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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/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/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.

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

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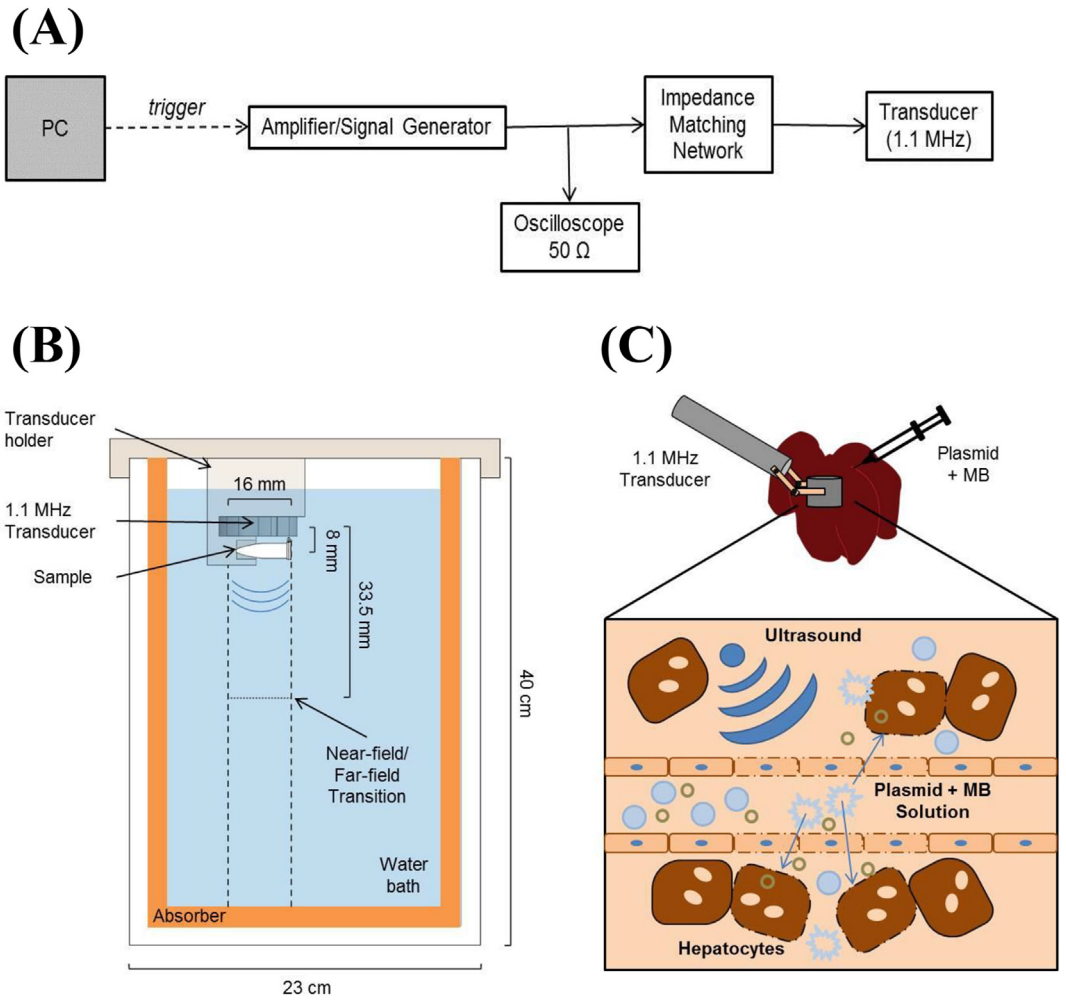

Fig. 1. Overview of *in vitro* and *in vivo* experimental apparatus and setup. (A) Block diagram of signal generation and real-time monitoring. (B) Schematic representation of *in vitro* experiments done in an anechoic water bath. Cell/pDNA/MB suspension is treated 8 mm from the face of the transducer 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.

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