Chapter 2

Optimization of ultrasound and microbubbles targeted 
gene delivery to cultured primary endothelial cells


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Abstract
Ultrasound and microbubbles targeted gene delivery (UMTGD) is a promising technique for local gene delivery. As the endothelium is a primary target for systemic UMTGD this study aimed at establishing the optimal parameters of UMTGD to primary endothelial cells. For this, an in vitro ultrasound (US) setup was employed in which individual UMTGD parameters were systematically optimized. The criteria for the final optimized protocol were 1) relative high reporter gene expression levels, restricted to the ultrasound exposed area and 2) induction of not more than 5% cell death. US frequency and timing of medium replacement had a strong effect on UMTGD efficiency. Furthermore, ultrasound intensity, DNA concentration and total duration of US all affected UMTGD efficiency. Optimal targeted gene delivery to primary endothelial cells can be accomplished with Sonovue® microbubbles, using 20 μg/ml plasmid DNA, a 1 MHz US exposure of Ispta 0.10 W/cm² for 30 sec with immediate medium change after UMTGD. This optimized protocol resulted in both an increase in the number of transfected cells (more than 3 fold) and increased levels of transgene expression per cell (170%).

Introduction
Although gene therapy has been used in several clinical trials, it is still not approved for regular clinical use. In about 70 % of the clinical trials, recombinant viruses have been used as a gene delivery vector. As viruses have the innate ability to infect host cells, they are efficient vectors for gene delivery. However, the drawbacks of viral gene transfer are the possible immunogenic, inflammatory, cytotoxic and in the case of retroviruses, oncogenic responses. Furthermore, the costs of large-scale production of such viruses are generally high. For these reasons, non-viral vectors have received considerable attention. These non-viral vectors have the potential to be relatively safe, due to their low inflammatory, non-infectious properties and may be produced at a large scale with relatively low costs. However, the main drawback of non-viral vectors is their limited efficiency, limiting their clinical usefulness.

It has been demonstrated that ultrasound (US) and microbubbles targeted gene delivery (UMTGD) is a promising non-viral vector for local gene delivery. UMTGD has several advantageous properties; the method is relatively cheap, requires minimal invasive procedures, can be applied locally and repeatedly to tissues. Furthermore, microbubbles are commercially available and are approved by the regulatory authorities for use as contrast agent in US imaging. Clinical practice demonstrates that microbubbles are well tolerated as severe adverse side effects are rarely observed.

The ultimate goal for systemic UMTGD, would be to inject microbubbles coated with DNA into the circulation and after subsequent exposure to US, confine gene delivery to the tissues exposed to US. The endothelium is a primary target tissue for this systemic approach of UMTGD and plays an important role in several vascular pathologies, including hypertension, arteriosclerosis, arterial restenosis and thrombosis. To date, UMTGD has...
already been successfully applied in several experimental cardiovascular disease models to promote angiogenesis\textsuperscript{12-14}, reduce neointima formation\textsuperscript{15} and augment endothelial function\textsuperscript{16}.

Despite these studies, there is limited data on the parameters of UMTGD that influence transfection efficiency in endothelial cells. It has been shown for several cell lines, including endothelial cells, that addition of microbubbles improves US mediated delivery of naked DNA\textsuperscript{17, 18}. Further the importance of several other UMTGD parameters were identified in immortalized cell lines\textsuperscript{19-21}. However, as primary endothelial cells and immortalized cells differ substantially in physiology and ability to take up DNA\textsuperscript{22, 23}, specific UMTGD settings may be required for primary endothelial cells. Therefore, the aim of this study is to determine the optimal parameters for an maximal UMTGD to primary endothelial cells using commercially available Sonovue\textsuperscript{9} microbubbles. The hypothesis is that important UMTGD parameters, i.e. ultrasound (US) intensity, total time of US exposure, US frequency, DNA concentration and timing of medium change will affect transfection efficiency and cell viability of the endothelial cells. Therefore, these parameters were systematically changed to establish an optimal UMTGD protocol.

