analysis (Figure 5) and the whole-image analysis (Figure 3), we found that the geometric means of the histograms generated by single-cell analysis are all 25 - 30% of pDNA in the nucleus. These values are similar to the results from the whole-image analysis, which shows that the two analysis methods are generally self-consistent.

Further examination of the histograms from single-cell analysis shows that almost all of the cells with pDNA uptake had at least some pDNA in the nucleus, which is consistent with findings from the whole-image analysis (Figure 3). However, the single-cell analysis further shows that 30 min after sonication, cells had a broad and relatively flat range of pDNA delivery efficiency into the nucleus, ranging from 0% to 80% of intracellular pDNA (Figure 5a). Over time, the distribution became more bimodal, such that cells either had little or no pDNA in the nucleus or they had most or all pDNA in the nucleus cells (Figure 5b-d).

Figure 5. Histograms showing the fraction of intracellular Cy3-labeled pDNA in the cell nucleus in individual cells. Cells were examined by confocal microscopy at (a) 30 min ($n = 29$ replicates), (b) 8 h ($n = 50$), (c) 16 h ($n = 19$) and (d) 24 h ($n = 25$) after sonication.

3.5. Quantitative analysis of pDNA trafficking in cytoplasm after sonication

We previously noted that pDNA delivered into the cytoplasm was typically found segregated and accumulated in the perinuclear region and hypothesized that the pDNA may be sequestered within organelles. To assess this hypothesis, we used specific antibodies to label early endosomes, late endosomes and lysosomes separately in cells incubated for up to 24 h after sonication, and looked for the co-localization of pDNA with these labeled organelles.

Confocal microscopy showed little or no co-localization between pDNA and early endosomes (Figure 6a) or late endosomes (Figure 6b) at all the time points after sonication. In most cases, pDNA was also not found co-localized with lysosomes (Figure 6c), except for two co-localization cases seen among in the ~700 cells imaged (Figure 6d). These results indicate that US-mediated delivery of pDNA did not appear to involve endocytic processes.

We also used LysoTracker to broadly label acidic organelles including endosomes/lysosomes and autophagosomes/autophagolysosomes. Co-localization between these organelles and pDNA was found within 30 min after US exposure and then increased over time (Figure 7). These observations indicate that pDNA in the cytoplasm became entrapped by autophagosomes/autophagolysosomes and possibly transported or degraded by them, since pDNA was not found to co-localize with endosomes or lysosomes (Figure 6).

Figure 6. Representative confocal microscopy images of Cy3-labeled pDNA (red) and antibody-labeled early endosomes (a), late endosomes (b) and lysosomes (c, d) (all green) in cells 8 h after sonication. The yellow color indicates co-localization of pDNA and endosomes/lysosomes. Scale bars are 10 μ m.

Figure 7. Representative confocal microscopy images of Cy3-labeled pDNA (red) and LysoTracker®-labeled endosomes/lysosomes and autophagosomes/autophagolysosomes (green) in cells 30 min, 2 h, 4 h and 24 h after sonication. The yellow color indicates co-localization of pDNA and labeled vesicles (also indicated by arrows).

We determined the fraction of cytosolic pDNA co-localized with autophagosomes/autophagolysosomes and combined these results with the fraction of pDNA co-localized with the cell nuclei (Figure 8). This combined analysis shows that 30 min after sonication, $27.4 \pm 2.2\%$ of pDNA was co-localized with cell nuclei, $27.4 \pm 19.0\%$ was co-localized with autophagosomes/autophagolysosomes, and $45.2 \pm 10.6\%$ was “free” in the cytoplasm. Between 4 h and 16 h after sonication, the fraction of pDNA co-localized with cell nuclei remained at $\sim 30\%$, but the fraction of pDNA co-localized with autophagosomes/autophagolysosomes increased to $\sim 90\%$, leaving only $\sim 10\%$ “free” in the cytoplasm. Finally, after 24 h, the fraction of pDNA co-localized with cell nuclei was still $\sim 30\%$ and all the rest of the pDNA was co-localized with autophagosomes/autophagolysosomes (i.e., there was essentially no pDNA “free” in the cytoplasm).

