Chapter 3

Ultrasound and microbubble mediated gene therapy: effectiveness of siRNA versus plasmid DNA delivery

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Abstract
The enhanced delivery of nucleic acids and the favorable safety profile may transform ultrasound and microbubbles targeted delivery (UMTD) into a valuable therapeutic tool in the treatment of vascular disease. Recently, we demonstrated that in UMTD of primary endothelial cells, small molecules enter the cell mainly through transient pores while larger molecules are more dependent on endocytosis. Based on the molecular weights and cellular targets of siRNA and plasmid DNA, we hypothesized that UMTD of siRNA is more efficient in changing gene expression than UMTD of plasmids. Therefore, the aim of this study is to evaluate the efficiencies of both strategies in changing the expression of the moderately expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cultured endothelial cells.

UMTD of GAPDH siRNA resulted in transfection of 97.9±1.5% of all endothelial cells, with labeled siRNA both in the nucleus and cytoplasm immediately after UMTD. Transfection of siRNA decreased GAPDH protein levels by approximately 70%. In contrast, following UMTD of GAPDH plasmid DNA, only 2.0 ± 0.7% of cells expressed the transgene, which failed to increase overall GAPDH protein levels. Importantly, labeled plasmid DNA was delivered to 43.0 ± 4.2% of all cells and was detected in endosomes positive for clathrin and caveolin immediately and 24 hours after UMTD.

In conclusion, UMTD of siRNA is more effective than UMDT of plasmid in modifying protein levels of the moderately expressed gene GAPDH in cultured endothelial cells, suggesting that UMTD of siRNA may become a valuable therapeutic strategy in the treatment of vascular disease.

Introduction
The vascular endothelium is an attractive target for gene therapy because of its accessibility and its importance in a wide range of cardiovascular pathologies, including hypertension, arteriosclerosis, arterial restenosis and thrombosis. Gene therapy may be used as a therapeutic strategy to alter the homeostatic balance of endothelial cells by expressing transgenes or by silencing endogenous target genes. Upregulation of genes may be accomplished by the administration of DNA encoding for the gene of choice, while downregulation is generally achieved by interference at the post-transcriptional level employing gene specific synthetic antisense oligonucleotides, such as oligodeoxynucleotides (ODNs) or siRNA.

Ultrasound contrast microbubbles are a promising vehicle in gene therapy, due to their low toxicity and immunogenicity, non-invasive nature, local application and its cost-effectiveness. Moreover, molecular imaging and therapeutic compound delivery may be performed simultaneously, in an efficient way.

Ultrasound and microbubbles targeted delivery (UMTD) of nucleic acids targeting the endothelium has been applied in vitro as well as in vivo. In vitro, UMTD of plasmids to endothelial cells reached transfection efficiencies of 5-20%. Furthermore, UMTD of plasmid in-vivo targeting the endothelium was successfully applied in models of
cardiovascular disease to augment endothelial function (eNOS) or promote angiogenesis (VEGF, HGF, FGF), although the exact transfection efficiencies were not determined. Only a limited number of studies have reported on ultrasound and microbubble mediated delivery of siRNA. In vivo, UMTD of siRNA has been performed in the tibialis muscle of mice. In addition, several studies demonstrated UMTD of siRNA in in-vitro models.

Recently, we investigated the mechanisms underlying UMTD in primary endothelial cells. There, we demonstrated that endocytosis is a key mechanism in UMTD besides transient pore formation, with the contribution of endocytosis being dependent on molecular size. Although siRNA and plasmid DNA are both oligonucleotides, they differ substantially in size (~15 kDa versus ~3500 kDa). Therefore, siRNA may enter the cell mainly through transient pores and deliver the siRNA directly to its target, the mature mRNA residing in the cytosol. The uptake of plasmids would be mediated mainly through endocytosis. For transcription of the plasmids, the plasmid DNA needs to escape the endosome with subsequent active transport to the nucleus. As it is unlikely that UMTD promotes these latter two processes, we hypothesized that UMTD mediated delivery of siRNA is more effective in changing gene transcription than UMTD mediated delivery of plasmids.

