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Prolonging pulse duration in ultrasound-mediated gene delivery lowers acoustic pressure threshold for efficient gene transfer to cells and small animals

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Abstract

While ultrasound-mediated gene delivery (UMGD) has been accomplished using high peak negative pressures (PNPs) of 2 MPa or above, emerging research showed that this may not be a requirement for microbubble (MB) cavitation. Thus, we investigated lower-pressure conditions close to the MB inertial cavitation threshold and focused towards further increasing gene transfer efficiency and reducing associated cell damage. We created a matrix of 21 conditions ($n = 3/\text{cond.}$) to test in HEK293T cells using pulse durations spanning 18 μs –36 ms and PNPs spanning 0.5–2.5 MPa. Longer pulse duration conditions yielded significant increase in transgene expression relative to sham with local maxima between 20 J and 100 J energy curves. A similar set of 17 conditions ($n = 4/\text{cond.}$) was tested in mice using pulse durations spanning 18 μs –22 ms and PNPs spanning 0.5–2.5 MPa. We observed local maxima located between 1 J and 10 J energy curves in treated mice. Of these, several low pressure conditions showed a decrease in ALT and AST levels while maintaining better or comparable expression to our positive control, indicating a clear benefit to allow for effective transfection with minimized tissue damage versus the high-intensity control. Our data indicates that it is possible to eliminate the requirement of high PNPs by prolonging pulse durations for effective UMGD *in vitro* and *in vivo*, circumventing the peak power density limitations imposed by piezo-materials used in US transducers. Overall, these results demonstrate the advancement of UMGD technology for achieving efficient gene transfer and potential scalability to larger animal models and human application.

Keywords

Gene transfer; Ultrasound; Microbubble; Liver; Nonviral gene delivery

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1. Introduction

Nonviral gene therapy confers appreciable benefits over viral methods including lower risk of immunopathogenicity, greater flexibility in vector construction, and better spatial and temporal control. Delivery of plasmid DNA (pDNA) is particularly attractive as manipulation of the host genome can be avoided and the vector can more easily be engineered for episomal persistence and long-term promoter activation. Ultrasound (US)-mediated gene delivery (UMGD) has long been recognized [1–9] as a potential method to perform minimally invasive, nonviral gene transfer of pDNA. Effective UMGD requires the presence of microbubbles (MBs), which has been demonstrated to significantly enhance gene transfer efficiency, resulting in increased transgene expression. Under appropriate acoustic pressures and applied frequencies, spontaneous formation of gas cavities, termed cavitation, may occur. MBs serve as our cavitation nuclei and can oscillate radially and collapse when exposed to a driving pressure field. Although the precise mechanism is not yet known, MB cavitation and/or destruction during therapeutic sonication is shown to facilitate transient pore formation along the cell membrane [10,11]. Acoustic cavitation of MBs may also increase permeability of endogenous barriers such as the cell membrane or vessel wall to allow normally impermeable materials (e.g., drugs or macromolecules) to cross via diffusion.

Other nonviral gene therapies developed include systemic exposure to lipid nanoparticles carrying genetic material or direct injection of gene vectors to tissue-specific sites (e.g., intraparenchymal or intramuscular) [12–14]. However, use of lipid or polymer encased pDNA may be hindered by difficulty in packaging, expelling genetic load, and avoiding cytoplasmic degradation. In addition, direct injection to tissue-specific sites faces the challenge of traversing the plasma membrane of target cells. Alternatively, systemic administration of genetic vectors is also challenged by the multiple barriers hindering entry of pDNA into cells. The vectors must first cross the endothelium, the basement membrane, and smooth muscle layer before overcoming the outer cell membrane of the target cells and finally the nuclear membrane for efficient gene expression. UMGD may potentially overcome some or all of these barriers to significantly improve gene transfer efficiency targeting specific tissue/cells. Our incorporation of UMGD methods with nonviral gene therapy is motivated by the aim of treating genetic diseases, such as hemophilia, that is safe with comparable efficiency to viral gene transfer methods.

The liver is an ideal target for gene therapy in hemophilia A patients as it is a predominant site of factor VIII production, and where deficiency of the protein is responsible for the hemophiliac phenotype. We have shown previously that UMGD can significantly enhance gene transfer in livers of both small and larger animal models [1–4]. In our mouse models, MBs and pDNA encoding a luciferase reporter gene, pGL4, were co-administered by injection into the portal vein (PV) while US was simultaneously applied to the liver lobes. We were required to modify our treatment protocol in rats to accommodate the larger liver lobes by injecting the pGL4/MBs into individual liver lobes through a PV branch. Similar to our rat studies, we achieved a nearly 100-fold increase in average luciferase expression relative to our sham when translating the single lobe injection strategy to our dog models. In agreement with our small animal studies, we concluded from our dog model that a peak

negative pressure (PNP) of about 2.7 MPa is required for effective gene transfection with minimal liver tissue damage. However currently, our surgical procedure requires opening the cavity of the animal model to treat the surface of the liver. One important milestone is to develop a minimally invasive surgical procedure that could still facilitate effective UMGD. Therefore, many technical issues must still be overcome when considering the translation of these technologies towards human application.