Materials and methods

Cell culture
Primary bovine aorta endothelial cells (BAECs, Cell Applications, San Diego, CA, USA) were cultured in DMEM supplemented with 1 g/l glucose, 4 mM L-glutamine, 25mM HEPES, 110 mg/l pyruvate (Gibco BRL, Invitrogen, Groningen, the Netherlands), 10 % of Fetal Bovine Serum (PAA laboratories, Pasching, Germany), 100 units/ml of penicillin and 100 μg/ml streptomycin (Gibco BRL, Invitrogen, Groningen, the Netherlands) in a humidified incubator at 37°C and 5% CO\textsubscript{2}. When cells reached confluence, they were subcultured in a 1:10 ratio employing trypsin EDTA (Gibco BRL, Invitrogen, Groningen, the Netherlands). Cells between passage 3 and 6 were used for UMTGD experiments.

Plasmids
The 4.7 kb pEGFP-N1 (Clontech, Mountain view, CA, USA) and the 4.8- kb pGL3-basic (Promega, Madison, WI, USA) plasmids, encoding GFP and luciferase respectively, were amplified using E.coli JM109. Plasmids were isolated using the plasmid giga isolation kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s instructions. DNA concentrations and purity was determined using the nanodrop spectrometer ND-1000 (Isogen Lifescience, Usselstein, the Netherlands).

Ultrasound exposure setup
The experimental acoustic setup was similar to the one described by van Wamel et al.\textsuperscript{24} and consisted of a 2.25 MHz or 1 MHz unfocused 14 mm single-element transducer (Panametrics, Waltham, MA, USA) mounted at an angle of 45 degrees in a tank filled with PBS (Invitrogen, Groningen, the Netherlands) at 37°C. Cells were grown in Opticell\textsuperscript{TM} cell culture chambers (Biocrystal, Westerville, OH, USA), in which cells were adherent to one of
the two gas-permeable membranes enclosing a 10 ml chamber. Opticell™ chambers were mounted in the experimental setup as shown in figure 1A. The membranes of the Opticell™ caused no change in the characteristics of the US (data not shown). US was generated by a computer controlled waveform generator (33220A, Agilent, Palto Alto, CA, USA) and amplified by a linear power amplifier (150A100B, Amplifier Research, Bothell, WA, USA). The amplified signal was monitored by a synchronized digital oscilloscope (GOULD DSO 465, Valley View, OH, USA). The peak to peak and peak negative acoustic pressure generated at the region-of-interest were measured with a calibrated hydrophone (PVDFZ44-0400, Specialty Engineering Associates, Soquel, CA, USA). The peak negative acoustic pressure was 0.33 MPa for the 2.25 MHz transducer and 0.22 MPa for the 1 MHz transducer.

**Preparation of Sonovue® microbubbles / DNA suspension**

Sonovue® microbubble contrast agent (Bracco, High Wycombe, UK) was reconstituted in 5 ml of saline solution, according to manufacturer’s protocol, resulting in a solution containing $2 \cdot 10^6 - 5 \cdot 10^7$ microbubbles/ml. For the transfection of one Opticell™, 125 μl of Sonovue® microbubble suspension was transferred to a new vial and 100 μg pGL3 basic and 100 μg of pEGFP-n1 was added, resulting in a total volume of approximately 250 μl. After thorough mixing, the mixture was incubated for 5 minutes at room temperature, before being injected into the Opticells™.

Figure 1: Experimental setup and US parameters. A. Diagram showing experimental US setup. An unfocused 14 mm single-element US transducer (1MHz or 2.25 MHz) was mounted at an angle of 45 degrees in a tank filled with PBS. Endothelial cells were cultured in Opticell™ cell culture chambers. B. Diagram showing the US protocol used to transfect endothelial cells. PRP = pulse repetition period is the time from the beginning of a pulse to the beginning of the next pulse.
Optimization of ultrasound and microbubbles targeted gene delivery.

**Initial UMTGD protocol**

Based on literature and pilot experiments the following initial protocol was established. One day before gene delivery, cells were seeded at 33% confluence in Opticell™ cell culture chambers. Prior to US exposure, culture medium was replaced with 10 ml of medium without fetal bovine serum and microbubbles and DNA suspension was added to the medium in the Opticells™. The microbubbles were homogenously distributed throughout the medium. Opticell™ chambers were subsequently placed horizontally to allow microbubbles to rise to the surface of the cells and mounted in the experimental acoustic set up. Microbubbles and cells were exposed to sinusoidal US waves with a frequency of 2.25 MHz with a pulse repetition period (PRP) of 50 ms with 10 000 cycles per pulse for 120 seconds (figure 1B). After US exposure, Opticell™ chambers were incubated in a humidified incubator at 37°C and 5% CO₂. Serum free medium was replaced with normal culture medium 16 hours after US exposure. Twenty-four hours after US exposure, GFP expression and cell detachment were assessed by fluorescent and phase-contrast microscopy. Subsequently, luciferase activity was measured to quantify gene delivery efficiency.