4. Discussion

4.1. *pDNA Trafficking in cells associated with transfection*

We studied the ability of US to deliver pDNA into cell nuclei by quantitative analysis of confocal micrographs and, in some cases, flow cytometry. The results support our hypothesis that pDNA introduced into the cytoplasm during sonication is either transported into the nucleus within 30 min or removed by autophagosomes/autophagolysosomes, and pDNA that reaches and remains in the nucleus is efficiently transcribed and translated to produce its expression product. We found that within 30 min after sonication, up to $\sim 30\%$ of cells had pDNA uptake and most had a portion of the pDNA already in the nuclei (Figure 3). Overall, $\sim 30\%$ of intracellular pDNA was in the nuclei, $\sim 30\%$ was in autophagosomes/autophagolysosomes and the rest was “free” in the cytoplasm (Figure 8).

Figure 8. Fraction of Cy3-labeled pDNA that co-localized with cell nuclei (black bar), co-localized with autophagosomes/autophagolysosomes (grey bar) and were “free” in the cytoplasm (white bar). Data points represent the average $\pm$ SD of 3 biological samples with ≥ 5 fields of cells imaged from each sample and a total of >100 cells analyzed at each time point.

After 4 h, the percentage of cells with pDNA uptake decreased to $\sim 20\%$ and roughly half of those cells (i.e., $\sim 10\%$ of all cells) exhibited expression of the reporter protein GFP (Figure 3). The percentage of intracellular pDNA remained $\sim 30\%$ in the nuclei, but increased to $\sim 60\%$ in autophagosomes/autophagolysosomes, leaving just $\sim 10\%$ “free” in cytoplasm (Figure 8). At later times up to 24 h, the percentage of cells with pDNA uptake decreased to $\sim 10\%$, with most of these cells exhibiting GFP expression (Figure 3). pDNA continued to be distributed $\sim 30\%$ in the nuclei and most or all of the rest in autophagosomes/autophagolysosomes (Figure 8).

US was found to deliver pDNA into cell nuclei within 30 min in our study and other studies [52]. This could explain the rapid gene transfection observed within 4 h after sonication, compared to the kinetics of gene transfection mediated by chemical vectors like liposomes, which typically takes ~ 3 times longer before seeing gene expression [49]. Over time, we observed a decrease in the fraction of cells containing pDNA, possibly due to pDNA degradation and cell division. The maximum GFP expression was observed 8 h after sonication and then transfection efficiency decreased significantly after 24 h, possibly due to pDNA degradation, cell division and the limited lifetime of GFP in mammalian cells [53].

These results suggest that pDNA delivery into the nucleus during and shortly after sonication were critical to gene transfection hours later. It appears that if pDNA got into the nucleus initially, it was transcribed and translated (Figure 3) and pDNA initially in the cytoplasm appeared not to be transported into the nucleus later, but was instead cleared by autophagosomes/autophagolysosomes (Figure 8).

4.2. Role of autophagosomes/autophagolysosomes

Although pDNA transfection mediated by US did not involve endocytosis (Figure 6), pDNA was believed to be entrapped in large vesicles like autophagosomes and autophagolysosomes (Figure 7), which were generally thought to originate randomly throughout the cytoplasm from the endoplasmic reticulum [54], the post-Golgi compartment or the phagophore [55].

