# LANGMUR Cite This: Langmuir 2019, 35, 10128–10138

Article

# Engineered 3D Microvascular Networks for the Study of Ultrasound-Microbubble-Mediated Drug Delivery

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ABSTRACT: Localized and targeted drug delivery can be achieved by the combined action of ultrasound and microbubbles on the tumor microenvironment, likely through sonoporation and other therapeutic mechanisms that are not well understood. Here, we present a perfusable in vitro model with a realistic 3D geometry to study the interactions between microbubbles and the vascular endothelium in the presence of ultrasound. Specifically, a three-dimensional, endothelial-cellseeded in vitro microvascular model was perfused with cell culture medium and microbubbles while being sonicated by a single-element 1 MHz focused transducer. This setup mimics the in vivo scenario in which ultrasound induces a therapeutic



effect in the tumor vasculature in the presence of flow. Fluorescence and bright-field microscopy were employed to assess the microbubble-vessel interactions and the extent of drug delivery and cell death both in real time during treatment as well as after treatment. Propidium iodide was used as the model drug while calcein AM was used to evaluate cell viability. There were two acoustic parameter sets chosen for this work: (1) acoustic pressure: 1.4 MPa, pulse length: 500 cycles, duty cycle: 5% and (2) acoustic pressure: 0.4 MPa, pulse length: 1000 cycles, duty cycle: 20%. Enhanced drug delivery and cell death were observed in both cases while the higher pressure setting had a more pronounced effect. By introducing physiological flow to the in vitro microvascular model and examining the PECAM-1 expression of the endothelial cells within it, we demonstrated that our model is a good mimic of the in vivo vasculature and is therefore a viable platform to provide mechanistic insights into ultrasoundmediated drug delivery.

# INTRODUCTION

Microbubbles are micrometer-sized (typically  $1-10 \ \mu m$ ) gaseous spheres stabilized by lipid, protein, or synthetic shells.<sup>1–8</sup> While they are traditionally used as ultrasound contrast agents, researchers have discovered the potential of using microbubbles to achieve enhanced drug delivery, particularly in anticancer and gene delivery applications. The primary therapeutic mechanism identified at the cellular level is sonoporation, which is defined by the nonlethal, temporary, and reversible permeabilization of the plasma membrane after treatment with ultrasound and microbubbles.<sup>9–13</sup> When exposed to ultrasound, microbubbles exhibit volumetric oscillations around the equilibrium radius and may undergo inertial collapse leading to fragmentation. The bubble oscillations are typically nonlinear, and their frequency spectrum includes harmonics, subharmonics, and ultraharmonics of the excitation frequency.<sup>14,15</sup> These microbubble behaviors can have profound biophysical and biological influences on neighboring cells; along with the forces they exert on the neighboring cells, they produce not only sonoporation but biological responses such as intracellular  $Ca^{2+}$  transients,  $^{16-21}$  endocytosis,  $^{22-24}$  and cytoskeletal rearrangement.<sup>25,26</sup> All of these cellular modulations can be exploited to increase the uptake of therapeutic agents in vivo,

especially ones that have low permeability to cells. On the tissue level, the organization of vascular endothelium may also be disrupted by microbubbles, thus creating intercellular pathways for macromolecules to access the interstitium and cancer cells. By the enhancement of drug uptake in cells and tissues, the use of microbubbles and ultrasound can allow for reduction of dosage, yielding lower side effects caused by toxic chemotherapeutic agents. Researchers across a variety of disciplines have demonstrated promising results in the past couple of decades in the use of ultrasound for minimizing systemic toxicity.<sup>27–35</sup>

While the efficacy of microbubble-facilitated drug delivery and gene transfer has been widely demonstrated, researchers have yet to obtain a thorough understanding of the mechanisms underlying these processes. One of the main challenges is the absence of a suitable biological model. Up until now, researchers have mostly employed in vivo models (mice, rat, pig, or primates) $^{36-47}$  or oversimplified in vitro

Received: September 27, 2018 Revised: November 27, 2018 Published: December 12, 2018

Special Issue: Microbubbles: Exploring Gas-Liquid Interfaces for **Biomedical Applications** 