To test this hypothesis, we aimed at changing the gene expression levels of the moderately expressed gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cultured endothelial cells by UMTD using GAPDH siRNA and a GAPDH expression plasmid. For this, we employed our optimized protocol for delivery of plasmid DNA to cultured primary endothelial cells.

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, Netherlands), 10 % of Fetal Bovine Serum (FBS, PAA laboratories, Pasching, Germany), 100 units/ml of penicillin and 100 µg/ml streptomycin (Gibco BRL, Invitrogen, Groningen, Netherlands) in a humidified incubator at 37°C and 5% CO₂. When cells reached confluence, they were subcultured in a 1:10 ratio employing trypsin EDTA (Gibco BRL, Invitrogen, Groningen, Netherlands). Cells between passage 3 and 7 were used for UMTD experiments. 48 Hours prior to ultrasound exposure, cells were seeded at 33% confluency in Opticell™ cell culture chambers (Biocrystal, Westerville, OH, USA), in which cells could adhere to one of the two gas-permeable, ultrasound transparent membranes.

Preparation of Sonovue® microbubbles

Sonovue® microbubble contrast agent (Bracco, High Wycombe, UK) was reconstituted in 5 ml of saline solution according to manufacturer’s protocol. Reconstitution of the Sonovue® microbubbles resulted in a preparation containing 2·10⁸ – 5·10⁸ microbubbles/ml.
Ultrasound exposure setup

The experimental acoustic setup used was similar to a described before\(^5\). In short, the ultrasound setup consisted of a 1 MHz unfocused 14mm single-element transducer (Panametrics, Waltham, MA, USA) mounted at an angle of 45 degrees in a tank filled with PBS (Invitrogen, Groningen, Netherlands) at 37°C. US was generated by a computer controlled waveform generator (33220A, Agilent, Palo 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).

UMTD protocol

For the evaluation of the relative efficiencies of UMTD of siRNA and plasmid DNA, we used the previously optimized protocol for the delivery of plasmid DNA to cultured primary endothelial cells\(^5\). There, we determined that a plasmid DNA concentration of 20 µg/ml was optimal in UMTD of plasmids in endothelial cells. siRNA concentrations ranged between 2.25 nM and 2.25 µM. For the transfection of one Opticell, 125 µl of Sonovue microbubble suspension was transferred to a new vial and either plasmid or siRNA was added in a volume 125 µl at a concentration of 80 times the end-concentration. After thorough mixing, the mixture was incubated for 5 min at room temperature. Addition of 9.75 ml medium without FBS resulted in the desired end-concentration and the mixture was subsequently injected into the Opticells\(^\text{TM}\). Microbubbles and cells were exposed to sinusoidal US waves with a frequency of 1 MHz with a pulse repetition period of 50 ms with 3111 cycles per pulse during 30 seconds. Peak negative acoustic pressure generated at the region-of-interest was 0.22 MPa as measured with a calibrated hydrophone (PVDFZ44-0400, Specialty Engineering Associates, Soquel, CA, USA).

Plasmids and fluorescent microscopy

The 5.7 kb plasmid CMV-sport6 encoding bovine GAPDH (LGC promochem, Teddington, UK) and the 4.7 kb plasmid EGFP-N1 encoding green fluorescent protein (GFP; Clontech, Mountain view, CA, USA) were amplified using Escherichia coli JM109. Plasmids were isolated using the nucleobond pc10000 isolation kit (Machery Nachel, Düren, Germany) according to the manufacturer’s instructions. DNA concentrations and purity were determined using a nanodrop spectrometer ND-1000 (Isogen Lifescience, IJsselstein, Netherlands). Functionality of both plasmids was established in human 293 cells by transfection of the plasmids using lipofectamine (invitrogen). After 24 hours, the expression of EGFP and bovine GAPDH was established by fluorescent microscopy and western-blot, respectively (data not shown).