The co-localization of pDNA and autophagosomes/autophagolysosomes was observed as early as 30 min after sonication (Figure 7 and 8), where 27% of pDNA was co-localized with autophagosomes/autophagolysosomes and 45% of pDNA appeared to be “free” in the cytoplasm (Figure 8). Four hours after sonication, almost all of the pDNA in the cytoplasm was co-localized with autophagosomes/autophagolysosomes. We expect that autophagosomes/autophagolysosomes degrade and clear pDNA from the cell [56], which is detrimental to transfection. Although autophagosomes/autophagolysosomes are reported to move bi-directionally along microtubules towards and away from the cell nuclei with the help of the motor protein dynein [57], our study does not indicate the co-localization of pDNA and autophagosomes/autophagolysosomes facilitates the active transport of pDNA to the cell nucleus, as suggested in other studies that gene carriers could possibly be transported to the cell nucleus along microtubules [8, 58, 59]. Further study is needed to better understand how co-localization of pDNA and autophagosomes/autophagolysosomes affects the pDNA trafficking and transfection efficiency in US-mediated gene transfection.

4.3. *Strategies to increase pDNA transfection*

This study suggests that there are a number of rate-limiting steps that could be addressed to increase pDNA transfection efficiency by sonication:

- (i) pDNA was delivered into only ~30% of cells exposed to US.
- (ii) Only ~30% of intracellular pDNA reaches the nucleus, and this level of efficiency is highly variable between cells.
- (iii) Over time, pDNA disappears from the nucleus and transfection levels drop correspondingly.

These observations suggest opportunities to increase pDNA transfection by US.

- (i) pDNA transfection can be improved by increasing the fraction of cells that receive intracellular pDNA and survive. As shown in previous studies, it is difficult to achieve efficient uptake while maintaining high cell viability [60], apparently due to the cavitation mechanism of US-mediated transfection, which leads to heterogeneous bioeffects. It is believed that cells proximal to cavitating microbubbles are mechanically destroyed, cells at mid-distances from microbubbles exhibit reversible poration and pDNA uptake, and cells far from microbubbles remain unaffected [61, 62]. Given the known trade-off between increased uptake and cell viability, strategies to protect cell viability under conditions that deliver pDNA effectively into cells may be useful. Some studies have shown the possibility to increase cell viability after US exposure by chelation of intracellular Ca^{2+} and supplementation with ATP energy [62, 63].
- (ii) It appears that there is a competition between pDNA transported to the nucleus and pDNA sequestration and clearance by autophagosomes/autophagolysosomes. Thus, methods that increase/target pDNA trafficking to the nucleus by enhancing active transport [59, 64], and nuclear import [65, 66] and methods that decrease clearance by inhibiting autophagosomes/autophagolysosomes [67] may be useful. Whether these approaches can enhance US-mediated gene transfection needs further investigation [45, 68].
- (iii) Disappearance of pDNA from the nucleus may be associated with loss of nuclear structural integrity during cell division. This is suggested by pDNA loss from the nucleus on a time scale of 24 h, which is comparable to time scale of the DU145 cell cycle [69]. Thus, slowly dividing cells in the body (as opposed to rapidly dividing cancer cell culture used in this study) may retain DNA in the nucleus longer and thereby sustain expression levels longer.

4.4. Significance of findings more broadly

This study was carried out in DU145 prostate cancer cells using a representative ultrasound condition that causes transient cavitation. We hypothesize that the general findings of trafficking mechanisms discussed here may be relevant more broadly to other mammalian cells exposed to other ultrasound conditions that cause transient cavitation. This is because the literature shows that ultrasound causes intracellular delivery and DNA transfection in many different cell types over a similar range of ultrasound conditions [70-72]. Transfection has also been reported at conditions with little or no cavitation or with stable cavitation [73, 74]; DNA uptake and trafficking mechanisms are more likely to be different under these conditions and may involve endocytosis [10, 11]. Additional research is needed to assess how broadly the observations made here can be applied.

4.5. Comments on experimental methods

Flow cytometry is expected to be more sensitive at measuring the fraction of cells with pDNA uptake, compared to microscopy, where uptake cells were identified by the naked human eye. This could explain the difference between uptake efficiencies by flow cytometry and confocal microscopy reported in Figure 3a. To determine the fraction of cell nuclei containing pDNA (Figure 3b), three-dimensional image analysis was used to quantify the co-localization and therefore provided a better method than counting cell nuclei by eye.

