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● *Original Contribution*

ENHANCEMENT OF NON-INVASIVE TRANS-MEMBRANE DRUG DELIVERY USING ULTRASOUND AND MICROBUBBLES DURING PHYSIOLOGICALLY RELEVANT FLOW

FARAH E. SHAMOUT, ANTONIOS N. POULIOPOULOS, PATRIZIA LEE, SIMONE BONACCORSI, LEILA TOWHIDI, ROB KRAMS, and JAMES J. CHOI

Department of Bioengineering, Imperial College London, London, UK

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Abstract—Sonoporation has been associated with drug delivery across cell membranes and into target cells, yet several limitations have prohibited further advancement of this technology. Higher delivery rates were associated with increased cellular death, thus implying a safety–efficacy trade-off. Meanwhile, there has been no reported study of safe *in vitro* sonoporation in a physiologically relevant flow environment. The objective of our study was not only to evaluate sonoporation under physiologically relevant flow conditions, such as fluid velocity, shear stress and temperature, but also to design ultrasound parameters that exploit the presence of flow to maximize sonoporation efficacy while minimizing or avoiding cellular damage. Human umbilical vein endothelial cells (EA.hy926) were seeded in flow chambers as a monolayer to mimic the endothelium. A peristaltic pump maintained a constant fluid velocity of 12.5 cm/s. A focused 0.5 MHz transducer was used to sonicate the cells, while an inserted focused 7.5 MHz passive cavitation detector monitored microbubble-seeded cavitation emissions. Under these conditions, propidium iodide, which is normally impermeable to the cell membrane, was traced to determine whether it could enter cells after sonication. Meanwhile, calcein-AM was used as a cell viability marker. A range of focused ultrasound parameters was explored, with several unique bioeffects observed: cell detachment, preservation of cell viability with no membrane penetration, cell death and preservation of cell viability with sonoporation. The parameters were then modified further to produce safe sonoporation with minimal cell death. To increase the number of favourable cavitation events, we lowered the ultrasound exposure pressure to 40 $kPa_{pk\text{-}neg}$ and increased the number of cavitation nuclei by 50 times to produce a trans-membrane delivery rate of 62.6% ± 4.3% with a cell viability of 95% ± 4.2%. Furthermore, acoustic cavitation analysis showed that the low pressure sonication produced stable and non-inertial cavitation throughout the pulse sequence. To our knowledge, this is the first study to demonstrate a high drug delivery rate coupled with high cell viability in a physiologically relevant *in vitro* flow system. (E-mail: j.choi@imperial.ac.uk)

Key Words: **Microbubbles, Ultrasound, Trans-membrane drug delivery, Sonoporation, Endothelial cells, EA.hy926.**

INTRODUCTION

Focused ultrasound (FUS) techniques are being developed to drive acoustic cavitation—acoustically driven microbubble activity—and produce therapeutic biological effects at specified locations (Dalecki 2004; Stride and Coussios 2010). Amongst a multitude of methods, trans-membrane molecular delivery has been of great interest due to its potential to address one of the most pressing challenges with pharmaceuticals: drug transport into cells. The application of FUS and microbubbles facilitates trans-membrane transport by generating acoustic cavitation at or near the cell membrane, but the exact physical and biological mechanisms involved remain unknown (Schlicher et al. 2006; Zeghimi et al. 2012). Proposed routes of molecular delivery include the formation of short-lived pores in the membrane, also known as sonoporation (Yu and Xu 2014), and endocytosis (Geers et al. 2011; van Wamel et al. 2006). In addition, increased trans-membrane delivery efficiency is associated with increased damage and cell death (Qiu et al. 2010, 2012), and this efficacy–safety trade-off has limited the technology's development; hence, it remains unutilized in the clinic as a drug delivery tool.

Address correspondence to: James J. Choi, Noninvasive Surgery and Biopsy Laboratory, Department of Bioengineering, Imperial College London, London, SW7 2AZ, UK. E-mail: j.choi@imperial.ac.uk

Ultrasonic trans-membrane delivery requires microbubbles undergoing acoustic cavitation to act near or on the surface of the cell membrane (Ohl et al. 2006; Prentice et al. 2005; Zhou et al. 2012). Ultrasound is typically generated from a focused transducer outside the body and can propagate several centimeters into the body to converge to a small focal volume. Microbubbles, which are typically 1–10 μm in diameter, are coated with a protein, polymer or lipid shell and filled with a stabilized gas core, are then systemically administered so that they circulate throughout the body's diverse vasculature. Microbubbles are clinically approved and being used as contrast agents in ultrasound imaging (Cosgrove 2006). However, their use is extended to therapeutic applications such as sonoporation and targeted drug delivery (Ferrara et al. 2007). Microbubbles can flow at speeds varying from 20 cm/s in the middle of the vessel to a few mm/s near the vessel wall, which correlates well with the velocity of red blood cells (Levine et al. 1984). When exposed to ultrasound, microbubbles respond in a complex range of behaviors known as acoustic cavitation (Apfel 1997). Ultrasound exposure parameters, such as center frequency, peak-rarefactional pressure, pulse length, pulse repetition frequency *etc.* can be adjusted to control the cavitation magnitude, duration and distribution (Choi and Coussios 2012; Dalecki 2004).

