ICA 2013 Montreal
Montreal, Canada
2 - 7 June 2013

Biomedical Acoustics
Session 3aBAa: Delivery of Nucleic Acids (DNA, siRNA, antisense oligos)

3aBAa5. Small interfering ribonucleic acid delivery with phase-shift nanoemulsions
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Acoustic cavitation offers a unique approach to small interfering RNA (siRNA) delivery compared to current methods. Typically, preformed microbubbles are used as cavitation nuclei to permeabilize cells and facilitate siRNA entry into the cytoplasm. However, microbubbles are restricted to the vasculature space and suffer from stability issues that limit their applicability. Alternatively, phase-shift nanoemulsions (PSNE) possess the long circulation and extravasation properties of nanoparticles, while also serving as cavitation nuclei in tissue upon acoustic droplet vaporization. Here we report the use of PSNE for delivery of siRNA engineered to knockdown green fluorescent protein (GFP) expression. A cell suspension (5x10^6 cells/mL) of GFP expressing breast adenocarcinoma cells was exposed to 5 MHz pulsed ultrasound (4 MPa peak negative pressure, 3 cycles, 250 Hz, 100 second exposure duration) in the presence of PSNE (~10^9/mL) and free siRNA (1.8 µM). Flow cytometry was used to quantify GFP expression and cell viability. There was 20% (p<0.05, n=6) reduction in GFP fluorescence for cells treated with GFP siRNA and 80% (+/- 6.4%) cell survival. This work highlights the potential for PSNE to serve as interstitial cavitation nuclei for siRNA delivery in tissue. [Supported in part by NIH grants R25CA153955 and R03EB015089.]

Published by the Acoustical Society of America through the American Institute of Physics
INTRODUCTION

Phase-shift nanoemulsions (PSNEs) provide a unique platform for many ultrasound-mediated drug delivery applications (Matsunaga 2012, O’Neill 2011). PSNEs are composed of a liquid perfluorocarbon core surrounded by a biocompatible shell, which is usually composed of lipids, polymers, or proteins. Drugs and other bioactive molecules can be incorporated into the shell along with targeting moieties. Due to their small size and biocompatibility, they are long circulating and able to extravasate from the vascular space and accumulate in solid tumors. Upon the application of ultrasound, PSNEs can be nucleated to a gaseous core in a process called acoustic droplet vaporization (Kripfgans 2004). The newly formed microbubbles can then be driven acoustically to facilitate bioeffects such as sonoporation (Wu 2008). In effect, PSNEs serve as cavitation nuclei that expand the potential of ultrasound-mediated drug delivery to outside the vasculature space.

Here we present the feasibility of PSNEs to deliver small interfering ribonucleic acids (siRNAs) engineered to knockdown enhanced green fluorescent protein (eGFP) expression. Similar studies have shown that microbubbles can be used to deliver siRNA intracellularly and may provide a unique approach to siRNA delivery (Kinoshita 2005). Current delivery methods rely on cationic nanocarriers to facilitate uptake through endocytosis, which can degrade the siRNA if it is not released from the endosome. Mechanical disruption of the cell membrane using acoustic cavitation provides a direct route of entry and bypasses the complexity of the endosomal pathways. PSNEs provide a distinctive delivery system that could have advantages over current siRNA delivery systems.

MATERIALS & METHODS

Nanoemulsion Preparation & Characterization

Lipid coated perfluorocarbon nanoemulsions were prepared using a three step hydration, emulsification, extrusion procedure. Lipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE PEG2000) (Avanti Polar Lipids, Alabaster, AL) were weighed out into a glass vial at a molar ratio of 90:10 (DPPC:DPPE PEG2000) and then dissolved in chloroform. The chloroform was evaporated and the remaining lipid film was hydrated with phosphate-buffered saline (PBS). The lipid vesicles were then sonicated using a high power sonication tip (Model VC505, Sonic & Materials, Newton, CT) for 1 minute to produce a clear solution of small unilamellar vesicles at a lipid concentration of 1 mg/mL. Submicron perfluorocarbon emulsions were made by adding 100 µL of dodecafluoropentane (DDFP) (FluoroMed, L.P., Round Rock, Texas) to 3 mL of lipid solution and sonicating with the high power sonication tip in an ice water bath. This opaque suspension was then added to 7 mL of PBS and extruded 10 times through 200 nm polycarbonate filters using a LIPEX™ Extruder (Northern Lipids Inc., Burnaby, British Columbia). Excess lipid vesicles were removed by three centrifugal washes for 5 minutes at 10,000 g. Each time the supernatant was removed and the nanoemulsion pellet was resuspended in PBS. Tunable resistive pulse sensing (TRPS) was used to determine the size and concentration of PSNEs. TRPS was carried out using the qNano system (Izon Science, Christchurch, New Zealand), which allows particle-by-particle sizing and counting.