It is also worth noting that the Cy3/Cy5 labeling process could alter pDNA and its trafficking, localization, uptake and transfection [51]. For instance, the fluorophores were randomly attached to the plasmids with an alkylating aromatic nitrogen mustard [75] and the bulky fluorophores that coat the plasmid may block transcription factors from binding to the pDNA [76]. This could explain the fact that transfection efficiency of unlabeled pDNA was higher than labeled pDNA (Figure 3c). The use of a peptide nucleic acids designed to bind specifically to a certain sequence of pDNA might be able to address this problem [51].

Finally, this study only assessed cell viability by exclusion of propidium iodide shortly after US exposure. It is known that there can be longer-term effects of US exposure, such as apoptosis [63, 77]. The possible effects of these longer-term events on DNA trafficking was beyond the scope of this study.

4.6. Summary of findings

Although further study is needed, these data suggest the following sequence of events. Sonication initially delivers pDNA into ~30% of cells exposed to US. In many of those cells, significant amounts of pDNA reach the nucleus within 30 min. Overall, ~30% of pDNA that is delivered into cells makes it into the nucleus within 30 min and the rest is removed from the cytoplasm by autophagosomes/autophagolysosomes. Over time, the fraction of cells containing pDNA decreases to 10% after 24 h. Most cells that contain pDNA in the nucleus express that pDNA, which peaks 8 h after sonication.

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References

- [1] H. Pollard, G. Toumaniantz, J.L. Amos, H. Avet-Loiseau, G. Guihard, J.P. Behr, D. Escande, Ca²⁺-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids, *J Gene Med*, 3 (2001) 153-164.
- [2] D. Lechardeur, K.J. Sohn, M. Haardt, P.B. Joshi, M. Monck, R.W. Graham, B. Beatty, J. Squire, H. O'Brodovich, G.L. Lukacs, Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer, *Gene Ther*, 6 (1999) 482-497.
- [3] G.L. Lukacs, P. Haggie, O. Seksek, D. Lechardeur, N. Freedman, A.S. Verkman, Size-dependent DNA mobility in cytoplasm and nucleus, *J Biol Chem*, 275 (2000) 1625-1629.
- [4] E.E. Vaughan, J.V. DeGiulio, D.A. Dean, Intracellular trafficking of plasmids for gene therapy: mechanisms of cytoplasmic movement and nuclear import, *Curr Gene Ther*, 6 (2006) 671-681.
- [5] C. Pichon, L. Billiet, P. Midoux, Chemical vectors for gene delivery: uptake and intracellular trafficking, *Curr Opin Biotech*, 21 (2010) 640-645.