A clinically applicable procedure would involve transcutaneous UMGD. Given the 2.7 MPa threshold necessary for effective gene transfer into the liver cells however, acoustic pressures beyond the capabilities of the piezo-material would be required due to the loss of acoustic energy passing through several tissue layers. While UMGD has historically been achieved through high PNPs of 2 MPa or greater, emerging evidence suggests it may not be a requirement for MB cavitation [15–17]. In this study, we investigated lower-pressure conditions for UMGD, close to the MB inertial cavitation threshold. We therefore created a matrix of pulse durations and acoustic pressures used in HEK293T cells and mice. These conditions were focused towards achieving comparable, and possibly further increasing, gene transfer efficiency while reducing associated cell damage. We discovered lower pressure, longer pulse duration conditions within a range of acoustic energies that produced gene expression levels similar to or better than our previously reported results without significantly increasing tissue damage. Thus, these prolonged pulse duration US protocols have potential for improving US transducer design to readily achieve US intensity threshold with increased treatment volumes that could lead to effective gene transfer transcutaneously. Our results provide a guideline for overcoming current challenges in broad applications of UMGD and transducer design to facilitate clinical translation.

2. Methods

2.1. Plasmid preparation

The luciferase reporter plasmid pGL4.13 [luc2/SV40] (Promega, Madison, WI) was produced by GenScript Inc. (Piscataway, NJ) according to standard techniques. The green fluorescent protein (GFP) reporter plasmid p2X-GFP (pGFP) was prepared as previously described [5] using an Endo-Free Maxi-Prep kit (Qiagen Inc., Valencia, CA), according to the manufacturer's manual.

2.2. Microbubble preparation

RN18 microbubbles were prepared according to the previously described protocol [18]. The MB shells were comprised of lipids at a 82:10:8 M ratio of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphate (DSPA), and N-(Carbonylmethoxypolyethyleneglycol 5000)-1,2-distearoyl-*sn*-glycero-e-phosphoethanolamine (MPEG-5000-DSPE) purchased from Avanti® Polar Lipids, Inc. (Alabaster, AL). Before use, MBs were produced by vigorous agitation of the lipid emulsion for 45 s using a Vialmix™ (Lantheus Medical Imaging, N. Billerica, MA), yielding an average concentration of $2\text{--}5 \times 10^9$ MBs/mL.

2.3. Ultrasound conditions

The experimental ultrasound setup for both cell culture and murine experiments is summarized in Fig. 1A. A laptop was used to control a signal-generating amplifier via serial interface (Models: RFG-1000 and RFG-1500BB, JJ&A Instruments, Duvall, WA) that was connected to an impedance matching network and subsequently to a single-element, 16 mm diameter, unfocused transducer (Model H158, Sonic Concepts, Bothell, WA). A broad range of ultrasound conditions was tested in both HEK293T cells (Table 1) and mice (Table 2). All experiments used a center frequency of 1.1 MHz, pulse repetition frequency (PRF) of 14 Hz, and 20 cycles of a 1 s *ON*, 2 s *OFF* pulse train to yield 60 s total treatment time. US treatment monitoring was performed by capturing current, voltage, and calculated power from a gated, triggered waveform using a high sample-rate oscilloscope (44MXs-B, Teledyne Lecroy, Chestnut Ridge, NY).

The acoustic energy (E) is defined here as the product of spatial average intensity (I_{avg}) and exposure time (τ):

$$I_{avg} = \frac{P_{avg}}{A} \quad (1)$$

$$\tau = PD \cdot PRF \cdot T \quad (2)$$

$$E = I_{avg} \cdot \tau \quad (3)$$

where P_{avg} is the average power output, A is the active cross-sectional area of the beam, PD is the pulse duration, PRF is the pulse repetition frequency and T is the insonation time. The exposure time (τ) is defined here as the time length in which the ultrasound is actively on, whereas the insonation time (T) is defined as the full time duration of pulsed ultrasound treatment from start to finish [19].

2.4. In vitro UMGD

Human embryonic kidney (HEK)293 T cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagles medium (DMEM) (Mediatech, Inc., Manassas, VA) with 10% fetal bovine serum (FBS) (Atlanta Biologicals Inc., Lawrenceville, GA), 1% HEPES buffer (Mediatech, Inc), 1% Penicillin/Streptomycin (Mediatech, Inc), and 1% L-Glutamine (Mediatech, Inc). For transfection experiments, the cells were suspended in 1.7 mL complete media and 3.2 μ g of pGFP plasmid were added and then transferred into TPX microcentrifuge tubes (Diagenode Inc., Denville, NJ) leaving a small headspace of gas necessary to avoid MB destruction when capping. Subsequent steps were performed serially by each tube to prevent passive decay of diluted MBs. 0.1% (v/v) (17 μ L) of activated RN18 were added into each tube, capped immediately, and mounted in the acoustic water bath.