Plasmid labeling

For fluorescent microscopy experiments, plasmid DNA was covalently labeled with the fluorophore Cy3 using the Label IT kit (MIRUS, Madison, WI) according to the
manufacturer’s instructions. On average, one Cy3 molecule was bound per 71 bp. Immediately and 24 hours after UMTD, fluorescent microscopy (LSM 410, Carl Zeiss, Sliedrecht, Netherlands) images were made using a 40x or 100x magnification oil-immersion lens (Carl-Zeiss, Sliedrecht, Netherlands).

**siRNA and fluorescent microscopy**

Double stranded siRNA directed toward the mRNA target for bovine glyceraldehyde 3-phosphate dehydrogenase (sense strand 5’-CCACUUUGUCAAGCUCAUUTT-3’, antisense strand 5’-AAUGAGCUUGACAAAGUGGTT-3’, Eurogentec, Maastricht, Netherlands) was used. As a control, the Eurogentec universal negative control sequence (scrambled siRNA) was used. To study its cellular localization, siRNA was labeled with a 5’ TAMRA label (Eurogentec, Maastricht, Netherlands) and used at a final concentration of 2.2 µM. Immediately after UMTD, fluorescent and bright-field images were recorded.

**Co-localisation of plasmids with endocytosis markers clathrin and caveolin-1**

Directly following UMTD of Cy3-labeled plasmid, regions-of-interest were cut from the Opticell (approximately 1.5 cm2) and placed in PBS. Cells were fixed in 4% formaldehyde 10 minutes at room temperature. Cells were washed three times with PBS, permeabilized for 5 minutes in 0.05% Triton X-100 (Sigma-Aldrich) in PBS, followed by three washes in PBS-Tween (0.5%, Tween 20; Sigma-Aldrich). Cells were incubated with polyclonal goat anti-clathrin heavy chain, a marker for clathrin-mediated endocytosis (Santa Cruz, the Netherlands) or with monoclonal mouse anti-caveolin-1 (Clone C060; BD Biosciences, Breda, the Netherlands), a marker for caveolin-mediated endocytosis; both antibodies were diluted 1:100. Cells were incubated overnight at 4ºC, washed three times with PBS-Tween, and incubated with both Cy3-labeled rat-anti-mouse and Cy5-labeled donkey-anti-goat secondary antibodies (1:100, Molecular Probes) 30 minutes at room temperature in the dark. Cells were washed twice with PBS-Tween, washed once with PBS and mounted on a microscope slide with mounting-medium containing DAPI nuclear stain (VectashieldTM, Vector Laboratories, Burlingame, CA, USA). 3-Dimensional (3D) images were acquired using a ZEISS Axiovert 200M MarianaSTM inverted microscope (I.I.I) equipped with a motorized stage (stepper-motor z-axis increments: 0.2 micron). Images were taken using a 63x oil-immersion lens (Carl-Zeiss). A cooled CCD camera (C1280x1024 pixels; Cooke Sensicam, Cooke, Tonawanda, NY), recorded images with true 16-bit capability. The camera is linear over its full dynamic range (up to intensities of over 4000) while dark/background currents (estimated by the intensity outside the cells) are typically <100. The microscope, camera, data viewing-processing were conducted/controlled by SlideBookTM. This software was also used to deconvolve the 3D image stacks in order to remove out-of-focus light, as well as to quantify the extent of co-localization for plasmid with clathrin or caveolin by calculating Pearson’s correlation factor. Pearson’s correlation factor lies between +1 and −1. A positive value implies a positive correlation, thus co-localization,
'0' implies no correlation, and a negative value implies an inverse correlation. As a control, the correlation factor between clathrin (Cy5)/DAPI and caveolin (Cy3)/DAPI was determined.

