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● *Original Contribution*

MECHANISTIC INSIGHT INTO SONOPORATION WITH ULTRASOUND-STIMULATED POLYMER MICROBUBBLES

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Abstract—Sonoporation is emerging as a feasible, non-viral gene delivery platform for the treatment of cardiovascular disease and cancer. Despite promising results, this approach remains less efficient than viral methods. The objective of this work is to help substantiate the merit of polymeric microbubble sonoporation as a non-viral, localized cell permeation and payload delivery strategy by taking a ground-up approach to elucidating the fundamental mechanisms at play. In this study, we apply simultaneous microscopy of polymeric microbubble sonoporation over its intrinsic biophysical timescales–with sub-microsecond resolution to examine microbubble cavitation and millisecond resolution over several minutes to examine local macromolecule uptake through enhanced endothelial cell membrane permeability–bridging over six orders of magnitude in time. We quantified microbubble behavior and resulting sonoporation thresholds at transmit frequencies of 0.5, 1 and 2 MHz, and determined that sonic cracking is a necessary but insufficient condition to induce sonoporation. Further, sonoporation propensity increases with the extent of sonic cracking, namely, from partial to complete gas escape from the polymeric encapsulation. For the subset that exhibited complete gas escape from sonic cracking, a proportional relationship between the maximum projected gas area and resulting macromolecule uptake was observed. These results have revealed one aspect of polymeric bubble activity on the microsecond time scale that is associated with eliciting sonoporation in adjacent endothelial cells, and contributes toward an understanding of the physical rationale for sonoporation with polymer-encapsulated microbubble contrast agents. (E-mail: villanuevafs@upmc.edu; website: http://www.imagingtherapeutics.pitt.edu) © 2017 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Sonoporation, Microbubbles, High-speed imaging, Endothelial cells, Sonic cracking.**

INTRODUCTION

Gene therapy is becoming an increasingly attractive therapeutic approach to modulating molecular determinants of disease pathogenesis, such as in cancer or cardiovascular disease. A range of different strategies have been applied to cancer gene therapy, including delivery of tumor suppressor genes (Roth 2006; Ventura et al. 2007), immunotherapy (Rosenberg et al. 2004) and gene-directed enzyme pro-drug therapy (Dachs et al. 2005). Cardiovascular molecular targets employed for therapeutic intervention include vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) to promote therapeutic angiogenesis in ischemia applications (Cao et al. 2003; Isner et al. 1996; Losordo et al. 1998), and genes responsible for calcium homeostasis (Jaski et al. 2009; Miyamoto et al. 2000). Despite these results, effective strategies to deliver nucleic acids intra-vascularly to the site of disease and traverse the endothelial cell membrane are limited. Viral vectors, although efficient, elicit specific inflammatory and anti-viral immune responses (Otake et al. 1998; Verma and Somia 1997). Promising non-viral vectors, including liposomal (Audouy et al. 2002) and polymer-based nanoparticle (Leong et al. 1998; Salem et al. 2003)