**Optimization protocol**

The UMTGD parameters, ultrasound intensity (spatial peak temporal average intensity (lspa)), DNA concentration, timing of changing transfection medium for culture medium, total time of ultrasound and frequency, were systematically changed. The most optimal parameter setting, as determined by luciferase expression and cell detachment was carried forward in the subsequent steps of the optimization. For obtaining an optimal UMTGD protocol the following criteria were used: 1) Transfection should be mediated by US, therefore transfection should only occur in the region exposed to the US. 2) Cell death, determined by cell detachment, should be less than 5% in the final optimized protocol.

**Cell detachment**

The percentage of cell detachment was scored and ranked in a scale from 0 to 3. Score 0: less than 5% cell detachment, score 1: between 5 and 10% detachment, score 2: between 10 and 30% detachment, score 3: more than 30% cell detachment. Cell detachment was scored both inside and outside of the US exposed area to exclude potential culturing artefacts and toxicity of the transfection medium.

**Luciferase activity**

Using a template, a square of 3.24 cm² was cut from the Opticell™ membrane in the region exposed to US. Similarly, a section of unexposed membrane was cut from the same Opticell™. Subsequently, the cells on the excised membranes were lysed with 125 μl luciferase assay lysis buffer. Luciferase activity was measured per 40 μl sample according to the manufacturer’s instructions (Promega, Madison, WI, USA).
Chapter 2

Quantification of GFP expression
24 hours after UMTGD, five fields of 1.3 mm by 1.3 mm per Opticell™ were scanned in the area exposed to US using confocal microscopy (LSM 410, Carl Zeiss, Germany). The number of GFP positive cells per field and their intensity were determined with Image-Pro plus v 4.5 (Media Cybernetics, Silver Spring, MD, USA).

Microscopic observations of microbubbles during US exposure
The behaviour of the Sonovue® microbubbles was observed with a high speed CCD camera (LCL-902K, Watec America Corp., Las Vegas, NV, USA) mounted on top of a inverted microscope (Olympus, Zoeterwoude, the Netherlands) Recordings were made with 2000 frames per second

Statistics
Each transfection condition was evaluated in at least six-fold. Luciferase activity and fluorescent intensity data are presented as mean±SEM. The Student’s t-test was used to test differences between 2 groups, otherwise one way ANOVA with least squared differences post hoc analysis was used or in case of differences in variances between groups a Dunnett T3 post hoc analysis was used. Detachment scores and number of GFP positive cells were presented as median±IQR (interquartile range) and compared with a Kruskal-Wallis test followed by Mann-Whitney tests for individual group comparisons with a Bonferroni’s correction. A P-value lower than 0.05 was considered significant.

Results
Initial UMTGD protocol
UMTGD was evaluated with an initial protocol of sinusoidal US waves (peak negative acoustic pressure of 0.33 MPa) with a frequency of 2.25 MHz in a pulse repetition period (PRP) of 50 ms with 10 000 cycles per pulse for 120 seconds (figure 1B). After 24 hours, fluorescent microscopy of GFP expression demonstrated that the initial UMTGD protocol resulted in transfection of endothelial cells, which was restricted to cells exposed to US (data not shown). These data were confirmed by quantification of luciferase activity, as cells outside the US exposed area showed no significant luciferase activity (62.3 ± 2.8 relative light units per second [RLU/sec]) compared to untreated control cells (69.2 ±2.3 RLU/sec). Luciferase activity in the cells exposed to US was significantly increased to 273.0 ± 47.8 RLU/sec.Using the initial protocol, moderate to severe cell detachment was observed in the US exposed area (detachment score: 2.0 ± 0.3). Cell detachment was negligible in the area that was not exposed to US (detachment score: 0.0 ± 0.0.), indicating that the observed cell detachment in the US exposed area is caused by exposure to US. To reduce the level of cell detachment, we first aimed at reducing the US intensity, by reducing the number of cycles per pulse.
Optimization of ultrasound and microbubbles targeted gene delivery.