**Western blotting**

Cells were homogenized in RIPA buffer and protein concentration was determined according to the Bradford method (Sigma, Zwijndrecht, Netherlands), with bovine albumin as a standard. Protein expression was determined by Western blot analysis and expressed as ratio levels of GAPDH/ beta-actin. For this, denatured protein (20 µg) was separated by SDS-PAGE using 4-20% precise protein gels (Pierce, Rockford, IL, USA), transferred to nitrocellulose membranes (Stratagene, Amsterdam, Netherlands) and incubated with primary antibodies against GAPDH (Fitzgerald, Concord, MA, USA) and beta-actin (Sigma-Aldrich, Zwijndrecht, Netherlands). Horseradish peroxidase conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Heerhugowaard, Netherlands) was used as secondary antibody. Signals were detected by the ECL detection method (Amersham, Roosendaal, Netherlands) and quantified by densitometry.

**Immunofluorescence**

Cells were fixed in 4% formaldehyde (Klinipath, Duiven, Netherlands) for 10 minutes at room temperature and were subsequently washed three times in PBS. Cells were permeabilized for 5 min in 0.1% Triton-x (Sigma-Aldrich, Zwijndrecht, Netherlands) in PBS (Gibco BRL, Invitrogen, Groningen, Netherlands), followed by three washes with PBS. After cells were incubated with 3% bovine serum albumine (BSA) (Sigma-Aldrich, Zwijndrecht, Netherlands) in PBS for 30 minutes, a drop of 100 µL of dissolved rabbit monoclonal anti-GAPDH (1:150, Fitzgerald, Concord, MA, USA) in 3%BSA/PBS, was placed on parafilm. Cells were placed on top of the antibody mixture and incubated for 1 hour at room temperature, followed by three PBS wash steps. Thereafter, a drop of 100 µL PBS containing 3% BSA, FITC labeled goat-anti-rabbit secondary antibody (1:200, Molecular Probes, Leiden, Netherlands) was placed on parafilm. Cells were put on top of the secondary reaction mixture and incubated for 60 min at room temperature, in the dark. Subsequently, cells were washed 3 times with PBS and mounted on a microscope slide with mounting medium (Vectashield, Vector Laboratories) and sealed with nail polish. Fluorescent microscopy (LSM 410, Carl Zeiss, Sliedrecht, Netherlands) pictures were taken using a 100x oil-immersion lens (Carl-Zeiss, Sliedrecht, Netherlands). Cellular GAPDH levels were quantified using Image Pro plus, v 5.0 (Media Cybernetics, Silver Spring, MD, USA).

**Cell viability assay**

Using a metal mask, a section of the membrane carrying the cells exposed to ultrasound was cut from the Opticell™ cell culture chamber. Cells were harvested using trypsin and resuspended in 1 ml of medium. 50 ul of the cell suspension was transferred to a well of a 96-well plate. Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA).
Statistics and analyses
All data are shown as the mean±S.D. (n=5). Data were considered significant when P<0.05. The t-test was used to calculate statistical significance.

Results
To compare the efficiency of modulation of gene expression using UMTD, endothelial cells were transfected with GAPDH siRNA to reduce GAPDH protein levels, or with plasmid DNA encoding the bovine GAPDH gene to enhance its expression. Moreover, uptake of the gene constructs and cellular distribution were determined by fluorescently labeled nucleic acids. The previously optimized protocol for the delivery of plasmid DNA to cultured primary endothelial cells was employed.

Silencing of GAPDH after UMTD of GAPDH siRNA
UMTD of GAPDH siRNA to endothelial cells was performed to reduce GAPDH protein levels. Western blot demonstrated that GAPDH protein levels were decreased to 24.3±7.9% of controls using 2.25 µM GAPDH siRNA, while scrambled siRNA had no effect on GAPDH protein levels (figure 1A and 1B). To determine the optimal siRNA concentration, we varied GAPDH siRNA concentrations between 2.25 nM and 2.25 µM. All concentrations were equally effective in down-regulating GAPDH protein levels to about 30% of controls (figure 1C). No differences in cell viability were observed (data not shown).