**Figure 1.** Preparation and assembly of MVNs. (A-i) The top half of an MVN: liquid collagen I was injected into the space enclosed by a patterned PDMS stamp and a top acrylic housing. (A-ii) The bottom half of an MVN: a thin collagen layer was applied on top of a cover glass which sits above the window of a bottom acrylic housing. (A-iii) After combing the two MVN halves, HUVECs were introduced to the collagen scaffold. (A-iv) HUVECs attached to the collagen walls overnight and formed a vascular endothelium after 2–3 days of culture. (B) MVNs were perfused with microbubbles and PI by a syringe pump at a flow rate of 10  $\mu$ L/min. The MVN was exposed to ultrasound once steady flow was established. The transducer was in direct contact with the MVN pointing at a 45° angle and coupled with gel. A brightfield microscope captured videos of microbubble flow and ultrasound exposure.

models for sonoporation studies.<sup>10,48-55</sup> In vivo models, while similar to the human system physiologically, do not allow realtime examination of microbubble-vessel interactions, and the high variability between individual animals further complicates the task. The cost and ethical concerns associated with using test animals also make quantitative or parametric studies difficult to carry out, not to mention the time and care needed to prepare the animals. Comparatively, in vitro models such as cell-monolayers, cell suspensions, or OrganoPlate-based assays are much easier to prepare and have much lower variability, but there are three disadvantages in the use of these in vitro models. First, they fail to recreate the complex physical and biological environment of the human vasculature, which can vary dramatically both between vessel types and organ types. Second, existing in vitro models tend to confine microbubbles in a static environment while allowing them to come in direct contact with cancer cells. Such is not the case in the human body where microbubbles travel with the blood and must traverse through the vascular endothelium and soft tissue to reach the therapeutic targets. Lastly, the interactions between microbubbles and the vascular endothelium are far more dynamic and complex than the controlled oscillations observed in vitro. Considering the disadvantages of existing models, there exists a real need for a perfusable in vitro model that recapitulates the physical and physiological characteristics of the human vasculature. The successful development of such a model would enable researchers to investigate the physio-

logical response of cancer cells and the human vasculature to ultrasound-based therapy.

We previously developed an in vitro microvascular network (MVN) model that captured the spatial characteristics (tubular architecture and vessel branching), physical environment (flow and shear conditions), and biological interactions (intercellular and matrix-cell communication) found in vivo.<sup>56-58</sup> This collagen-based three-dimensional (3D) microvascular network was made using microfabrication and injection molding techniques and cellularized with human umbilical cord endothelial cells (HUVECs). The diameter of the microvascular channels (150  $\mu$ m) is comparable to that of a human arteriole or a dilated tumor capillary.<sup>59,60</sup> We have demonstrated that HUVECs in MVNs exhibit in vivo-like behaviors and provided evidence that they are suitable for studying vascular biology, pathology, and pharmacology. 56-58,61-63 Our main hypothesis is that the development of an in vitro model that closely resembles physiology will serve as an important research platform to study sonoporation. Here we have further utilized and exploited the MVN by perfusing it with microbubbles at physiological flow rates and interrogating it with ultrasound to observe in real-time the interactions between microbubbles and the vascular endothelium. As a research platform, the engineered MVNs boast several advantages over conventional in vivo models, including ease of handling, higher throughput, better reproducibility, and no bioethical concerns. Compared to conventional in vitro models

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used for sonoporation studies, the MVN offers a more biologically relevant microenvironment by allowing for blood flow and having endothelial cells embedded in vessel-like collagen channels (as opposed to flat monolayers).

In the present work, where for the first time microbubbles are introduced to the MVNs, we first present the characteristics and mechanics of the MVNs perfused with microbubbles in order to study sonoporation in a clinically relevant in vitro model. We evaluate microbubble supply rates, concentrations, and the delivered acoustic field to the microbubbles next to the endothelial cells. Finally, we report on proof-of-concept sonoporation results (the differences in sonoporation and cell death between two ultrasound conditions) of endothelial cells in MVNs.