The cell/pDNA/MB suspension was then exposed to US in the nearfield of the H158 transducer for 60 s, varying the US signals used as detailed in section 2.3. The experimental set-up can be visualized in Fig. 1B. After completion, cells were gently mixed by pipetting then plated in single-cell suspension into 6-well tissue culture plates. For each experiment, pDNA transfection controls were also generated to validate gene expression by directly transferring 1.7 mL of the cell and plasmid solution to an empty well of a 6-well plate and adding 2 μ L of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). An additional 1.7 mL of complete media was added to each well for a total volume of 3.4 mL. Cells were harvested at 48 h post-transfection for expression analysis via flow cytometry.

2.5. Flow cytometry analysis

A more robust method of assessing both viability and gene transfer efficiency was required. Flow cytometry allows the opportunity to simultaneously observe both experimental dimensions within a single experiment. Fluorescence is not a frequently reported output measure for gene transfer. Therefore, a correlation between GFP⁺ expression and luciferase activity was determined (Supplemental Fig. 1). A correlation of 0.857 was derived between GFP⁺ expression and luciferase activity, deeming satisfactory for comparative analysis.

HEK293T cells were stained with a viability dye, 7AAD (Biolegend®, San Diego, CA), and analyzed via flow cytometry for PE fluorescence. GFP expression was analyzed using the FITC channel. Viability was calculated as the percentage of all events that are GFP^{+/−} and 7AAD[−] events within a parent gate of singlet HEK293T cells. GFP expression was calculated as the percentage of GFP⁺/7AAD[−] events within a parent gate of singlet HEK293T cells, multiplied by the GFP MFI of the same population. Representative flow analysis and microscopy images are shown (Supplemental Fig. 2). For comparison across experiments performed on different dates, GFP⁺ expressions were normalized to a positive US control protocol (2 ms, 1.1 MPa).

2.6. In vivo UMGD

Eight-week-old C57/BL6 male mice were purchased from the Jackson Lab (Bar Harbor, ME) and maintained at a specific pathogen-free vivarium. All animal experiments were performed in accordance with IACUC-approved protocol and guidelines for animal care of both National Institutes of Health and Seattle Children's Research Institute. Prior to surgery, mice were anesthetized by continuous inhalation of isoflurane. A midline incision was made to expose the liver and portal vein in mice. Mouse livers were transduced, varying the US signals used as detailed in section 2.3. Murine UMGD was performed as previously described [3]. Briefly, a 400 μ L mixture containing 50 μ g of pGL4.13, RN18 MBs, and 5% glucose in PBS was injected into the liver via the portal vein. US treatment was performed simultaneously to injection, and continued for 30 s after completion of injection for a total treatment time of 60 s. Hemostasis was applied and the incision site closed, and the mice were allowed to recover from anesthesia.

2.7. Evaluation of luciferase gene expression

All liver lobes were harvested from the animal 24 h after gene delivery to analyze luciferase gene expression. The liver was subsequently processed and assayed as previously described

[1]. Briefly, supernatant was collected from homogenized liver that underwent three freeze-thaw cycles. Luciferase activity in the lysate was measured by using a commercial kit (Luciferase Assay System, Promega) and luminometer (Victor 3; Perkin-Elmer, Wellesley, MA), and then normalized to protein levels measured using a BCA assay kit (Bio-Rad, Hercules, CA). Luciferase expression data are expressed as relative light units per mg protein (RLU/mg protein).

2.8. Transaminase assay

Blood samples were collected by retro-orbital bleeding prior to euthanizing the animal and used to assess liver damage by measuring alanine aminotransferase (ALT or SGPT) and aspartate aminotransferase (AST or SGOT) levels using commercial assay kits (Teco Diagnostics, Anaheim, CA). Normal, untreated mice were used as controls.

2.9. pDNA stability assessment

The stability of the pGL4.13 during US and in the presence of MBs was assessed using the same experimental set-up as the *in vitro* UMGD experiments. A 1.7 mL solution mixture containing 5% glucose, 50 µg of pDNA, and 5% (v/v) RN18 MBs in 1 × PBS was prepared in a 1.7 mL TPX microcentrifuge tube. Using transducer H158, the mixture was exposed to US using a pulse duration of 18 µs at 2.5 MPa for 60 s. The condition was repeated without MBs, and again without US + MBs. The pDNA for each condition was then linearized with *Bgl*II and analyzed alongside the undigested form on a 1% (v/v) agarose gel.

2.10. Cavitation experiments

17 µL of activated RN18 MBs was added to 1.7 mL of PBS in TPX microcentrifuge tubes. These tubes were then exposed to ultrasound identically to our *in vitro* protocol. The MBs used for each experimental tube were drawn from the same pool of activated RN18 MBs. After completion of ultrasound exposure, MBs were analyzed using flow cytometry and their sizes and concentrations were calibrated using known dilutions of polybeads (Polysciences Inc., Warrington, PA) on the same day. Run times were fixed and the number of events gathered was allowed to vary. Total events of US exposed MBs were compared to a control tube without US exposure. Consistent cytometer flow rate was verified by comparing dilutions of polybeads and untreated RN18 MBs from the start and end of each experiment.

2.11. Statistical analysis

All statistical analyses were performed using a two-tailed Student's *t*-test with unequal variance.