Figure 2: Optimization of UMTGD to endothelial cells.

Individual UMTGD parameters were optimized in the following order: A) cycles per pulse, B) DNA concentration, C) timing of medium change, D) total time of US exposure and E) US frequency. For each round of experiments the most optimal parameter combination from the previous round, was carried forward into the next experimental series. Asterisks show significant differences compared to default setting of each experimental. Optimization started with a protocol of sinusoidal US waves.
(peak negative acoustic pressure of 0.33 MPa) with a frequency of 2.25 MHz in a pulse repetition period (PRP) of 50 ms with 10000 cycles per pulse for 120 seconds, DNA concentration 20µg DNA/ml. A. The use of 7000 cycles per pulse (Ispta 0.23 W/cm$^2$) resulted in a significant increase in luciferase activity compared to the default setting of 10 000 cycles per pulse (0.32 W/cm$^2$) (p < 0.05).

B. The default DNA concentration of 20 µg/ml was more efficient than 4, 8 and 14 µg/ml of plasmid DNA (p < 0.05). C. Direct change of transfection medium for culture medium results in a better efficiency over changing the medium after 16 hours (p < 0.001). D. Exposure of the cells to 30 seconds of ultrasound shows a significant increase in luciferase activity compared to the default setting of 120 seconds of ultrasound exposure p < 0.05. E. The use of ultrasound with a frequency of 1 MHz, with an ultrasound intensity of Ispta 0.23 and 0.1 W/ cm$^2$, resulted in a significant increase in UMTGD efficiency over 2.25 MHz (Ispta 0.23 W/cm$^2$)

Ultrasound intensity
Varying the ultrasound intensity, by varying the number of cycles per pulse, in the initial UMTGD protocol resulted in a 2.5 fold increase in transfection efficiency at 7000 cycles per pulse (Ispta 0.23 W/cm$^2$) over the initial protocol of 10 000 cycles per pulse (Ispta 0.32 W/cm$^2$) (figure 2A). Cell detachment scores for 1000 (Ispta 0.03 W/cm$^2$), 5000 (Ispta 0.16 W/cm$^2$) and 7000 cycles per pulse (Ispta 0.23 W/cm$^2$) were similar (1.0±0.0, 1.0±0.0 and 1.0±0.0, respectively), but were decreased compared to 10 000 cycles per pulse (detachment score: 2.0 ± 0.3). For further optimization, 7000 cycles per pulse was carried forward into the next experimental series.

DNA concentration
To study the effect of DNA concentration, we studied plasmid DNA concentrations from 4 to 40 µg/ml. As shown in figure 2B, decreasing the plasmid DNA concentration from the default 20 µg/ml resulted in reduced luciferase activities. Increasing the DNA concentration to 40 µg/ml did not result in a significant change in luciferase activity. Cell detachment scores for the DNA concentrations of 4, 8, 14, 20 and 40 µg/ml were 0.0±0.0, 0.0±0.0, 1.0±0.5, 1.0±0.8, 3.0±0.0 respectively. A DNA concentration of 20 µg/ml was used in subsequent experiments.

Timing of replacement of transfection medium
In the experiments described above, cells were incubated with transfection medium for 16 hours after UMTGD. Transfection efficiency was increased by about 7.5 fold when the transfection medium was replaced with culture medium immediately after UMTGD (figure 2C). This was accompanied by a significant decrease in cell detachment score from 1.0±0.8 to 0.0±0.0. Therefore, transfection medium was immediately replaced with normal culture medium after US exposure in all subsequent experiments.
Optimization of ultrasound and microbubbles targeted gene delivery.

Total duration of US exposure
Further, the effect of the total duration of US exposure on UMTGD efficiency was studied (figure 2D). Compared to the default of 120 seconds of US exposure, luciferase activity was significantly increased to 175% using an US duration of 30 seconds. Detachment scores were 0.0 ±0.0 for all durations. In the subsequent experiments, cells were exposed to 30 seconds US.