#### EXPERIMENTAL SECTION

**Endothelial Cell Culture.** HUVECs used for this work were acquired from the American Type Culture Collection (ATCC; Manassas, VA, U.S.A.). The HUVECs were maintained in culture flasks with endothelial cell growth media (EGM; Lonza, Basel, Switzerland) in a 37 °C incubator supplemented with 5% CO<sub>2</sub> and ambient O<sub>2</sub>. Trypsin-EDTA (0.05%) (Thermo Fisher Scientific, Waltham, MA, USA) was used to harvest HUVECs for subculture and MVN cellularization.

Fabrication and Cellularization of in Vitro Microvascular **Networks.** Detailed descriptions of the MVN fabrication process can be found in our previous publications.<sup>56,61</sup> Briefly, the collagen scaffold of a MVN was prepared in a sandwich-like manner. Figure 1A illustrates the cross sections of an MVN before (Parts i and ii) and after (Parts iii and iv) assembly. The top half of the MVN (Figure 1Ai) was prepared by injecting collagen I solution (7.5 mg/mL) into a space enclosed by a top acrylic housing and a polydimethylsiloxane (PDMS) stamp that has a protruded pattern of the desired microvascular network. The bottom half of the MVN (Figure 1A-ii) was prepared in the following manner: after placing a 22 mm × 22 mm coverslip atop the square window of a bottom acrylic housing, a thin layer of collagen I solution (7.5 mg/mL) was applied to the top of the coverslip and flattened by a thin PDMS slab. The two MVN halves were incubated at 37 °C for 30 min (or until collagen I has gelled sufficiently) and combined afterward. The assembled MVN was then incubated again at 37 °C to ensure the proper gelation and formation of the bare-collagen microvascular network. The MVN used in this study consists of 12 parallel channels (Figure 1B) each with a length of 7 mm and height and width of 150  $\mu$ m, which is approximately the diameter of a human arteriole or a dilated tumor capillary.<sup>59,60'</sup> The MVN branches at the inlet and converges at the outlet.

HUVECs between passages 5 and 7 were used for MVN cellularization. The cells were trypsinized from culture flasks and resuspended in EGM to a concentration of  $7 \times 10^6$  cells/mL. This cell suspension was then pipetted into the MVN through its inlet while the cells spread out across the bare-collagen microvascular network under gravity flow. Once the cells were distributed evenly throughout the network, the MVN was put into culture at 37 °C with 5% CO<sub>2</sub> and ambient oxygen, allowing the HUVECs to adhere to the vessel walls. Typically, cell adhesion occurs overnight while a complete vascular endothelium can be observed after 3 days of culture.

Immunofluorescent Staining and Imaging of HUVECs in Microvascular Network. Hoechst and CD31/PECAM-1 (an endothelial cell specific marker) immunostaining were performed on a subset of MVNs to demonstrate the formation of vascular endothelium within our devices. To describe the procedure, mature MVNs were fixed for 20 min with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, U.S.A.) and then washed three times with 1× phosphate-buffered saline (PBS; Mediatech, Manassas, VA, U.S.A.). The MVNs were then incubated for 1 h in a mixed solution of 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, U.S.A.) to permeabilize the plasma membrane and block any nonspecific antibody binding. Subsequently, Hoechst 33342 (1:250 v/v dilution) and monoclonal mouse-antihuman CD31/PECAM-1 antibody (1:100 v/v dilution; Abcam, Cambridge, U.K.) were added to the MVNs, which were kept in dark at 4 °C overnight. On the following day, the MVNs were first washed three times with 1× PBS and then added with Alexa Fluor 488 goat-antimouse secondary antibody (1:100 v/v dilution; Thermo Fisher Scientific, Waltham, MA, U.S.A.). The MVNs were then kept in the dark at room temperature for 2 h, after which the staining was complete. Prior to imaging, the MVNs were washed three times with 1× PBS to reduce background fluorescence. All antibodies and reagents were added to the MVNs through simple pipetting. All images of the MVNs were captured with an inverted fluorescent microscope (Eclipse Ti-U; Nikon, Minato, Tokyo, Japan).