- [6] M. Golzio, J. Teissié, M.-P. Rols, Direct visualization at the single-cell level of electrically mediated gene delivery, *Proc Natl Acad Sci USA*, 99 (2002) 1292-1297.
- [7] G.L. Wilson, B.S. Dean, G. Wang, D.A. Dean, Nuclear import of plasmid DNA in digitonin-permeabilized cells requires both cytoplasmic factors and specific DNA sequences, *J Biol Chem*, 274 (1999) 22025-22032.
- [8] E.E. Vaughan, R.C. Geiger, A.M. Miller, P.L. Loh-Marley, T. Suzuki, N. Miyata, D.A. Dean, Microtubule acetylation through HDAC6 inhibition results in increased transfection efficiency, *Mol Ther*, 16 (2008) 1841-1847.
- [9] M. Duvshani-Eshet, L. Baruch, E. Kesselman, E. Shimoni, M. Machluf, Therapeutic ultrasound-mediated DNA to cell and nucleus: bioeffects revealed by confocal and atomic force microscopy, *Gene Ther*, 13 (2006) 163-172.
- [10] D.M. Paula, V.B. Valero-Lapchik, E.J. Paredes-Gamero, S.W. Han, Therapeutic ultrasound promotes plasmid DNA uptake by clathrin-mediated endocytosis, *J Gene Med*, 13 (2011) 392-401.
- [11] B.D. Meijering, L.J. Juffermans, A. van Wamel, R.H. Henning, I.S. Zuhorn, M. Emmer, A.M. Versteilen, W.J. Paulus, W.H. van Gilst, K. Kooiman, N. de Jong, R.J. Musters, L.E. Deelman, O. Kamp, Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation, *Circ Res*, 104 (2009) 679-687.
- [12] R. Suzuki, Y. Oda, N. Utoguchi, K. Maruyama, Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles, *J Control Release*, 149 (2011) 36-41.
- [13] C.S. Yoon, J.H. Park, Ultrasound-mediated gene delivery, *Expert Opin Drug Del*, 7 (2010) 321-330.
- [14] V. Zderic, Ultrasound-enhanced drug and gene Delivery: A Review, *IEEE Eng Med Bio*, (2008) 4472-4472.
- [15] A.J. Fischer, J.J. Stanke, G. Omar, C.C. Askwith, R.W. Burry, Ultrasound-mediated gene transfer into neuronal cells, *J Biotechnol*, 122 (2006) 393-411.
- [16] V.G. Zarnitsyn, M.R. Prausnitz, Physical parameters influencing optimization of ultrasound-mediated DNA transfection, *Ultrasound Med Biol*, 30 (2004) 527-538.
- [17] H.D. Liang, Q.L. Lu, S.A. Xue, M. Halliwell, T. Kodama, D.O. Cosgrove, H.J. Stauss, T.A. Partridge, M.J. Blomley, Optimisation of ultrasound-mediated gene transfer (sonoporation) in skeletal muscle cells, *Ultrasound Med Biol*, 30 (2004) 1523-1529.
- [18] M.S. Michel, P. Erben, L. Trojan, A. Schaaf, K. Kiknavelidze, T. Knoll, P. Alken, Acoustic energy: a new transfection method for cancer of the prostate, cancer of the bladder and benign kidney cells, *Anticancer Res*, 24 (2004) 2303-2308.