3. Results

3.1. HEK293T UMGD

In order to establish a reference for ultrasound parameters in our *in vitro* experiments, we tested a matrix of 36 conditions in HEK293T cells. These conditions spanned pulse durations of 18 µs–36 ms and PNPs of 0.5–2.5 MPa (intensities of 5–140 W/cm²). Group sizes were *n* = 2–8 samples per condition, with a mode size of *n* = 5 samples. GFP

expression was quantified as the population of live, expressing cells and adjusted to incorporate the MFI of the population. To assess the effectiveness of varying US parameter combinations, the resulting GFP expression was plotted against pulse duration and PNP as shown in Fig. 2A. It was observed that conditions with pulse durations 150 μ s and below displayed minimal increase in expression compared to sham-treated controls (no significant difference, P -value = 0.05) at all tested powers. Across most other tested pulse durations, one or more corresponding PNPs did not produce significantly different expression levels from the global expression maximum (located at 22 ms, 0.5 MPa).

With increasing pulse duration, maximum expression was found to occur at progressively lower PNPs. Increasing PNP beyond the local expression maximum for pulse durations on the millisecond scale does not enhance expression any further; rather, expression appears to decrease. Additionally, increasing pulse duration resulted in a narrower range of optimal pressures. The majority of high-expressing conditions delivered total treatment energies greater than or equal to 60 J. Expression from various US protocols appears to be separated by two treatment energy ranges. Results from US transfected HEK 293 T cells were pooled into two separate groups: cells treated with acoustic energies of greater than or equal to 60 J, or cells treated with acoustic energies below 60 J. The pooled data from each group was averaged and compared. Analysis revealed that points of expression equal to or above the 60 J treatment energy curve were significantly greater than points that lie below the 60 J energy curve ($P < 0.0005$, Fig. 2C). Mean values and standard deviation evaluations for GFP expression may be found in Supplemental Fig. 3A.

3.2. HEK 293T viability

Cell viability was quantified using flow cytometry populations selected via a 7-AAD stain. Fig. 2B shows the resulting empirical distribution of cell viability over the chosen pulse duration-PNP parameter space. The control US protocol (2 ms, 1.1 MPa) resulted in 88% cell viability. Shorter pulse durations within the microsecond scale did not affect cell viability even when increasing PNP. Cell viability begins to decrease gradually when increasing pulse duration beyond the microsecond scale. The global viability minimum occurs at a condition of 1 ms, 1.1 MPa with 50% of viable cells remaining. When plotted against total treatment energy across the parameter space, cell viability appears to decrease with increasing treatment energy. Mean values and confidence interval evaluations are shown in Supplemental Fig. 3B.

3.3. Murine UMGD

The effects of 32 unique ultrasound conditions were examined in C57/BL6 mice using the existing UMGD protocol. The conditions were selectively narrowed from the HEK 293 T transfection experiments and upper limits of 22 ms for pulse duration and 2.5 MPa for PNP were determined. Group sizes ranged from $n = 2$ –14 samples per condition, with a mode size of $n = 5$ samples. Our reference condition comprised of $n = 37$ mice (18 μ s, 2.5 MPa), and was used to verify stationarity across experimental days.

Luciferase expression results from our experimental mice are summarized in Fig. 3A. Mean values and standard deviation evaluations of luciferase expression are shown in

Supplemental Fig. 4A. In general, at shorter pulse durations, a higher PNP value is required for efficient gene delivery. For increasingly long pulse durations, the best expression was found at decreasing PNP values. The range of acceptable pressures also appeared to decrease with increasing pulse duration. Increasing the PNP at longer pulse durations did not increase expression. This is indicated by the more rapid falloff in expression with respect to pressure at longer pulse durations. However, increasing pulse duration beyond the microsecond range at low pressures appeared to enhance gene transfer, while decreasing toxicity. Similar to UMGD experiments performed on HEK 293 T cells, luciferase expression at varying US parameter combinations appears to organize close to an equi-energy curve. It was found that the majority of high-expressing conditions in this model fell on or near a curve close to 5 J of total treatment energy. These high-expressing conditions were not significantly different from the positive control (Supplemental Fig. 5A). Expression from various US protocols appears to be separated by three treatment energy ranges. The results from US-treated mice were pooled into three groups: mice treated with acoustic energies < 1 J, mice treated with acoustic energies between 1 J and 10 J, or mice treated with acoustic energies above 10 J. The pooled data from each group was averaged and compared. Analysis revealed that the points that lie between the 1–10 J treatment energy band were significantly greater than points that lie below the 1 J energy curve or points above the 10 J energy curve ($P < 0.005$, Fig. 3B). There was no significant difference comparing the grouped points below 1 J and above 10 J treatment energies.

