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● *Original Contribution*

ACOUSTIC CAVITATION-MEDIATED DELIVERY OF SMALL INTERFERING RIBONUCLEIC ACIDS WITH PHASE-SHIFT NANO-EMULSIONS

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Abstract—Localized, targeted delivery of small interfering ribonucleic acid (siRNA) has been the foremost hurdle in the use of siRNA for the treatment of various diseases. Major advances have been achieved in the synthesis of siRNA, which have led to greater target messenger RNA (mRNA) silencing and stability under physiologic conditions. Although numerous delivery strategies have shown promise, there are still limited options for targeted delivery and release of siRNA administered systemically. In this *in vitro* study, phase-shift nano-emulsions (PSNE) were explored as cavitation nuclei to facilitate free siRNA delivery to cancer cells *via* sonoporation. A cell suspension containing varying amounts of PSNE and siRNA was exposed to 5-MHz pulsed ultrasound at fixed settings (6.2-MPa peak negative pressure, 5-cycle pulses, 250-Hz pulse repetition frequency (PRF) and total exposure duration of 100 s). Inertial cavitation emissions were detected throughout the exposure using a passive cavitation detector. Successful siRNA delivery was achieved (*i.e.*, >50% cell uptake) with high (>80%) viability. The percentage of cells with siRNA uptake was correlated with the amount of inertial cavitation activity generated from vaporized PSNE. The siRNA remained functional after delivery, significantly reducing expression of green fluorescent protein in a stably transfected cell line. These results indicate that vaporized PSNE can facilitate siRNA entry into the cytosol of a majority of sonicated cells and may provide a non-endosomal route for siRNA delivery. (E-mail: marktb@bu.edu) **© 2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Small interfering RNA, Acoustic cavitation, Sonoporation, Microbubbles, Phase-shift nano-emulsions, Ultrasound, Acoustic droplet vaporization, Nanomedicine.**

INTRODUCTION

Therapeutics based on RNA interference (RNAi) are in development for numerous diseases including cancer, viral infections and other genetic disorders (Davidson and McCray 2011). These double-stranded RNA molecules allow post-transcriptional modification of gene expression with remarkable potency and specificity (Vaishnaw et al. 2010). In particular, small interfering RNA (siRNA) has attracted much interest for its ability to knock down specific proteins central to disease progression (Lares et al. 2010). Once introduced into the cytosol of cells, siRNA molecules are incorporated into the RNAi pathway, where they lead to degradation of complementary messenger RNA (mRNA) molecules, which, in turn, leads to a reduction in targeted protein expression (Elbashir et al. 2001). In theory, this opens up the possibility of targeting any of the approximately 20,000 genes in the human genome with synthetic siRNA molecules. Proteins once considered undruggable using conventional small molecules could then be targeted, which could have a profound impact on the treatment of human diseases (Wu et al. 2014).

Effective siRNA delivery has remained an elusive challenge in the pursuit of its use as a systemically administered therapeutic. siRNA is large (~14 kDa), highly anionic, susceptible to enzymatic degradation and rapidly filtered from circulation by the kidneys (Tokatlian and Segura 2010). Progress has been made in improving the pharmacokinetic properties of siRNA therapeutics through chemical modifications of the siRNA molecule and packaging into nanoparticles (Behlke 2008; Whitehead et al. 2009; Zhang et al. 2007). Numerous lipid- and polymer-based nanoparticles are currently being developed for siRNA delivery, with a few in clinical trials (Burnett et al. 2011). These nanoparticles extend the half-life of siRNA in circulation and protect against degradation en route to diseased tissue. With respect to

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cancer therapies, nanoparticles are able to accumulate in tumors through the enhanced permeability and retention (EPR) effect (Fang et al. 2011). Nanoparticles are then internalized by cells through endosomal pathways, where endosomal escape must occur to release siRNA into the cytosol where it can access RNAi machinery. Gilleron et al. (2013) reported that endosomal escape of siRNA occurs at low efficiencies (1%–2%) with lipid-based nanoparticle delivery. Therefore, it may be beneficial to bypass the endosomal pathways and deliver siRNA directly into the cell's cytosol.