- [19] J.P. Christiansen, B.A. French, A.L. Klibanov, S. Kaul, J.R. Lindner, Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles, *Ultrasound Med Biol*, 29 (2003) 1759-1767.
- [20] T. Li, K. Tachibana, M. Kuroki, Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison, and Levovist in mice--initial results, *Radiology*, 229 (2003) 423-428.
- [21] S.V. Pislaru, C. Pislaru, R.R. Kinnick, R. Singh, R. Gulati, J.F. Greenleaf, R.D. Simari, Optimization of ultrasound-mediated gene transfer: comparison of contrast agents and ultrasound modalities, *Eur Heart J*, 24 (2003) 1690-1698.
- [22] X. Wang, H.D. Liang, B. Dong, Q.L. Lu, M.J. Blomley, Gene transfer with microbubble ultrasound and plasmid DNA into skeletal muscle of mice: comparison between commercially available microbubble contrast agents, *Radiology*, 237 (2005) 224-229.
- [23] Y. Manome, N. Nakayama, K. Nakayama, H. Furuhashi, Insonation facilitates plasmid DNA transfection into the central nervous system and microbubbles enhance the effect, *Ultrasound Med Biol*, 31 (2005) 693-702.
- [24] N. Sheikov, N. McDannold, N. Vykhodtseva, F. Jolesz, K. Hynynen, Cellular mechanisms of the blood-brain barrier opening induced by ultrasound in presence of microbubbles, *Ultrasound Med Biol*, 30 (2004) 979-989.
- [25] M. Shimamura, N. Sato, Y. Taniyama, S. Yamamoto, M. Endoh, H. Kurinami, M. Aoki, T. Ogihara, Y. Kaneda, R. Morishita, Development of efficient plasmid DNA transfer into adult rat central nervous system using microbubble-enhanced ultrasound, *Gene Ther*, 11 (2004) 1532-1539.
- [26] R. Bekeredjian, S. Chen, P.A. Frenkel, P.A. Grayburn, R.V. Shohet, Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart, *Circulation*, 108 (2003) 1022-1026.
- [27] D.P. Guo, X.Y. Li, P. Sun, Z.G. Wang, X.Y. Chen, Q. Chen, L.M. Fan, B. Zhang, L.Z. Shao, X.R. Li, Ultrasound/microbubble enhances foreign gene expression in ECV304 cells and murine myocardium, *Acta Biochim Biophys Sin (Shanghai)*, 36 (2004) 824-831.
- [28] S. Tsunoda, O. Mazda, Y. Oda, Y. Iida, S. Akabame, T. Kishida, M. Shin-Ya, H. Asada, S. Gojo, J. Imanishi, H. Matsubara, T. Yoshikawa, Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart, *Biochem Biophys Res Commun*, 336 (2005) 118-127.
- [29] C.H. Miao, A.A. Brayman, K.R. Loeb, P. Ye, L. Zhou, P. Mourad, L.A. Crum, Ultrasound enhances gene delivery of human factor IX plasmid, *Hum Gene Ther*, 16 (2005) 893-905.
- [30] H. Azuma, N. Tomita, Y. Kaneda, H. Koike, T. Ogihara, Y. Katsuoaka, R. Morishita, Transfection of NFkappaB-decoy oligodeoxynucleotides using efficient ultrasound-mediated gene transfer into donor kidneys prolonged survival of rat renal allografts, *Gene Ther*, 10 (2003) 415-425.
- [31] H. Koike, N. Tomita, H. Azuma, Y. Taniyama, K. Yamasaki, Y. Kunugiza, K. Tachibana, T. Ogihara, R. Morishita, An efficient gene transfer method mediated by ultrasound and microbubbles into the kidney, *J Gene Med*, 7 (2005) 108-116.
- [32] H.Y. Lan, W. Mu, N. Tomita, X.R. Huang, J.H. Li, H.J. Zhu, R. Morishita, R.J. Johnson, Inhibition of renal fibrosis by gene transfer of inducible Smad7 using ultrasound-microbubble system in rat UUO model, *J Am Soc Nephrol*, 14 (2003) 1535-1548.
- [33] Y.Y. Ng, C.C. Hou, W. Wang, X.R. Huang, H.Y. Lan, Blockade of NFkappaB activation and renal inflammation by ultrasound-mediated gene transfer of Smad7 in rat remnant kidney, *Kidney Int Suppl*, (2005) S83-91.
- [34] X. Gao, K.-S. Kim, D. Liu, Nonviral gene delivery: What we know and what is next, *AAPS J*, 9 (2007) E92-E104.
- [35] M. Giacca, S. Zacchigna, Virus-mediated gene delivery for human gene therapy, *J Control Release*, 161 (2012) 377-388.