3.4. Murine hepatotoxicity

Mouse plasma and livers were harvested 24 h following surgery to examine any potential hepatic injury. ALT and AST enzyme levels were examined in each UMGD experimental mouse to identify conditions with increased gene expression and minimal liver tissue damage. ALT results from the panel of mouse experiments are presented in Fig. 3C and similar AST results are presented alongside in Fig. 3D. Mean values and standard deviation evaluations of the ALT and AST results are shown in Supplemental Fig. 4B, 3C. We found that one particular condition (400 μ s, 1.1 MPa) resulted in comparable transgene expression, but decreased ALT levels (Supplemental Fig. 5B & C). It can be observed that total delivered treatment energy correlates with an increase in average ALT and AST values for each condition. Liver damage was within murine therapeutic norms for most conditions tested, though the sole condition tested lying on the 100 J energy curve began to elevate above tolerable levels with an accompanying drop in expression. As a result, fewer conditions in the higher 60–100 J range were tested in mice than in HEK293T experiments and the range was deemed the upper limit.

3.5. pDNA stability after UMGD

The integrity of pGL4.13 was assessed after UMGD to determine if loss of expression at certain US conditions could be attributed to DNA instability. For conditions in which the pDNA was exposed to US, a pulse duration of 18 μ s at 2.5 MPa was used for a total treatment time of 60 s. This condition set was used to compare against the results of the reference condition in the *in vivo* experiments.

Three conditions were tested: a control untreated pDNA mixture, pDNA exposed to US without MBs, and pDNA exposed to US in the presence of MBs. The undigested and digested forms of the pDNA for each condition were compared on a gel as shown in Fig. 4. In both conditions where the pDNA was exposed to US alone and US in the presence of MBs, the integrity of pGL4.13 remained unchanged relative to the untreated pDNA. The supercoiled structure of pGL4.13 was intact, and the overall size of the pDNA remained the same for all conditions.

3.6. MB destruction efficiency

To allow comparison of expression results versus MB activity, endpoint studies of MB concentration were conducted using flow cytometry. Conditions were selected as a representative subset of those previously tested in HEK293T cells. It was found that once again, delivered US energy closely predicted results, although the trend was not a simple linear correlation. A comparison of treatment energy versus the extent of MB destruction is shown in Fig. 5. For treatment energies above 5 J, there is a rapid drop-off in the average percent of MBs remaining after US exposure. The data is fitted by a single-decay, exponential curve.

4. Discussion

We have previously shown the potential for UMGD as means for a targeted and safer method of gene delivery [1–4,18]. Other methods incorporating delivery systems such as viral vectors or adenoviral vectors are hampered by immunotoxicity or poor genetic payload, respectively [20–23]. Therapeutic administration of UMGD however, has yielded spatial and temporal control over delivery in our animal experiments and is well-tolerated [3,4,18]. Our current surgical method of performing gene delivery requires opening the cavity of the subject to access the liver. However, the procedure is invasive and not ideal for clinical translation especially for hemophilia patients. Therefore, transcutaneous UMGD may be a possibility for minimally invasive treatment. In exchange for a safer surgical procedure however, the intensity of the acoustic wave may be attenuated via absorption by soft tissue when treating across several tissue layers. Moving forward with this consideration, we hypothesized a greater PNP would be required for sufficient gene delivery transcutaneously. Achieving this goal is currently constrained by the upper limits of the transducer materials, US parameter boundaries, and resulting adverse bioeffects.

To overcome the barrier imposed by the transducer limits, we investigated the effects of varying US parameter pairings – pulse duration and acoustic pressure. In our previous animal study, 2.7 MPa PNP was deemed appropriate for the desired transfection effect with minimal tissue damage. To perform hepatic UMGD transcutaneously, a more powerful transducer must be designed to meet the 2.7 MPa PNP requirement on the surface of the liver. Alternatively, we attempted to replicate the transfection efficiency of our previous results using other PNPs and pulse durations. A matrix of varying pulse durations and PNP values was generated to extend our previous US parametric explorations [3]. When tested *in vitro*, we found that increasing the pulse duration resulted in greater GFP expression compared to shorter durations. As the pulse duration was increased, the PNP required for

transfection decreased and the local GFP expression maximum occurred at progressively lower PNPs. Cell viability was maintained at 70% or greater for most conditions. We observed a narrowing range of optimal applied pressures with maximal expression as pulse duration was increased. Interestingly, it appeared the local maximums in each experimental pulse duration set clustered along a treatment energy curve upon mapping the expression results to PNP against pulse duration. Likewise, the decrease in cell viability trended towards clustering around specific energy curves. Other studies have found similar results to ours that suggest cell transfection and viability may correlate to ultrasound energy exposure [24,25]. They report optimal energy exposures in the range of 10 to 40 J/cm², using a low frequency of 24 kHz. Our observed energy levels required to significantly sonoporate HEK 293T cells are greater than what has been previously reported by other groups. This difference may be explained by how we have chosen to calculate our treatment energy wherein the duty factor has been incorporated. In addition, Helfield et al. has suggested that at fixed frequencies, longer pulse durations are required to increase the occurrence of bubble oscillation necessary for sonoporation *in vitro* [26]. Karshafian et al. have suggested sonoporation having a dependence on pulse center frequency instead [19]. This may be due to different sized MBs having varied resonant frequencies that allow them to oscillate [27]. We did not test differing driving frequencies since the chosen 1.1 MHz driving center frequency setting allowed 100% power transfer across the H158 transducer with 90% output efficiency. Thus, it may be possible that only a subset of the MB population is able to undergo cavitation and contribute to sonoporation. However, we show in our end-point MB cavitation experiments that for nearly the entire range of power and pulse duration, and thus treatment energy, an increase led to a majority of MBs being destroyed or fragmented. This would suggest most of the MBs were initially able to oscillate at the fixed frequency and could potentially contribute to sonoporation. We are also using a different cell line compared to other groups who have done similar work, which may affect the efficacy of sonoporation if membrane properties differed.