**Microbubble Preparation.** Custom-made microbubbles (similar to Definity) were used throughout this work and synthesized with a method described previously by De Temmerman et al.<sup>64</sup> The microbubble shell was composed of DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and DSPE-PEG (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] ammonium salt) (Avanti Polar Lipids Inc., Alabaster, AL, U.S.A.) in a 95:5 mol ratio. Perfluorobutane gas (C<sub>4</sub>F<sub>10</sub>) was used as the gas core of the microbubbles. The average diameter of the microbubbles was 1.41  $\mu$ m weighted by number, and the vial concentration was 1.8 × 10<sup>9</sup> bubbles/mL, as determined by a particle counter (Multisizer 3; Beckman Coulter, Brea, CA, U.S.A.). We further diluted the microbubbles by a factor of 10 (1.8 × 10<sup>8</sup> bubbles/mL) before introducing them to the MVNs.

Perfusion and Imaging of Microbubbles in Microvascular Network. Prior to perfusion, each MVN was incubated with Hoechst 33342 (2.2  $\mu$ M; Thermo Fisher Scientific, Waltham, MA, U.S.A.) at 37 °C for 30 min. Hoechst 33342 is a fluorescent agent that labels the HUVEC nuclei. A syringe infusion pump (EW-74900-00; Cole-Parmer; Vernon Hills, IL, U.S.A.) was used to introduce a perfusatean EGM mixture of microbubbles  $(1.8 \times 10^8 \text{ microbubbles/mL},$ confirmed by Multisizer 3) and propidium iodide (PI; 37  $\mu$ M; Thermo Fisher Scientific, Waltham, MA, U.S.A.)-to the MVN (Figure 1B). The MVN perfusion rate was set to 10  $\mu$ L/min to recreate physiological shear on the HUVECs in MVNs, as described in our previous works.<sup>56,65</sup> By making some simple assumptions about the cell sizes (10  $\times$  20  $\mu$ m) and about the distribution of the microbubbles in the 150  $\mu$ m diameter channels, we estimated that there were about 20 cells for every microbubble close to the vessel wall. This calculation seemed to be in agreement with our microscopic qualitative observations. While the microbubble concentration was suggested as a major determinant of the ultrasoundinduced bioeffects, we did not specifically study this parameter in the present work. PI, in this work, was used as an indicator of both cell death and ultrasound-microbubble-induced membrane perforation, or sonoporation. PI is impermeable to healthy cells but once it gains access to the nuclei of compromised cells, it will intercalate DNA and emit red fluorescence upon laser excitation.

The MVN was exposed to ultrasound once the flow had reached steady-state. The resultant microbubble-vessel-wall interactions were observed by bright-field imaging with an inverted microscope (Eclipse Ti-U; Nikon, Minato, Tokyo, Japan) at a frame rate of approximately 30 fps (Figure 1B). Ultrasound was generated by a 1 MHz, singleelement, lightly focused transducer (diameter = 2.01 cm, focal distance = 7.61 cm, focal gain = 2.64) coupled to the MVN with ultrasound transmission gel (Aquasonic; Parker Laboratories, Farfield, NJ, U.S.A.). The transducer was mounted at a 45° angle to the plane of the MVN to limit reflections. A more elegant analysis of the possibility of standing waves in such complex structures is offered in Beakers et al. where they also concluded that no standing waves were present in a similar geometry.<sup>55</sup> Ultrasound pulses were generated by a function generator (AFG3102C; Tektronix, Beaverton, OR, U.S.A.) and amplified by a power amplifier (2200L; Electronics and Innovation, Rochester, NY, U.S.A.). Two ultrasound conditions were used for this work: (1) acoustic pressure: 1.4 MPa, pulse length: 500 cycles, duty cycle: 5% and (2) acoustic pressure: 0.4 MPa, pulse length: 1000 cycles, duty cycle: 20%. In either condition, the frequency was 1 MHz and the treatment duration was 5s. These conditions were similar to those used in published in vitro studies.<sup>10,48-54</sup> The first condition was chosen to be highly bubble-destructive, possibly causing inertial cavitation, but with fewer cycles and lower duty cycle. The second condition was chosen to be less bubble destructive, possibly below the threshold of inertial cavitation, but with a longer exposure and higher duty cycle.