- [36] K.B. Kaufmann, H. Buning, A. Galy, A. Schambach, M. Grez, Gene therapy on the move, *EMBO Mol Med*, 5 (2013) 1642-1661.
- [37] M.A. Kotterman, T.W. Chalberg, D.V. Schaffer, Viral vectors for gene therapy: translational and clinical outlook, *Annu Rev Biomed Eng*, 17 (2015) 63-89.
- [38] T. Goodwin, L. Huang, Nonviral vectors: we have come a long way, *Adv Genet*, 88 (2014) 1-12.
- [39] M. Jafari, M. Soltani, S. Naahidi, D.N. Karunaratne, P. Chen, Nonviral approach for targeted nucleic acid delivery, *Curr Med Chem*, 19 (2012) 197-208.
- [40] D. Wang, G. Gao, State-of-the-art human gene therapy: part I. Gene delivery technologies, *Discov Med*, 18 (2014) 67-77.
- [41] E. Kimmel, Cavitation bioeffects, *Crit Rev Biomed Eng*, 34 (2006) 105-161.
- [42] M.W. Miller, D.L. Miller, A.A. Brayman, A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective, *Ultrasound Med Biol*, 22 (1996) 1131-1154.
- [43] D. Raz, U. Zaretsky, S. Einav, D. Elad, Cellular alterations in cultured endothelial cells exposed to therapeutic ultrasound irradiation, *Endothelium*, 12 (2005) 201-213.
- [44] M. Duvshani-Eshet, T. Haber, M. Machluf, Insight concerning the mechanism of therapeutic ultrasound facilitating gene delivery: increasing cell membrane permeability or interfering with intracellular pathways?, *Hum Gene Ther*, 25 (2014) 156-164.
- [45] Y. Liu, N.J. Bowen, L. Matyunina, J. McDonald, M.R. Prausnitz, Gene transfection enhanced by ultrasound exposure combined with drug treatment guided by gene chip analysis, *Int J Hyperthermia*, 28 (2012) 349-361.
- [46] I.A. Khalil, K. Kogure, H. Akita, H. Harashima, Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery, *Pharmacol Rev*, 58 (2006) 32-45.
- [47] J.J. Ludtke, M.G. Sebestyen, J.A. Wolff, The effect of cell division on the cellular dynamics of microinjected DNA and dextran, *Mol Ther*, 5 (2002) 579-588.
- [48] D. Lechardeur, G.L. Lukacs, Nucleocytoplasmic transport of plasmid DNA: a perilous journey from the cytoplasm to the nucleus, *Hum Gene Ther*, 17 (2006) 882-889.
- [49] S. Mehier-Humbert, T. Bettinger, F. Yan, R.H. Guy, Ultrasound-mediated gene delivery: kinetics of plasmid internalization and gene expression, *J Control Release*, 104 (2005) 203-211.
- [50] C.D. Ohl, M. Arora, R. Ikink, N. de Jong, M. Versluis, M. Delius, D. Lohse, Sonoporation from jetting cavitation bubbles, *Biophys J*, 91 (2006) 4285-4295.
- [51] J.Z. Gasiowski, D.A. Dean, Postmitotic nuclear retention of episomal plasmids is altered by DNA labeling and detection methods, *Mol Ther*, 12 (2005) 460-467.
- [52] M. Duvshani-Eshet, M. Machluf, Therapeutic ultrasound optimization for gene delivery: a key factor achieving nuclear DNA localization, *J Control Release*, 108 (2005) 513-528.
- [53] P. Corish, C. Tyler-Smith, Attenuation of green fluorescent protein half-life in mammalian cells, *Protein Eng*, 12 (1999) 1035-1040.
- [54] V. Deretic, Autophagy in innate and adaptive immunity, *Trends Immunol*, 26 (2005) 523-528.
- [55] G. Juhasz, T.P. Neufeld, Autophagy: a forty-year search for a missing membrane source, *PLoS Biol*, 4 (2006) e36.
- [56] E.L. Eskelinen, Maturation of autophagic vacuoles in mammalian cells, *Autophagy*, 1 (2005) 1-10.
- [57] L. Jahreiss, F.M. Menzies, D.C. Rubinsztein, The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes, *Traffic*, 9 (2008) 574-587.
- [58] R. Bausinger, K. von Gersdorff, K. Braeckmans, M. Ogris, E. Wagner, C. Brauchle, A. Zumbusch, The transport of nanosized gene carriers unraveled by live-cell imaging, *Angew Chem Int Ed Engl*, 45 (2006) 1568-1572.
- [59] K. Ogawa-Goto, K. Tanaka, W. Gibson, E. Moriishi, Y. Miura, T. Kurata, S. Irie, T. Sata, Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid, *J Virol*, 77 (2003) 8541-8547.