Sonoporation, or the use of acoustic cavitation for cell membrane disruption, has been found to facilitate non-endosomal delivery of large biomolecules, such as drugs and genetic material, into the cell cytosol (Lentacker et al. 2013). Acoustic cavitation can generate stresses on biological structures in close proximity as a result of microstreaming, bubble expansion/collapse, shock wave emission and microjetting (Fan et al. 2012; Kooiman et al. 2011; Ohl et al. 2006; Sundaram et al. 2003; van Wamel et al. 2006; Wu and Nyborg 2008; Zhou et al. 2012). Gas-filled ultrasound contrast agents (UCAs) used for diagnostic imaging are typically employed as cavitation nuclei to induce these stresses for enhancement of gene and drug delivery (Ferrara et al. 2007; Sirsi and Borden 2012). Kinoshita and Hynynen 2005 highlighted the possibility of using UCAs for intracellular delivery of siRNA, and other studies have since reported promising results (Negishi et al. 2008). However, UCAs are limited to the vascular system because of their size (1–10 μm) and are quickly cleared from circulation within minutes of injection by the mononuclear phagocyte system and dissolution (Garg et al. 2013; Unger et al. 2004). Therefore, researchers have begun to explore submicron cavitation nuclei for ultrasound-mediated gene delivery to solid tumors (Endo-Takahashi et al. 2012; Suzuki et al. 2011).

Phase-shift nano-emulsions (PSNE) present an attractive alternative to UCAs for gene and drug delivery to solid tumors. PSNE are superheated nanodroplets of liquid perfluorocarbon that are stabilized with a biocompatible lipid, polymer or protein shell. Ultrasound can be used to vaporize the perfluorocarbon core. The pressure threshold for vaporization depends on numerous factors, including the boiling point of the perfluorocarbon (Kawabata et al. 2005; Sheeran et al. 2012), ambient temperature (Fabiilli et al. 2009; Zhang and Porter 2010), ultrasound frequency (Kripfgans et al. 2000) and size of the droplet (Fabiilli et al. 2009; Kripfgans et al. 2004). In theory, PSNE vaporization could allow controlled initiation of acoustic cavitation in tissue where bubbles are difficult to form and control (Kopechek et al. 2013). PSNE combine the improved biocompatibility, long circulation and extravasation properties of lipid-based nanoparticles with the beneficial bioeffects of acoustic cavitation, such as sonoporation. This unique class of emulsion has been used for drug delivery applications (Adan et al. 2012; Rapoport et al. 2009; Wang et al. 2012), contrast-enhanced ultrasound imaging (Sheeran et al. 2013b; Williams et al. 2013) and bubble-enhanced heating for high-intensity focused ultrasound ablation (Kopechek et al. 2013; Phillips et al. 2013).

The objective of this study was to investigate the potential of PSNE as cavitation nuclei for delivery of free siRNA to *in vitro* cell suspensions. We hypothesized that vaporized PSNE can be used to transiently disrupt cell membranes in a manner similar to traditional UCAs and facilitate entry of siRNA into the cell cytoplasm. Therefore, we first examined the uptake of fluorescently tagged siRNA as a function of PSNE concentration. Cell suspensions containing siRNA and PSNE were exposed to short bursts of ultrasound with simultaneous detection of acoustic cavitation emissions. Cells were analyzed using flow cytometry, and the correlation between siRNA uptake and acoustic cavitation activity was assessed. The second part of the study explored the delivery of siRNA designed to knock down green florescent protein (GFP) in stably transfected cells. The PSNE concentration that yielded the highest uptake of siRNA was used along with varying doses of GFP-targeted siRNA. The subsequent sections outline the methodology used for PSNE fabrication, ultrasound experiments and analysis. Finally, results from the siRNA delivery experiments are discussed along with the implications for localized siRNA delivery using PSNE.

METHODS

Nano-emulsion preparation

Lipid-coated perfluorocarbon nano-emulsions were prepared using a three-step hydration, emulsification and extrusion procedure (Kopechek et al. 2012). The 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, USA) were weighed and dissolved in chloroform (Sigma-Aldrich, St. Louis, MO, USA) at a molar ratio of 9:1 (DPPC:DPPE-PEG2000). A thin lipid film was formed in a glass vial by evaporating the chloroform with a steady stream of argon. The vial was placed under vacuum overnight to remove any residual chloroform. Subsequently, the lipid film was hydrated for 1–2 h at 50°C with phosphate-buffered saline (PBS) (Boston Bioproducts, Ashland, MA, USA) with periodic vortexing and sonication in an ultrasonic bath (Cole-Parmer, Vernon Hills, IL, USA). The lipid vesicles were then sonicated using a high-power