After treatment, the MVN was incubated with calcein AM (1  $\mu$ M; Thermo Fisher Scientific, Waltham, MA, U.S.A.) at 37 °C for 30 min to assess cell viability. Calcein AM is a membrane-permeable agent that can be converted to its green-fluorescent active state by intracellular esterases upon entry into live cells. The presence of calcein indicates a viable cell. It is important to note that the colocalization of calcein and PI indicates live sonoporated cells whereas PI-only staining indicates cell death.

To evaluate the efficacy of our treatment, bright-field and fluorescence (Hoechst, PI) images of an MVN were taken before and after treatment with a Nikon Eclipse Ti-U microscope. Please note that calcein images were only taken after treatment. For each MVN, at least three identical regions of interest (ROIs) were examined before and after treatment. All images were captured by a digital sCMOS camera (ORCA-Flash4.0 LT; Hamamatsu, Bridgewater, NJ, U.S.A.) and postprocessed with ImageJ. A LED illumination source (X-Cite 110LED, Excelitas Technologies, Waltham, MA, U.S.A.) was used for fluorescence excitation.

Field Characterization of the Therapeutic Transducer. In order to estimate the acoustic pressure delivered to the MVN, acoustic field measurements of our 1 MHz treatment transducer were taken with a 0.4 mm membrane hydrophone (Precision Acoustics, Dorchester, U.K.) in a 10 gal water tank (Figure 2). A motorized



**Figure 2.** Experimental setup for acoustic field measurement. A microvessel housing piece is placed at the focal plane of the custommade transducer. A PVDF membrane hydrophone measures the acoustic pressure after passage through the microvascular housing.

micropositioning system (Newport Corporation, Irvine, CA, U.S.A.) was used to align the transducer to the hydrophone and to map the acoustic field. The 2D transverse beam profile was created by taking measurements using steps of 0.5 mm and 1 mm in the x- and y-direction, respectively. The distance from the transducer to the hydrophone was 2 cm. 2D transverse beam profiles were taken with and without the acrylic housing in between the transducer and hydrophone to determine sound attenuation through the housing. Assuming that the effect of the MVN is a simple acoustic pressure reduction mainly coming from the acrylic housing is an oversimplification. In addition to the acrylic, the 1 mm collagen and the presence of the 150  $\mu$ m channels also influence the field to some extent, as presented by Beakers et al.<sup>55</sup> However, here we only wanted to have an approximate delivered acoustic pressure value for the two selected acoustic conditions (0.4 and 1.4 MPa).

**Image Analysis and Determination of Permeabilization Efficiency.** The ImageJ "analyze particles" module was used to count in each acquired fluorescent image the total number of (1) HUVECs (based on Hoechst staining) and (2) HUVECs with PI uptake. We define permeabilization efficiency as the ratio of HUVECs with PI uptake to the total number of HUVECs.

Specifically, the number of PI-positive HUVECs counted in the ROI before treatment (existing dead cells in the system) was subtracted from the number of PI-positive HUVECs in the same ROI after treatment (both existing dead cells and impacted cells), then this difference was divided by the total number of Hoechst-stained HUVECs found before treatment. Please note that there exists a small difference between the pretreatment and post-treatment total HUVEC counts due to cell detachment after sonication. We chose the pretreatment total HUVEC count for our calculation to avoid the over-reporting of the bioeffect observed. The presented data was obtained from at least three independent MVNs with at least three independent ROIs.

**Estimation of Flow Velocity in Microvascular Network.** To estimate the flow velocity of microbubbles in MVN, bright-field recordings of MVN perfusion were acquired and analyzed with the ImageJ "manual tracking" module. The flow velocity was calculated based on the distance traveled by the microbubbles tracked and the frame rate of the recordings (30 frames per second). The result presented was obtained from recordings of seven perfused MVNs, each with at least three ROIs examined.