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approaches, face the endothelial barrier and must escape early endosomes to deliver their payload to the cell. To address this, our group and others have explored using ultrasound-stimulated microbubbles to potentiate the delivery of therapeutics, either co-injected (Aryal et al. 2013; Jordao et al. 2010; Kinoshita and Hynynen 2005; Treat et al. 2007) or anchored to the bubble itself (Carson et al. 2012; Christiansen et al. 2003; Kopechek et al. 2015; Leong-Poi et al. 2007; Shohet et al. 2000; Taniyama et al. 2002). By applying focused ultrasound to the target tissue during microbubble microvascular transit, localized acoustic cavitation can cause transient increases in plasma membrane (Bao et al. 1997; van Wamel et al. 2006) and vasculature permeability (Price et al. 1998; Skyba et al. 1998), aiding in site-specific therapeutic delivery. The efficiency of this platform, currently less than that of viral methods (Li and Huang 2006; Mehier-Humbert and Guy 2005), can be improved by a more complete understanding of microbubble physics and how it affects neighboring cell membrane permeability over longer time scales. To this end, our previous work with phospholipid-encapsulated agentcoupled ultrafast microscopy (16 million frames per second [Mfps]) and epifluorescence microscopy (15 fps) and simultaneously gathered information on single microbubble–cell systems over the two biophysically intrinsic time scales associated with sonoporation—ultrasound cavitation on the sub-microsecond time scale and cellular response (including macromolecule uptake/ diffusion *via* enhanced membrane permeability and plasma membrane reorganization) on the order of seconds to minutes (Helfield et al. 2016b). This work complements other sonoporation studies using lipid bubbles that examine biological consequences, including investigations into calcium signaling (Fan et al. 2010, 2012; Park et al. 2011), alterations in cell cycle (Chen et al. 2013a; Miller and Dou 2009; Zhong et al. 2011) and plasma membrane features (De Cock et al. 2015; Kudo et al. 2009; Leow et al. 2015; Schlicher et al. 2010; van Wamel et al. 2004).

As payload delivery vehicles, polymeric microbubbles can provide longer circulation times and increased drug/gene loading capacities within their encapsulation compared with lipid agents (Ferrara et al. 2007; Hernot and Klibanov 2008). Polymer-coated microbubbles, however, exhibit fundamentally different oscillation physics (Bloch et al. 2004) because of their relatively incompressible and rigid encapsulation. As opposed to lipid monolayer-based encapsulated agents, thick-shelled polymer agents can more easily withstand in-plane compression and can exhibit a globally non-spherical shape (Marmottant et al. 2011). Further, these microbubbles can undergo "sonic cracking," whereby the encapsulation material cracks and results in anisotropic free gas release (Bouakaz et al. 2005; Postema et al. 2005). This phenomenon may have particular application in drug release strategies that incorporate a therapeutic within the bubble itself (*e.g.,* in oil phase [Kooiman et al. 2009; Unger et al. 2002]) as opposed to being chemically anchored along the shell surface, a strategy that may aid in payload shielding (Lentacker et al. 2006; Tinkov et al. 2009).

Previous work has employed ultrafast microscopy to relate microbubble dynamics to sonoporation, conducted as separate correlative experiments (Kudo et al. 2009; van Rooij et al. 2016). There have also been multiple insightful studies employing coupled microscopy approaches to observe microbubbles on kilohertz timescales (*e.g.,* Fan et al. 2014). Our coupled approach with an ultrafast camera provides us the unique opportunity to directly observe and relate microbubble cavitation physics (sub-microsecond time scales) to neighboring endothelial cell permeability (millisecond time scales).

The specific objective of this work was to gain insight into mechanisms responsible for sonoporation with polymeric microbubbles. In this manner, rather than simply describing microbubble and ultrasound parameters that are empirically effective in causing sonoporation, our work attempted to discover fundamental governing physical principles of sonoporation with polymeric microbubbles.

METHODS

Cell culture

Primary human umbilical vein endothelial cells (HUVECs; No. C2519A, Lonza, Verviers, Belgium) were cultured in medium (EGM-2, No. C3162, Lonza) and incubated at 37°C with 5% CO_2. HUVECs were plated in an Opticell chamber (Thermo Scientific, Rochester, NY, USA), consisting of two 50-cm^2 gas-permeable, polystyrene membranes approximately 75 μm in thickness and separated by 2 mm. Experiments were conducted on HUVEC monolayers characterized by an approximate confluency of 80% to 90%, and all experiments were performed on cells with passage numbers $p \leq 10$ (number of times these cells were subcultured and a surrogate measure for cell age).

Sonoporation marker

Propidium iodide (PI; P1304 MP, Molecular Probes, Eugene, OR, USA) was employed as a surrogate drug and sonoporation marker, and was diluted in the culture medium at a concentration of 120 μM. PI is a non-fluorescent, cell-impermeant intercalating agent, with a molecular weight of 668.4 Da. On entry within a cell

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