Our studies and others [1,3,4,28,29] demonstrated that significant enhancement of UMGD efficiency resulted from membrane sonoporation by cavitation of the MBs upon US exposure. The efficiency is controlled mainly by US parameters and the characteristics of MBs. It was recently demonstrated that at low pressure such as 0.06 MPa, stable cavitation of MBs occur, whereas at pressure above 0.4 MPa, inertial cavitation of MBs can be induced and significantly enhanced gene transfection [28–31]. In our previous mouse studies, the *in vivo* gene transfer efficiency increased with increasing pressure and achieved a plateau at 2.5 MPa (1.1 MHz Frequency, 20 cycle pulses, 50 Hz pulse repetition frequency). Under these conditions, the total energy output from the transducer is only 1.4 J due to the very short pulse duration (18 μ s). However, very little enhancement was obtained at 0.4 MPa combined with 18 μ s pulse length. We hypothesize that higher gene transfer efficiency at low pressure such as 0.4 MPa may be achievable by increasing the treatment pulse duration during the *in vivo* dynamic condition of US treatment. Thus, the total energy output and duty factor are increased. We have not previously tested varying pulse durations extensively and have observed increasing pulse duration can improve sonoporation based on our *in vitro* studies. The result of our cell culture experiments provided a framework for translation of the US parameter matrix to small animals. We considered conditions above 22 ms may not

be necessary for sonoporation *in vivo* as the optimal condition found in culture was 22 ms, 0.5 MPa. Similarly, we have shown in our previous studies that 2.7 MPa is enough to induce sonoporation with minimal tissue damage. Therefore, we confined the upper limit of applied pressure and pulse duration to 2.5 MPa and 22 ms, respectively. We continued to use (2.5 MPa, 18 μ s), as our experimental control as we had in our cell culture studies for reference. As with our previous studies, we perfused a coadministration of pDNA and MBs through the portal vein while simultaneously treating with US on the surface of the liver. We anticipate whole MBs to cross the endothelial barrier upon permeabilization. In the case of treating hemophilia patients where transient large pores may result in a bleeding event, pretreatment with coagulation factors will be implemented to moderate bleeding during UMGD procedures. Similar to our cell experiments, we found that optimal gene expression in mice after UMGD appears to follow a treatment energy curve. However, we discovered that the range of energy curves that optimal gene expression occurs shifted towards lower energy ranges. The US parameter pairings with optimal luciferase expression and tolerable toxicity lies between 1 and 10 J. This may suggest an optimal treatment energy curve for sonoporation specific to the particular US settings and parameters defined. Within the 1 to 10 J treatment energy range, ALT and AST levels begin to rise above normal ranges. As treatment energy is increased beyond this range, the total average luciferase expression decreases and instead liver enzyme levels increase well outside of normal ranges, suggestive of tissue damage. In agreement with Karshafian et al., at the longest pulse duration settings (millisecond range), we saw increased treatment toxicity for pressure settings above 1 MPa [19]. However, the lowest pressure settings (< 1 MPa) using > 1 ms resulted in comparable ALT and AST values for shorter pulse duration, higher pressure settings. Overall, there is a general trend towards decreased tissue damage using lower PNP values at longer pulse durations. The *in vivo* results differed from transgene expression obtained *in vitro* where the longest pulse durations provided the best gene transfer. This may be explained by the MB-mediated sonoporation having irreversible effects on membrane recovery or induction of apoptosis *in vivo* at both longer pulse durations and higher pressure settings without pulse train [32–35]. Therefore, a compromise is made by using intermediate treatment energies to provide optimal UMGD with minimal toxicity *in vivo*. Lowered toxicity using longer pulse durations beyond 1 ms may be possible for treatment if a different pulse train setting is used. A pulse train would allow MBs to distribute further beyond the endothelial barrier and proximal hepatocytes before cavitating or being destroyed. Use of different pulse train settings during treatment will be considered for future studies. Although we had not tested higher pressures above 1.5 MPa at longer pulse durations, it is apparent that treatment with US above 1.5 MPa using pulse durations > 1 ms will decrease transfection efficiency. Using this knowledge as a guide, we can then translate the US parameter pairings to larger animal models. We can select longer pulse durations and lower applied pressures that are equally conducive to efficient gene transfer as short pulse duration, high pressure settings, while minimizing adverse bioeffects.