Ultrasound Set-up

A 5-MHz spherically focused transducer (Sonic Concepts, Bothell, Washington) was used to sonicate cell suspensions, which were held in a 1.5 mL microcentrifuge tube submerged in a tank of degassed deionized water at 37° C. The focal zone of the 5-MHz transducer (FWHM width of 0.32 mm and depth of 3.0 mm) was positioned inside the cell suspension using a three-axis translation system (Thorlabs Inc., Newton, NJ). A waveform generator (Agilent 33250A, Santa Clara, CA) connected to a 55 dB RF amplifier (ENI A150, Rochester, NY) was used to drive the transducer. A 15-MHz spherically focused transducer (Panametrics, Waltham, MA) aligned confocally with and 90° to the 5-MHz transducer was used to detect PSNE vaporization. The received signals were sent through a 10-MHz high-pass filter (Allen Avionics, Mineola, NY), amplified with a 20 dB pre-amplifier (Femto DHPVA, Berlin, Germany), and displayed on a digital oscilloscope (LeCroy WaveRunner, Chestnut Ridge, New York). Vaporization was detected as a significant increase in the received signal amplitude (i.e. > factor of 10) as compared to emissions using pressures below the vaporization threshold (Zhang and Porter 2010).
Small interfering RNA delivery

Human adenocarcinoma cells (MDA-MB-231) stably expressing eGFP were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 5% L-glutamine, and 1% penicillin streptomycin. Cells were maintained in a humidified atmosphere inside an incubator at 37°C with 5% CO₂. Cells were grown to confluence on 75 cm² tissue culture flasks before each experiment and harvested using trypsin-EDTA. A hemacytometer (Hausser Scientific, Horsham, PA) was used to count cells and adjust the concentration before experiments. For gene silencing experiments, anti-eGFP siRNA and non-targeted siRNA (Dharmacon Inc., Lafayette, CO) were used at a concentration of 1.8 µM. Cells (5x10⁶/mL) along with PSNEs (~10⁹/mL) were added to the suspension for a total volume of 50 µL in PBS. The ultrasound treatment consisted of 3-cycle pulses (4 MPa peak negative pressure) at a pulse repetition frequency of 250 Hz for a total exposure duration of 100 seconds. After the treatment, cells were washed using centrifugation and grown on 25 cm² tissue culture flasks. For viability experiments, the same procedure was followed as above but cells were immediately stained with propidium iodide after the exposure to detect non-viable cells. A total of 6 treatments were performed for siRNA knockdown experiments and 5 for viability experiments.

Flow cytometry was performed using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) system. Cells were harvested from the 25 cm² tissue culture flasks and suspended in PBS for analysis with flow cytometry. The cell population was gated and a total of 20,000 events were recorded. To determine percent eGFP knockdown, the mean fluorescence of the treated population was compared to control cells. For the viability experiments, the percent of non-viable cells were determined by gating the out non-viable cells and comparing it to control cells.

RESULTS & CONCLUSION

As shown in Figure 1, the PSNEs have a narrow size distribution with a mean diameter of 178.3 nm and a bulk concentration of 5.3x10¹¹ PSNEs/mL. The size of PSNEs can be controlled by selecting the appropriate filter during extrusion. This is important for applications were PSNEs must be able to extravasate through blood vessel walls and accumulate within neighboring tissue. For example, when targeting solid tumors via the enhanced permeability and retention effect (EPR) the upper limit of size may be from 200-800 nm depending on the case (Maeda 2000). So, it is important to have control over the size distribution during PSNE preparation.

**FIGURE 1.** Size distribution of phase-shift nanoemulsions (PSNEs). Tunable resistance pulse sensing was used to determine the size and concentration of PSNEs. The mean size of the PSNEs was 178.3 nm with a bulk concentration of 5.3x10¹¹ PSNEs/mL.
In this experiment we sought to determine whether PSNEs could be used to deliver siRNA to cells. Previous studies have shown that vaporized PSNE driven acoustically can facilitate the transport of small drugs such as chemotherapeutic agents across cell membranes. It has yet to be shown that larger biomolecules, such as oligonucleotides or proteins, may be delivered to cells using vaporized PSNE, or that the biomolecules remain biologically active. Figure 2 shows results that PSNEs can be used to deliver siRNA and that once delivered it remains active. There was a 20% reduction in eGFP fluorescent intensity for cells treated with anti-eGFP siRNA (p<0.05) and 80% (+/- 6.4%) cell survival. This shift was not observed in any of the control treatments so it can be assumed it was due to GFP knockdown and not because of toxicity or off-target side effects.

These results open up the possibility of using PSNEs as a siRNA delivery system. Future studies will explore experimental conditions that maximize siRNA delivery while limiting cell death. For in vivo applications the siRNA must be complexed to PSNEs using electrostatic or covalent interactions to protect the siRNA during circulation and insure both are co-localized in the tumor. Studies will also be performed to see if this has any effect on the delivery success compared to free siRNA.

FIGURE 2. siRNA knockdown of enhanced green fluorescent protein (eGFP). Experiments were performed using stably transfected MDA-MB-231 cells with eGFP siRNA, non-targeting siRNA, or no siRNA. Cells were analyzed with flow cytometry 48 hours after ultrasound treatment. The mean fluorescence of the gated cell population was compared to control cells that received no treatment. Data is expressed as mean +/- standard deviation (n=6, *p<0.05 vs. non-targeting siRNA treated cells).

ACKNOWLEDGMENTS

This work was funded in part by National Institute of Health grant numbers R25CA153955 and R03EB015089.

REFERENCES