#### RESULTS AND DISCUSSION

Biological Characteristics of the Engineered Microvascular Network. We successfully developed a perfusable 3D microvascular network (Figure 3) that was fabricated with biological materials, including collagen I and human endothelial cells. Collagen I is a key component of the native extracellular matrix and is used extensively for various tissue culture applications;<sup>56–58,61–63,66–69</sup> it provides good mechanical support to the vessels and allows biomolecular diffusion within it. In addition, the collagen I matrix allows HUVECs to exhibit in vivo-like cell-matrix interaction.<sup>69</sup> HUVECs embedded in the collagen scaffold form a continuous vascular endothelium throughout the microvascular network (Figure 3b). Immunostaining of PECAM-1 in the MVN suggests that the HUVECs retained their intercellular junctions and that they elongated along the long-axis of the microvascular channels under gravity driven flow. The 12-channel network pattern of the MVN used in this work was intended to be a simplified imitation of the branching human vasculature at the arteriole level or, in the case of tumor microvasculature, the dilated capillaries (which also have a single layer of endothelial cells).<sup>59,60</sup> By perfusing MVNs with microbubbles at physiological flow conditions, we effectively recreated the in vivo environment in which microbubbles circulate in the blood and interact with the microvasculature at a target site. In this specific case, the microbubbles were interacting with endothelial cells in a 150  $\mu$ m diameter vessel. Because the MVN is flow-enabled and has a window for direct optical observation, it presents a platform for the investigation of microbubble-vessel interaction in real time.

HUVECs were used for MVN seeding in this work because they are well-characterized and readily accessible through ATCC. Organ-specific endothelial cells can be used to further improve the physiological relevance of the model. We had previously used organ-specific endothelial cells to simulate the human kidney peritubular capillaries and the marrow vascular microenvironment.<sup>58,63</sup> In a future study, we may incorporate organ-specific endothelial cells into the MVN and investigate



**Figure 3.** (a) Bright-field image of the 12-channel microvascular network. The black linings of the channel are the vascular endothelium formed by the HUVECs. (b) PECAM-1 (red) staining of the HUVECs revealed that the cells formed intercellular junctions and elongated in the direction of the flow under gravity flow. Hoechst 33342 (blue) staining indicates the nuclei of the HUVECs.



Figure 4. 2D beam profiles of the 1 MHz transducer in (a) deionized water and (b) after passage through the microvascular housing. The acoustic field is attenuated and spatially distorted after passage through the microvascular housing. Peak pressure across the full width at half of the maximum intensity of the beam was reduced by approximately 30% (0.6–0.4 MPa).



Figure 5. (a) Microbubble flow in one of the 12 channels of the MVN. The darker edges confirm that the channel is endothelialized. (b) Tracing of a single bubble in a video loop gives the flow velocity.

whether the efficiency of ultrasound-mediated drug delivery efficiency is organ-dependent. As it stands, however, organspecific endothelial cells are donor-based and therefore limited in supply. They are also more variable in genotype and



Figure 6. Clustering of microbubbles in a microvascular channel. Clouds of microbubbles near the vessel wall aggregated and condensed into larger bubbles in the presence of ultrasound. The microbubbles were pushed in the direction of sound and against the vessel wall.

phenotype and much more difficult to culture. These challenges will need to be addressed before a sonoporation study of MVN with the use of organ-specific cells is practically feasible. The pattern of the microvascular network can be altered to simulate the tortuous vessels found in tumors or the interconnected microvascular beds found in most exchange interfaces.<sup>70–73</sup> In this work, we elected to use a simpler pattern for proof-of-concept.

Acoustic Field of the Therapeutic Transducer. Prior to treating the MVNs with a 1 MHz transducer, we studied how the MVN acrylic housing affected the transducer's acoustic field. Figure 4 shows the two-dimensional beam profiles of the transducer in free field and in the presence of an MVN acrylic housing. Notably, the acoustic field was spatially distorted by the MVN housing. The width of the ultrasound beam, defined as the full beam width at half of the maximum intensity, increased from 8 mm (in free field) to 10.5 mm (in MVN housing). Since the size of the microvascular network is approximately 10 mm<sup>2</sup>, we assumed that the sound beam covered the entire network and that all cells within the network experienced similar, if not identical, acoustic pressure during treatment. In terms of attenuation, we found that the MVN housing reduced the acoustic pressure by a spatial average of  $33 \pm 28\%$  across the area of the sound beam. When treating the MVNs, we adjusted the source pressure to account for this loss.