The success of sonoporation in both *in vivo* and *in vitro* settings depends on MB cavitation and destruction. We observe in our end-point MB cavitation studies that at energies above 20 J, the percentage of MBs remaining in the population is reduced to about 2%. We observe notable transfection to occur above 20 J in UMGD of HEK 293 T. This suggests MB

destruction plays a role in transfection of HEK 293 T cells in our experimental set-up. In mice however, MB destruction does not appear to be the sole factor in determining sonoporation. We observe treatment energies between 1 and 10 J that are most ideal for *in vivo* transfection using our US parameter settings. Treatment energies within the 1–10 J range destruct or fragment 45–90% of the MB population based on our end-point cavitation results. We are careful to note that our end-point cavitation study is not able to quantify the number of MBs that could have fragmented during US exposure [36,37]. The efficacy of UMGD *in vivo* may depend on additional factors that are not captured in the end-point cavitation study. One consideration that also affects microbubble behavior is the viscosity of the fluid as proposed by Helfield et al. and others [17,36–39]. Our *in vitro* studies were performed in water, which is a less viscid fluid than that of blood. At low applied pressures in water, MBs tend to fragment to smaller sized bubbles. However, given the higher viscosity of blood, the radial oscillations of MBs can be dampened and the occurrence of fragmentation reduced. This could increase the likelihood of and prolong multiple viable sonoporation events. As the *in vivo* setting is dynamic, blood flow also plays a role in translating the MBs downstream from the portal vein injection site, increasing the volume of tissue exposed to cavitating MBs. MBs can be translated by primary and secondary Bjerknes forces which also enhance treatment volume. Similar consideration can also be applied to the restricted dense space in the extravascular tissue area where MBs are in close contact with the target liver cells. These phenomena may contribute to the observation that efficient gene transfer occurred in lower energy band (1–10 J) in mice compared with higher energy band (20–100 J) in cell culture experiments. Other reasons for needing greater pressures, longer pulse durations, or energy settings *in vitro* are proposed by other groups such as difference in membrane properties of cells, MB and cell concentration, and cells being in suspension rather than adherent [31,40,41].

There are significant limitations to designing US transducers and protocols that will achieve efficient gene transfer in large animal models and humans due to US intensity attenuation through several tissue layers, increased treatment area/volume, and potential tissue damage via UMGD. The current study focused on defining US parameters that can (1) decrease potential damage to the tissue, and (2) decrease the requirement of high peak intensity of US for efficient delivery, thus rendering workable designs of effective US transducers within the limitation of piezo materials and further reducing potential tissue damage. We found that the US parameter pairings between the 1 and 10 J energy curves generated optimal luciferase expression levels and tolerable toxicity. Specifically, increasing pulse duration lowered the pressure threshold required for efficient gene transfer. Thus, it was demonstrated that feasible US protocols can minimize damage to tissue and maintain efficient gene transfer, while overcoming limitations in transducer design. Lowering US peak intensity will also allow flexibility in transducer design where highly focused, narrow beams may not be necessary and can therefore increase treatment area/volumes. This is especially important and beneficial when translating to larger animal models wherein adverse bioeffects are a concern and tissue volume is much greater. It should be noted that the conditions outlined as optimal in these experiments have been optimized under specific US settings and transducer design and may change depending on the design of the transducer or desired target tissue. However, the results and conclusions drawn from the very extensive exploration of US

parameters in this study, especially the correlation between transfection efficiency or tissue damage and US conditions including pulse length, peak negative pressure, and energy curves have significant implication on how to achieve successful translation of US technology from small animal experiments to large animals and human applications. We provide here a guideline for overcoming obstacles in UMGD due to limitations of transducer design or adverse bioeffects to facilitate broader applications of US-mediated delivery. We have also evaluated changes in beam patterns such as focused, unfocused, or cylindrically focused beams in transducer designs [42]. Other changes including number and configuration of elements, or driving center frequency will also be considered in future studies. Of further interest is during exploration of various US parameter settings, treatment energy appears to play a role in UMGD bioeffects and should be an additional consideration for future investigation. For later studies, we will continue to consider how longer pulse duration, lower pressure settings and the relation to treatment energy will translate to transcutaneous UMGD. Success in these coming studies will not only advance ultrasound technology, but also potential application of UMGD in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2018.04.012>.

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Fig. 1.

Overview of *in vitro* and *in vivo* experimental apparatus and setup. (A) Block diagram of signal generation and real-time monitoring. (B) Schematic representation of *in vitro* experiments done in an anechoic water bath. Cell/pDNA/MB suspension is treated 8 mm from the face of the transducer where maximal PNP occurs. (C) Representation of *in vivo* sonoporation experiments performed in livers of mice. A pDNA/MB solution is injected via the portal vein and the liver exposed to US simultaneously. US treatment on surface of liver was performed 8 mm from the face of the transducer.

Fig. 2.