US frequency
The final parameter investigated was the effect of US frequency on UMTGD efficiency. For this the US frequencies of 2.25 MHz was compared to 1 MHz, while keeping the US intensity identical for both frequencies (Ispta 0.23 W/cm²). UMTGD with 1 MHz US was 2.5 fold more efficient than US with a frequency of 2.25 MHz (figure 2E). However, large numbers of cells detached from the membrane after 1 MHz US exposure (detachment score 2.0 ± 0.0) whereas the cell detachment score for 2.25 MHz stimulation was 0.0 ± 0.0. Because of the severe cell detachment with 1MHz US (Ispta 0.23 W/cm²), the number of cycles was decreased from 7000 to 3111 cycles per pulse, resulting in lower US intensity (Ispta 0.1 W/cm²) but identical duty factors for both frequencies. The protocol of 1 MHz US with 3111 cycles per pulse resulted in a cell detachment score of 0.0±0.0. Despite lower US intensity (Ispta 0.10 W/cm²), UMTGD efficiency at 1 MHz was increased by 13-fold compared to 2.25 MHz.

Transfection efficiency and expression levels
Next we aimed at determining whether the increased UMTGD efficiency as measured by luciferase expression, was caused by increased transgene expression per cell or whether more cells were transfected. To study this, the number of GFP expressing cells per microscopic field and the intensity of GFP expression per positive cell for both the initial protocol and the optimized protocol were determined. The number of GFP expressing cells increased from 6.0 ± 2.6 cells per field (area 1.7 mm²) for the initial protocol to 20.8 ± 6.8 cells per field, amounting to 5% of all cells, for the optimized protocol (p<0.001). Fluorescent intensity increased from 100 ±10 arbitrary units per cell for the initial protocol to 177 ± 15 arbitrary units for the optimized protocol (p<0.001).

High speed recordings of microbubble behaviour
Furthermore, because the mechanism of transfection is thought to be linked to acoustic bioeffects created by microbubbles when sonified, high speed recordings were taken to monitor microbubble behavior during US exposure. These experiments showed that all microbubbles disappeared or fused to clusters of larger stable macrobubbles immediately after onset of US. All macrobubble clusters were subsequently pushed out of the area exposed to US within 5 seconds. Furthermore, no formation of gas bubbles larger than 1 μm occurred by dissolved gas during negative pressure cycles of the acoustic waves (data not shown).
Chapter 2

Discussion

This study demonstrates that targeted transfection of primary endothelial cells is possible using UMTGD. Furthermore, all the parameters studied, ultrasound intensity, exposure time and frequency of ultrasound, the DNA concentration and timing of medium change significantly influenced UMTGD efficiency in endothelial cells. Optimal parameter settings increased the total number of transfected cells as well as the expression levels of the transgene per cell.

In all of our experiments, luciferase activity was restricted to the cells exposed to US. Therefore our data demonstrate that specific targeting of UMTGD to endothelial cells is possible using Sonovue® microbubbles. The observation that UMTGD is confined to the cells in the US exposed area may be dependent on the type of microbubble used. A recent in vivo study demonstrated that Optison® microbubbles effectively transfected mouse skeletal muscle without US exposure, whereas transfection with Sonovue® microbubbles was dependent on US exposure\(^2\). Obviously, this ability of Optison\(^\text{TM}\) to transfect cells without US exposure would seriously affect the preferred local confinement of gene delivery to tissues. Although these data were obtained by intramuscular injection of DNA and microbubbles, it indicates that Sonovue® microbubbles may be more suitable for local UMTGD following systemic administration.

Using our initial protocol, moderate to severe cell death was observed in the US exposed area. Cell detachment was negligible in the area that was not exposed to US, indicating that the observed cell detachment in the US exposed area is caused by exposure to US. To reduce the level of cell detachment, we first aimed at reducing the US intensity, by reducing the number of cycles per pulse. Reducing the number of cycles per pulse from 10000 to 7000, 5000 and 1000, resulted in decreased cell detachment, confirming that the observed cell detachment in the US exposed area is caused by exposure to US. A significant increase in UMTGD efficiency was observed only at 7000 cycles per pulse, indicating that a narrow optimal intensity of US for UMTGD is present close to the US intensity causing severe cell detachment. In contrast to our findings, Rahim et al.\(^\text{20}\) reported that duty factor does not significantly affect UMTGD efficiency in CHO cells. However, Rahim et al.\(^\text{20}\) did not investigate US intensities close to the levels causing severe cell detachment and therefore the narrow optimum in US intensity may have been missed.