Sonoporation of HUVECs in Perfused Microvascular Networks. To establish physiological flow in the MVN, we perfused it at a volumetric flow rate of 10  $\mu$ L/min. According to our measurement, microbubbles flowed through microvascular channels at an average velocity of 0.34 ± 0.13 mm/s (Figure 5), which is similar to the average human capillary blood flow velocities measured by Bollinger et al. and Ivanov et al.<sup>74,75</sup>

When MVNs were treated with ultrasound, several notable observations were made. First, sometimes, the microbubbles flowing in the microvascular channels formed big bubble clusters (Figure 6) and were pushed against the vessel wall in the presence of ultrasound, likely due to acoustic radiation and secondary Bjerknes forces.<sup>76</sup> Microbubble clustering and pushing have been well-described in the literature and purposefully used to enhance therapeutic efficiency.<sup>76,77</sup> In fact, most bioeffects (indicated by positive PI staining) in our system occurred immediately next to large clusters of microbubbles, which were mostly destroyed after sonication.

This proximity effect was also reported by Qin et al., who suggested that there exists a threshold bubble-to-cell distance at which sonoporation occurs.<sup>53</sup> In terms of immune-histology, we noticed that Hoechst staining was less uniform after treatment than before treatment (Figure 7). This likely resulted from the HUVECs detaching from vessel walls due to mechanical and acoustic radiation forces. In fact, a small number of floating cells were observed in the MVN after sonication. The extent of cell detachment was greater for the high amplitude acoustic condition and very little for the low amplitude condition. For the high amplitude condition, many treated cells were observed in the microvascular channels with cell death being more pronounced than sonoporation (Figure 7A-viii).

Correlation between HUVEC Membrane Permeabilization and Acoustic Conditions. To give insights into how various ultrasound parameters affect sonoporation, we compared quantitatively the permeabilization efficiency between two different ultrasound conditions. The first condition is 1.4 MPa, 500 cycles, 5% duty cycle, and the second condition is 0.4 MPa, 1000 cycles, 20% duty cycle. As expected, the two acoustic conditions elicited very different cellular responses. The first acoustic condition (short highpressure pulses) led to a permeabilization efficiency of 16  $\pm$ 16% (n = 7) while the second condition (long low-pressure pulses) yielded 4.4  $\pm$  8.2% (n = 3) (Figure 8). Although permeabilization efficiency varied among the MVNs treated with the same acoustic condition, the first acoustic condition was noticeably more effective and disruptive. As mentioned previously, HUVEC detachment was observed (albeit at a relatively small number compared to the total number of cells) at 1.4 MPa (first condition) while most cells remained viable and attached at 0.4 MPa (second condition). The enhanced permeabilization at higher pressure can be explained by what the computation models in the literature have predicted: greater acoustic pressure leads to greater stress on the plasma membrane.<sup>78,79</sup> Chen et al. also observed the formation of liquid jets (from asymmetric microbubble collapses) at peak negative pressures of 1.5 MPa and above, with greater acoustic pressure leading to greater vessel deformation and displacement.<sup>80</sup> Although these studies focused on single bubble interactions with the vessel wall in small tubes (about ten times smaller than the microvascular channels), they provided valuable insights to what may have happened when the MVNs were sonicated. Increased bioeffects at higher acoustic



Figure 7. Bright-field and fluorescence images of MVN ROIs before and after ultrasound-microbubble treatment. (A) Corresponds to treatment with the first acoustic condition (1.4 MPa, 500 cycles, 5% DC) while (B) corresponds to treatment with the second acoustic condition (0.4 MPa, 1000 cycles, 20% DC). The arrows in bright-field images point to microbubble clusters that were slowly moving through the microvascular channels before they were destroyed by ultrasound. Hoechst (blue) staining indicates HUVEC nuclei; PI (red) staining indicates cells with compromised plasma membrane, which can occur by sonoporation or pure cell death. Calcein (green) staining indicates viable cells. Panel viii shows composite images of Hoechst, DAPI, and PI.