- [60] Y. Liu, J. Yan, M.R. Prausnitz, Can ultrasound enable efficient intracellular uptake of molecules? A retrospective literature review and analysis, *Ultrasound Med Biol*, 38 (2012) 876-888.
- [61] H.R. Guzman, D.X. Nguyen, S. Khan, M.R. Prausnitz, Ultrasound-mediated disruption of cell membranes. II. Heterogeneous effects on cells, *J Acoust Soc Am*, 110 (2001) 597-606.
- [62] R.K. Schlicher, H. Radhakrishna, T.P. Tolentino, R.P. Apkarian, V. Zarnitsyn, M.R. Prausnitz, Mechanism of intracellular delivery by acoustic cavitation, *Ultrasound Med Biol*, 32 (2006) 915-924.
- [63] J.D. Hutcheson, R.K. Schlicher, H.K. Hicks, M.R. Prausnitz, Saving cells from ultrasound-induced apoptosis: quantification of cell death and uptake following sonication and effects of targeted calcium chelation, *Ultrasound Med Biol*, 36 (2010) 1008-1021.
- [64] E.E. Vaughan, D.A. Dean, Intracellular trafficking of plasmids during transfection is mediated by microtubules, *Mol Ther*, 13 (2006) 422-428.
- [65] D.A. Dean, B.S. Dean, S. Muller, L.C. Smith, Sequence requirements for plasmid nuclear import, *Exp Cell Res*, 253 (1999) 713-722.
- [66] R. Cartier, R. Reszka, Utilization of synthetic peptides containing nuclear localization signals for nonviral gene transfer systems, *Gene Ther*, 9 (2002) 157-167.
- [67] V. Sanz, C. Tilmaciu, B. Soula, E. Flahaut, H.M. Coley, S.R.P. Silva, J. McFadden, Chloroquine-enhanced gene delivery mediated by carbon nanotubes, *Carbon*, 49 (2011) 5348-5358.
- [68] M. Duvshani-Eshet, H. Keren, S. Oz, I.S. Radzishhevsky, A. Mor, M. Machluf, Effect of peptides bearing nuclear localization signals on therapeutic ultrasound mediated gene delivery, *J Gene Med*, 10 (2008) 1150-1159.
- [69] K.R. Stone, D.D. Mickey, H. Wunderli, G.H. Mickey, D.F. Paulson, Isolation of a Human Prostate Carcinoma Cell Line (Du 145), *International Journal of Cancer*, 21 (1978) 274-281.
- [70] C.S. Yoon, J.H. Park, Ultrasound-mediated gene delivery, *Expert Opin Drug Deliv*, 7 (2010) 321-330.
- [71] A. Delalande, S. Kotopoulis, M. Postema, P. Midoux, C. Pichon, Sonoporation: mechanistic insights and ongoing challenges for gene transfer, *Gene*, 525 (2013) 191-199.
- [72] D.J. Wells, Electroporation and ultrasound enhanced non-viral gene delivery in vitro and in vivo, *Cell Biol Toxicol*, 26 (2010) 21-28.
- [73] I. Lentacker, I. De Cock, R. Deckers, S.C. De Smedt, C.T. Moonen, Understanding ultrasound induced sonoporation: definitions and underlying mechanisms, *Adv Drug Deliv Rev*, 72 (2014) 49-64.
- [74] H. Yu, L. Xu, Cell experimental studies on sonoporation: state of the art and remaining problems, *J Control Release*, 174 (2014) 151-160.
- [75] A.M. Belikova, V.F. Zarytova, N.I. Grineva, Synthesis of ribonucleosides and diribonucleoside phosphates containing 2-chloroethylamine and nitrogen mustard residues, *Tetrahedron Lett*, 37 (1967) 3557-3562.
- [76] P.S. Slattum, A.G. Loomis, K.J. Machnik, M.A. Watt, J.L. Duzeski, V.G. Budker, J.A. Wolff, J.E. Hagstrom, Efficient in vitro and in vivo expression of covalently modified plasmid DNA, *Mol Ther*, 8 (2003) 255-263.
- [77] W.K. Bai, E. Shen, B. Hu, The induction of the apoptosis of cancer cell by sonodynamic therapy: a review, *Chin J Cancer Res*, 24 (2012) 368-373.

Cell with no
pDNA

Cell with pDNA
outside the
nucleus

Cell transfected
with pDNA