To determine the efficiency of siRNA delivery, we performed UMTD with TAMRA labeled siRNA. Immediately after UMTD, 97.9±1.5% of the cells was positive for TAMRA labeled siRNA. Next, the cellular localization of the TAMRA-labeled siRNA was determined by high magnification confocal microscopy. Immediately after UMTD, labeled siRNA was detected in the cytosol, nucleus and occasionally in large vesicles (figure 2A). 3D confocal imaging of syto-green labeled nuclei and TAMRA labeled siRNA confirmed the nuclear delivery of siRNA after UMTD (data not shown). TAMRA-labeled siRNA could not be detected after 24 hours.

Finally, we investigated whether GAPDH protein levels were decreased in all cells which received the GAPDH siRNA. Unfortunately, fluorescent siRNA could not be detected 24 hours after UMTD, making a direct comparison between siRNA delivery and GAPDH protein expression in individual cells impossible. However, a frequency diagram of the GAPDH protein expression levels before and after siRNA delivery indicates that GAPDH protein levels were decreased in virtually all cells (figure 3).

Over-expression of GAPDH after UMTD of a GAPDH expression plasmid
To enhance GAPDH protein levels, an optimal dose (20 µg) of plasmid DNA encoding the bovine GAPDH gene was delivered to endothelial cells by UMTD. Twenty-four hours after UMTD, GAPDH protein levels were determined by western blotting. Following UMTD of a GAPDH expression plasmid, no significant increase in GAPDH protein levels was found (figure 1D).
We questioned whether the failure to increase GAPDH protein levels using UMTD of GAPDH plasmid was the result of inefficient plasmid delivery or caused by inefficient plasmid expression. To examine this, we performed a UMTD cotransfection experiment of both a Cy3 labeled plasmid and an unmodified GFP expression plasmid (10µg/ml of each). Immediately after UMTD, 43.0 ± 4.2% of all cells was positive for Cy3. However, 24 hours after UMTD, only 2.0±0.7% of the Cy3 positive cells were also positive for GFP (figure 4). No GFP expression was observed in cells negative for Cy3. Finally, the cellular localization of the Cy3-plasmid was determined. Immediately after UMTD, Cy3-labeled plasmid DNA was mainly found in cytoplasmic vesicles and no Cy3-labeled plasmid was detected in the nucleus (figure 2B). Immediately after UMTD these vesicles appeared to be clustered within the cytoplasm. After 24 hours, the vesicles appeared to be distributed more evenly within the cytoplasm (figure 2C).

Co-localization of endocytosis markers and labeled plasmid
To further investigate the nature of the vesicles positive for labeled plasmid, we investigated whether internalized labeled plasmid co-localizes with clathrin and/or caveolin-1, which are established markers for two main routes of endocytosis. Figure 5 shows clear co-localization of labeled plasmid with clathrin (figure 5A) and with caveolin-1 (figure 5B), as demonstrated by the yellow/orange color in the merged images of plasmid (red) and clathrin/caveolin-1 (green). The extent of co-localization was determined using Pearson’s correlation factor. A positive correlation was found for plasmid with clathrin was 0.28±0.03 (p<0.01, compared to hypothetical value of 0.0), for plasmid with caveolin 0.32±0.12 (p<0.01). As control, no correlation was found for DAPI (nuclei) with either Cy3 (0.01±0.01, p=0.3) or Cy5 channel (-0.01±0.02, p=0.7) (both secondary antibodies).
Figure 1: GAPDH protein levels 24 hours after UMTD of siRNA and plasmid. A) Western blot showing typical result after UMTD of GAPDH plasmid and siRNA. B) UMTD of GAPDH siRNA resulted in significantly decreased GAPDH levels, 24 hours after UMTD. UMTD in the absence of siRNA or in the presence of scrambled siRNA did not affect GAPDH levels. C) GAPDH siRNA concentrations ranging from 2.25 nM to 2.25 µM were equally effective in reducing GAPDH protein levels. D) UMTD of GAPDH plasmid DNA did not increase basal GAPDH levels. UMTD in the absence of plasmid DNA and UMTD of GFP plasmid did not affect GAPDH levels. (* indicates p<0.01 vs control).
Figure 2: Cellular localization of siRNA and plasmid DNA. A. TAMRA labeled siRNA, immediately after UMTD. B. CY3 labeled plasmid immediately after UMTD. C. CY3 labeled plasmid 24h after UMTD.