Figure 8. Difference in HUVEC permeabilization efficiency (% PI uptake) between ultrasound treatment conditions.

pressures have also been reported in previous in vitro studies.<sup>48,49,52,54,81</sup> De Cock et al. reported an increase in dextran (2 MDa and 4 kDa) uptake by human melanoma cells with increased acoustic pressure from 0.1 to 0.5 MPa. They also noted elevated cell toxicity at higher acoustic pressures. Similarly, van Rooij et al. reported an increase in HUVEC sonoporation rate (from 5% to 40%) and cell death percentage (up to 30%) with increased acoustic pressure (from 0.15 to 0.5 MPa). Lai et al. and Karfashian et al. also reported similar trends in sonoporation of HeLa cells and murine fibrosarcoma (KHT-C) cells, respectively. Acoustic pressure is just one of the many parameters that influence sonoporation; therefore, a parametric study of ultrasound conditions and microbubble properties is required to identify the right balance between therapeutic effect and tissue disruption. It is also important to note that nonacoustic factors like vessel size, fluid viscosity, and microbubble shell material may all dramatically affect cell membrane permeabilization or disruption.<sup>78-80</sup> Here, our main objective was to establish MVNs as a viable platform for the study of sonoporation and report on the methods to develop and operate such a platform. In future works, we plan to perform a detailed and in-depth parametric study for cell permeabilization. The use of endothelialized MVNs will help bridge the gap between in vitro studies and in vivo studies.

Our selection of only two ultrasound conditions was such that we could make qualitative comparisons between higher and lower pressures (inertial vs nonintertial cavitation) and shorter and longer cycles. To a certain extent, this selection was done in an ad-hoc fashion, and while the conditions were very similar to what has already been published in this area, we remind the reader that our main objective was to introduce the MVNs to the sonoporation research community and demonstrate how they can be perfused with microbubbles and exposed to ultrasound. In vivo drug delivery studies also used acoustic pressures in a similar range (0.2-0.5 MPa) to maximize delivery without incurring tissue damage.<sup>82,83</sup>

The MVN is a versatile platform that can be modified and used in a variety of ways. With specific modifications to the microvascular network dimensions, the MVN can be used to study drug extravasation and drug delivery in vascular networks that are  $10-1000 \ \mu$ m, i.e., ranging from normal human capillaries to the feeding arterioles for tumors. By coculturing endothelial cells with tumor cells and inducing angiogenesis in the microvascular system, we can test the combined effects of

ultrasound, microbubbles, and cytotoxic or antiangiogenic drugs on slowing cancer progression.

## CONCLUSIONS

We have successfully developed a 3D endothelialized microvascular network that is perfusable with physiological flow rates. The addition of microbubbles in such a network has led to the visualization of the flow and has facilitated the sonoporation of endothelial cells. The analysis of video loops enabled the estimation of the flow velocities and the selection of flow conditions similar to those of microvessels of comparable size in vivo. Ultrasound-induced oscillations of microbubbles have resulted in the sonoporation of the endothelialized microvessels.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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#### Funding

This work was cofunded by the Life Sciences Discovery Fund (Project: 329512), National Institute of Health (NIH) Awards (1DP2DK102258 and UH2/UH3 TR000504), and the U.S. Department of Defense fund (CA160415/PRCRP).

## Notes

The authors declare no competing financial interest.

#### ACKNOWLEDGMENTS

We gratefully acknowledge the experimental support of Kiet Phong, former B.S. bioengineering student in the Zheng lab of University of Washington.

# ABBREVIATIONS

MVN, microvascular network; 3D, three-dimensional; HUVEC, human umbilical vein endothelial cells; ATCC, American Type Culture Collection; EGM, endothelial cell growth media; PDMS, polydimethylsiloxane; PBS, Phosphatebuffered saline; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] ammonium salt; PI, propidium iodide; ROI, region of interest

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