Transgene expression after UMGD in HEK293T cells and analysis of results grouped by treatment energy bands. (A) Expression and (B) viability results across pulse durations (18 μ s–36 ms) and PNPs (0.5–2.5 MPa). Expression was measured 48 h post-transfection using flow cytometry and quantified as product of MFI and percentage Live/GFP⁺. Expression values are normalized relative to the highest performing condition by average expression. Increasing GFP expression corresponds to a shift in color shade from light to dark. Viability was measured via 7-AAD staining using flow cytometry, quantified as the ratio of 7AAD⁻/Total Single Cells. Increasing viability corresponds to a shift in color from red to green. Each circle represents one group of cell culture experiments at a distinct condition ($n = 2$ –8 experiments/group), with color linearly related to GFP expression or viability. Equi-energy curves (1, 10, 20, 60, and 100 Joules (J)) are plotted for both (A) and (B). Statistical distribution and analysis of statistical significance can be found in Supplemental Fig. 2. (C) US parameter pairings that produce treatment energies at or above 60 J resulted in significantly greater overall transfection than pairings that produce treatment energies below 60 J. Error bars indicate standard deviation. ** $P < 0.005$, *** $P < 0.0005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Transgene expression after UMGD in C57/BL6 mice and analysis of results grouped by treatment energy bands. (A) Luciferase expression across 32 conditions, varying pulse durations (18 μ s–36 ms) and PNPs (0.5–2.5 MPa). Liver tissue was harvested 24 h post-treatment, and expression was evaluated via luciferase assay of excised tissue. Results were normalized against protein content of each sample as determined by Bradford protein assay. Increasing luciferase expression corresponds to a shift in color shade from light to dark. (B) US parameter pairings that produce treatment energies between 1 and 10 J resulted in

significantly greater overall transfection than pairings that produce treatment energies below 1 J or above 10 J. Group comparison of conditions that lie below 1 J or above 10 J show no statistical significance. Error bars indicate standard deviation. $**P < 0.005$, $***P < 0.0005$. RLU, relative light unit. (C) ALT levels, and (D) AST levels in plasma of treated mice following UMGD. Increasing levels in ALT and AST correspond to a shift in color from green to red. Plasma was isolated 24 h post-treatment for examining the hepatotoxicity by a colorimetric AST/ALT assay. Results were quantified using a plate-reader and calibrated to known standards for each run. Plotted lines are equi-energy curves (1, 10, 20, 60, and 100 Js). Each circle represents one group of mice at a distinct condition ($n = 5-10$ mice/group), with color linearly related to luciferase expression. Statistical distribution and analysis of statistical significance can be found in Supplemental Fig. 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

pDNA stability tested by exposing pGL4.13 [sv40/luc2] to US in the presence of MBs. A pulse duration of 18 μ s at 2.5 MPa was used with a treatment time of 60 s. RN18 MBs were used. Following US treatment, plasmid was linearized using *Bgl* II. Lane 1 is a 1kbp ladder, lanes 2–4 are pGL4.13 digested by *Bgl* II, lanes 5–7 are undigested pGL4.13. Lanes 4 & 7 indicate that neither US nor MB exposure affected the integrity of the DNA sequence or structure.

Fig. 5. Cavitation versus ultrasound treatment energy. RN18 MBs (approx. 1×10^9 MB/mL) were diluted 1:1000 in PBS and vortexed prior to 60 s of ultrasound exposure in the *in vitro* setup. The remaining percentage of MBs with respect to an increase in US treatment energy follows an exponential decay trend. A range of conditions (18 μ s–36 ms and PNPs 0.5–2.5 MPa) were tested with $n = 2$ for each condition. MB destruction was quantified using gated counts of MBs from flow cytometry, relative to sham-exposed MBs. Each circle represents one group with a distinct US condition.

Table 1

Summary of HEK293T experimental conditions.

Pulse Duration	PNP (MPa)	Power (W)	n
18 μ s	2.5	280	8
50 μ s	0.5	10	3
	1.1	50	3
	1.5	100	4
	2.1	200	4
150 μ s	0.7	20	5
	1.1	50	5
	1.5	100	7
	2.1	200	7
400 μ s	0.5	10	5
	0.7	20	5
	1.1	50	5
	1.5	100	6
	2.1	200	7
1 ms	1.3	71	5
	2.2	210	5
2 ms	0.5	10	3
	0.7	20	2
	0.8	30	2
	1.1	50	5
	1.6	110	5
	2.0	180	5
4 ms	0.5	10	2
	0.7	20	7
	0.8	30	2
	1.1	50	6
	1.4	89	5
5 ms	0.6	14	5
7 ms	0.5	10	5
	1.1	51	5
11 ms	0.7	19	6
14 ms	0.6	15	5
18 ms	0.7	20	5
22 ms	0.5	10	5
24 ms	0.6	15	5
36 ms	0.5	10	5

Table 2

Summary of murine experimental conditions.

Pulse Duration	PNP (MPa)	Power (W)	n
18 μ s	0.7	20	5
	1.1	50	5
	1.5	100	5
	2.1	200	5
	2.5	280	41
50 μ s	0.5	10	5
	0.7	20	5
	1.1	50	5
	1.5	100	5
	2.1	200	5
150 μ s	0.7	20	5
	1.1	50	5
	1.5	100	5
	2.1	200	5
400 μ s	0.5	10	5
	0.7	20	5
	1.1	50	10
	1.5	100	5
1 ms	0.5	10	5
	0.7	20	10
	0.8	30	10
	1.1	50	5
	1.5	100	4
2 ms	0.5	10	5
	0.7	20	4
	0.8	30	10
	1.6	108	6
4 ms	0.5	10	4
	0.7	20	9
	1.1	54	5
	1.4	94	5
7 ms	0.5	10	4
22 ms	0.5	10	10