Figure 3: histogram of GAPDH levels per cell. A frequency histogram was constructed from the cellular GAPDH staining intensity of 75 cells after UMTD of GAPDH siRNA and scrambled siRNA.
Figure 4: Colocalization of CY3 labeled plasmid and expression of GFP. Cells were transfected by UMTD using Cy3 labeled plasmid and an unmodified GFP expression plasmid in equal amounts. After 24 hours, the localization of CY3 labeled plasmid (red signal) and the expression of GFP (green signal) were evaluated using confocal microscopy.

Figure 5: Colocalization of CY3 labeled plasmid with endocytosis markers. A) Colocalization between CY3 plasmid (red) with clathrin (green). Arrows indicate cells positive for both markers. B) Colocalization between CY3 plasmid (red) with caveolin-1 (green).
Discussion

Recently, we demonstrated that in UMTD of primary endothelial cells, small molecules enter the cell mainly through transient pores while larger molecules are more dependent on endocytosis\textsuperscript{16}. Based on the molecular weights and cellular targets of siRNA and plasmid DNA, we hypothesized that UMTD of siRNA is more efficient in changing gene expression than UMTD of plasmids in cultured endothelial cells. To test this hypothesis, we compared the efficiencies of both strategies using the moderately expressed gene GAPDH as a common target.

The previously optimized protocol for UMTD mediated delivery of plasmid DNA\textsuperscript{5} was used to deliver siRNA to primary endothelial cells. This protocol resulted in siRNA delivery to almost all cells, resulting in GAPDH protein down-regulation in approximately all transfected cells. Therefore, our optimized protocol for UMTD of plasmid DNA is also very efficient in delivering siRNA to primary endothelial cells. High transfection efficiency (\textasciitilde50\%) and effective knockdown of target mRNA was also observed after UMTD of siRNA in cultured mesenchymal stem cells\textsuperscript{14}. UMTD of siRNA is therefore comparable to the transfection efficiency of bovine aorta endothelial cells using lentiviruses, adenoviral and retroviral transfection which are between 50-100\%\textsuperscript{17}. The efficiency of vascular delivery of siRNA through UMTD of siRNA is still largely unknown. Intraventricular co-injection of siRNA-GFP and microbubbles with concomitant ultrasonic exposure resulted in substantial reduction in EGFP expression in the coronary artery in EGFP transgenic mice\textsuperscript{18}. Furthermore, it has been demonstrated that in UMTD, siRNA could be delivered to the tibial muscle, skin and heart of mice although the delivery to the kidney failed\textsuperscript{12}. These in-vivo studies all demonstrated knock-down of reporter genes and therefore the efficiency of UMTD siRNA therapy in vascular pathology remains to be established.

Confocal microscopy demonstrated that labeled siRNA was homogeneously distributed in the cytoplasm and nucleus immediately after UMTD. Occasionally, siRNA was observed in large vesicular structures in the cytoplasm. These data support our general finding that in UMTD, small molecules enter the cell mainly through transient pores\textsuperscript{16}. Therefore UMTD of siRNA efficiently delivers siRNA to its target, the mature GAPDH mRNA residing in the cytosol. Furthermore, the nuclear delivery of siRNA may indicate that UMTD of siRNA may also be effective in the knock-down of transcripts restricted to the nucleus\textsuperscript{19}. However, there is no evidence that UMTD enhances the transfer of nucleic acids over the nuclear membrane. Therefore, the efficiency of the nuclear delivery of siRNA may depend primarily on the rapid diffusion of small nucleic acids throughout the cell\textsuperscript{20}.