Depending on the ultrasound parameters employed, the physical composition of the microbubbles under ultrasound exposure and the environment surrounding the microbubble, different types of cavitation can occur. Microbubbles undergoing acoustic cavitation are expanding and contracting in response to the compressional and rarefactional phases of the ultrasound wave (Apfel 1997; Helfield and Goertz 2013). At low acoustic pressures, microbubbles recurrently oscillate around an equilibrium radius in a behavior known as stable cavitation (Apfel 1997; Dalecki 2004; Morgan et al. 2000). Transient cavitation describes short-lived phenomena, which can be caused by fragmentation of the shell, dissolution of the core gas or other mechanisms (Apfel 1997; Chomas et al. 2001; Newman and Bettinger 2007). One cause of a transient response is inertial cavitation, which arises when the rarefactional phase of the ultrasound pulse results in a radial expansion, followed by a violent collapse dominated by the inertia of the surrounding liquid medium (Church and Carstensen 2001; Flynn 1982).

The different types of acoustic cavitation have been demonstrated to produce diverse cellular effects (Fan et al. 2014; Mehier-Humbert et al. 2005; Qiu et al. 2010). First, it has been shown that the interaction between the microbubble oscillation and the cell membrane can produce short-lived pores that allow drug uptake. These pores are up to 1 μm in size and have been shown to reseal within seconds (Hu et al. 2013; Newman and Bettinger 2007; Qiu et al. 2010; Zhou et al. 2009), minutes (Schlicher et al. 2006) or even hours (Zhao et al. 2008). Inertial cavitation produces higher rates of intra-cellular delivery (Fan et al. 2013; Karshafian et al. 2009; Park et al. 2011; Qiu et al. 2010) and is also more likely to disrupt or damage the cell than stable cavitation (Newman and Bettinger 2007). Furthermore, at diagnostic frequencies and acoustic pressures, endothelial damage can occur when contrast agents are under ultrasound exposure due to the generation of inertial cavitation. Such cavitation resulted in severe cell erosion from *in vitro* static monolayers (Brayman et al. 1999) and in cell lysis (Ward et al. 1999). Thus, non-inertial cavitation is deemed safer in comparison to inertial cavitation, due to its reduced effect on cell viability (Qiu et al. 2012).

Different ultrasound exposure parameters for sonoporation have resulted in different cellular effects (Fan et al. 2014). These parameters include the center frequency of the ultrasound transducer, the acoustic pressure, the pulse length, the pulse repetition frequency *etc.* As the number of ultrasound pulses increased, cell viability decreased (Park et al. 2011). Severe cell damage was also associated with the acoustic pressure of the applied pulses. As the acoustic pressure increases, damage increases in a generally linear fashion (Brayman et al. 1999; Qiu et al. 2010). Thus, an ultrasound pulse sequence must be designed to fulfill the requirement of both minimum cell damage and maximum desired bioeffect.

Previous studies have utilized a diverse range of experimental setups, ultrasound parameters, microbubble formulations and cell types, which resulted in large variations in delivery rates (Yu and Xu 2014). Intra-cellular drug delivery in human umbilical vein endothelial cells (HUVECs) was shown to depend on the experienced shear conditions, with the observed rates being below 10% in the physiologically relevant scenario (Park et al. 2011). Previous research that focused on sonoporation applied to modulate gene expression in HUVECs led to a transfection rate of 25% (Guo et al. 2004). More recently, GFP reporter plasmids were delivered to HEK294 cells with an efficiency of up to 80% (Tlaxca et al. 2010). A similar delivery rate was achieved at high pressures in the DNA transfection of MCF-7 cells (Qiu et al. 2010). However, high delivery rates were accomplished at the expense of cell viability, with extended cell death being observed at high pressure sonication ($P_{pk\text{-}neg} > 500$ kPa); in contrast, low acoustic pressures ($P_{pk\text{-}neg} < 100$ kPa$_{pk\text{-}neg}$) were proved to be safer, but resulted in transfection efficiency below 15% (Qiu et al. 2012). A common feature of most of the aforementioned