Similar to the optimal US intensity the optimal DNA concentration of 20 μg/ml was close to the concentration causing severe cell detachment (40 μg/ml). In general, our and previous studies\(^\text{19-21}\) demonstrate that relatively high DNA concentrations are needed for efficient UMTGD. As only a small fraction of the DNA binds to the Sonovue® bubbles (data not shown), UMTGD seems dependent on DNA that is dissolved in the transfection medium. When injected into the circulation, the DNA and microbubbles would separate quickly. Therefore, with Sonovue® microbubbles, systemic UMTGD will depend on a technique that injects both microbubbles and DNA directly upstream of the target tissue.

In initial experiments, cells were left on transfection medium (without fetal bovine serum) for 16 hours after UMTGD. Direct replacement of the transfection medium for culture
Optimization of ultrasound and microbubbles targeted gene delivery.

medium resulted in a 7.5 fold increase in transfection efficiency. This is likely due to a reduction in cell death and enhanced transcription as a result of removal of residual components in the transfection medium and/or addition of FCS. Moreover, the increase of transfection by immediate change of medium following US exposure suggest that transfection of endothelial cells by UMTGD is mainly accomplished during the US exposure. This is an agreement with a study by van Wamel et al. 

In this study optimal UMTGD efficiency was achieved with an US duration of 30 seconds. Other studies indicate that the optimal duration of US exposure depend on the type of cell studied. For MCF7 cells the optimum duration is between 1 and 2 minutes, while for CHO cells no clear optimum could be established. The exact mechanisms of UMTGD are still not completely understood, but are thought to be linked to a bioeffect called sonoporation. This process is defined as the transfer of extracellular molecules through transient pores in the cell membrane which are created as a consequence of acoustic cavitation. Our results showed a significant increase in UMTGD efficiency when cells are exposed to 30 sec of ultrasound compared to 5 sec (p< 0.05), even though no microbubbles are existing anymore in the area exposed to ultrasound within 5 sec. Consequently, our data suggest that in addition to acoustic cavitation, other mechanisms are involved in increasing efficiency of UMTGD by prolonged exposure to US. Recently, suggested that the collapse of the microbubbles destabilizes the cell membrane and that continued US enhances the membrane destabilization, increasing the efficiency of UMTGD. Alternatively, suggested that gas bodies capable of cavitation still exist after destruction of microbubbles. However, using high speed camera recordings, we could not support the latter hypothesis as we did not detect any gasbubbles larger than 1 μm after 5 seconds of US exposure. A better understanding of the mechanism(s) of UMTGD to endothelial cells may further improve UMTGD efficiency and increase its potential for systemic UMTGD gene therapeutic use.

UMTGD with 1 MHz US was more efficient than US with a frequency of 2.25 MHz, although cells detachment increased. Lowering the US energy for 1MHz US, further increased UMTGD efficiency while decreasing cell detachment to acceptable levels. From this we conclude that US of 1MHz is the optimal frequency for UMTGD to endothelial cells in our experimental setup. However, the most efficient frequency for UMTGD may depend on the type of microbubble being used. Although we found more efficient UMTGD delivery with Sonovue® using US of 1 MHz over 2.25 MHz, Larina et al. demonstrated improved UMTGD efficiency with 3 MHz US over 1 MHz US using Optison™. These differences may originate from factors that influence the cavitational behavior of microbubbles, such as the composition of the microbubble shell and bubble size distribution. Furthermore Larina et al. also observed more cell damage using 1 MHz US than for 3 MHz over a wide range of US intensities. Possibly the non-cavitational bioeffects of US may be involved in increased cell damage using 1MHz US.

In summary, optimal targeted gene delivery of endothelial cells can be accomplished using Sonovue® microbubbles with 20 μg/ml of plasmid DNA and ultrasound exposure (Ispta 0.10
W/cm²) with a frequency of 1 MHz for 30 sec and direct medium change after UMTGD. In this study discrepancies were noted compared to other optimization studies with respect to the influence of specific parameters on UMTGD efficiency, including ultrasound intensity, duration and frequency. This may be explained by the variability between experimental set-ups between labs. However, a previous study demonstrated that UMTGD vary substantially between different cancer cell lines using one experimental setup, which was likely caused by differences in physiology between the cancer cell lines. Therefore, it is more conceivable that differences in physiology and uptake of DNA between endothelial cells and immortalized cell lines may play an important role in the differences found in the influence of specific parameters on UMTGD efficiency.
Optimization of ultrasound and microbubbles targeted gene delivery.

References

Chapter 2