Despite the strong fluorescence of TAMRA-labeled siRNA immediately after UMTD, no fluorescent signal could be detected 24 hours after UMTD. This is in contrast to a study in gastrointestinal cancer cells showing TAMRA-labeled siRNA in vesicular structures at 24 hours after transfection\textsuperscript{21}. However, these vesicular structures most likely represent stable aggregates of labeled siRNA and may not reflect active siRNA in the cytosol.

Furthermore our study showed a 70% downregulation of the targeted protein by a broad range of siRNA concentrations (2.5 nM and higher) without a clear siRNA dose-dependent
effect. This is in contrast to a previous study demonstrating that the expression of a luciferase plasmid could be inhibited by UMTD of siRNA in a dose dependent manner using siRNA concentrations ranging from 5 to 50 nM. However, this study was performed using COS-7 cells transfected with a luciferase plasmid, resulting in high luciferase mRNA levels. The absence of a siRNA dose-dependent effect on the knockdown of the moderately expressed GAPDH gene in endothelial cell, suggest that the maximal effect of GAPDH-siRNA was already achieved at the lowest concentration investigated by us (2.5 nM).

All investigated siRNA concentrations in UMTD of siRNA had no effect on cell-viability, indicating the absence of cytotoxicity. This is in-line with a previous study investigating UMTD of siRNA in COS-7 cells. This is in contrast to UMTD of plasmid DNA, as demonstrated in a previous study by our group. There, deviation from the optimal DNA concentration (20 µg/ml) by a factor of two, resulted in either decreased transfection (10 µg/ml) or severe cell death (40 µg/ml). Therefore, these results indicate that UMTD of siRNA is more efficient over a larger concentration range than UMTD of plasmid DNA.

In contrast to UMTD of siRNA, UMTD of a GAPDH plasmid did not increase GAPDH levels over endogenous GAPDH protein levels. Although post-transcriptional control may be an underlying mechanism for maintaining GAPDH protein levels at fixed levels, our data obtained with the GFP expression vector indicate that poor transfection efficiencies of plasmid UMTD is the main cause for the inability to upregulate GAPDH protein levels. This study however demonstrates that poor delivery of the GAPDH plasmid is not the underlying mechanism for the inability to enhance GAPDH protein levels. Cotransfection of a Cy3-labeled plasmid with a GFP reporter vector demonstrated that a substantial number of cells took up the Cy3-labeled plasmid (43.0 ± 4.2%). However, of these positive cells, only 2% expressed the GFP reporter protein. Similar delivery efficiency of plasmid was observed in rat mammary carcinoma cells, showing UMTD of YOYO-1 labeled plasmid to transfect approximately 27% of all cells immediately after UMTD. Previously, we demonstrated that UMTD of large molecules (500 kDa dextran) is completely dependent on endocytosis and the delivered molecules were mainly localized in endosomes positive for clathrin and to a lesser extent in endosomes positive for caveolin. In the present study, we demonstrate that labeled plasmid was delivered equally to both endosomes positive for clathrin and to endosomes positive for caveolin-1, indicating that plasmid DNA is taken up by both routes of endocytosis. The subsequent escape from these endosomes and transport to the nucleus is therefore the most likely bottleneck for efficient transcription of plasmid DNA after UMTD. In accord, attachment of nuclear localization peptides to the plasmid does not enhance the efficiency of UMTD of plasmid DNA.

Despite the higher efficiency of siRNA to change the protein levels of GAPDH, this does not mean that UMTD mediated delivery of plasmids has no therapeutic potential. Several in-vivo studies employing UMTD of plasmids have been successfully completed. However, these studies used plasmids encoding potent paracrine factors, avoiding the need for a highly efficient vector able to transfect the majority of all target cells.
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In summary, this study demonstrates that UMTD of GAPDH siRNA is more effective than UMDT of GAPDH plasmid in modifying protein levels of the moderately expressed gene GAPDH in cultured endothelial cells. UMTD of siRNA was effective over a broad range of siRNA concentrations without cytotoxic effects, suggesting that UMTD of siRNA may become a valuable therapeutic strategy in the treatment of vascular disease.
References
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