Published in final edited form as:

Ultrasound Med Biol. 2019 March ; 45(3): 833–845. doi:10.1016/j.ultrasmedbio.2018.11.011.

Ultrasound Imaging of Microbubble Activity During Sonoporation Pulse Sequences

Sara Keller¹, Matthew Bruce², Michalakis A. Averkiou¹

¹Department of Bioengineering, University of Washington, Seattle

²Applied Physics Laboratory, University of Washington, Seattle

Abstract

Ultrasound-mediated drug delivery using the mechanical action of oscillating and/or collapsing microbubbles has been studied in many different experimental platforms, both *in vitro* and *in vivo*; however, the mechanisms remain to be elucidated. Many groups use sterile, enclosed chambers, such as Opticells or Clinicells, to optimize acoustic parameters *in vitro* needed for effective drug delivery *in vivo*, as well as for mechanistic investigation of sonoporation, or the use of sound to permeate cell membranes. In these containers, cell monolayers are seeded on one side and the rest of the volume is filled with a solution containing microbubbles and a model drug. Ultrasound is then applied to study the effect of different parameters on model drug uptake in cell monolayers. Despite the simplicity of this system, the field has been unable to appropriately address what parameters and microbubble concentrations are most effective at enhancing drug uptake and minimizing cellular toxicity. In this work, a common *in vitro* sonoporation experimental setup was characterized through quantitative analysis of microbubble-dependent acoustic attenuation in combination with high frame rate and high resolution imaging of bubble activity during sonoporation pulse sequences. The goal was to visualize the effect that ultrasound parameters have on microbubble activity. It was observed that under literature-derived sonoporation conditions (0.1 to 1 MPa, 20 to 1000 cycles, and 10k to 10M microbubbles per mL), there is strong and nonlinear acoustic attenuation, as well as bubble destruction, gas diffusion, and bubble motion resulting in spatiotemporal pressure and concentration gradients. Ultimately, it was shown that the acoustic conditions in common *in vitro* sonoporation setups are much more complex and confounding than often assumed.

Keywords

sonoporation; cavitation imaging; drug delivery; microbubble attenuation

Corresponding Author: Michalakis A. Averkiou, Department of Bioengineering, University of Washington, Box 355061, N107 William H. Foege Building, 3720 15th Ave NE, Seattle, WA 98195-5061, maverk@uw.edu, Telephone: (206) 616-9558.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Beyond diagnostic imaging, contrast-enhanced ultrasound has shown immense promise in the field of drug delivery (Kooiman et al. 2014; Mitragotri 2005). With the growing clinical relevancy of delivering macromolecular therapeutics, including proteins (Bekeredjian et al. 2005) and nucleic acids (Suzuki et al. 2011), ultrasound and microbubbles can provide a means for overcoming traditional barriers defining intracellular drug uptake (Qin et al. 2018). In the presence of an acoustic wave, microbubbles undergo volumetric oscillations and eventual destruction, which can provide both an acoustic signal for contrast enhancement through resonant scattering as well as localized microscale forces that can cause increased cell membrane permeation through a process known as sonoporation (Ferrara et al. 2007).

However, despite over 10 years of promising studies both *in vitro* (De Cock et al. 2015; Karshafian et al. 2009) and *in vivo* (Iwanaga et al. 2007; Shapiro et al. 2016), the acoustic parameters that lead to effective sonoporation remain ambiguous. Peak negative pressure, pulse length, frequency, pulse repetition frequency, and microbubble concentration are all important parameters that have been studied, yet there is not conclusive agreement for these parameters between different studies. It has been generally accepted that increasing acoustic pressure may increase sonoporation efficiency, but at the expense of cell viability (Helfield et al. 2016; van Rooij et al. 2016). Additionally, several groups suggest that microbubble-cell distance can also affect the success of particle uptake (Wang et al. 2018), while others suggest that rather it is microbubble displacement that governs membrane poration (Fan et al. 2014; van Rooij et al. 2016). The choice of whether to send a single pulse (van Rooij et al. 2016) or a series of repeated pulses (De Cock et al. 2015) can add an additional layer of complexity. The resultant ambiguity in parameter space can obfuscate understanding of biophysical mechanism. We hypothesize that an additional confounding factor *in vitro* is due to the lack of standardization in experimental setup and macroscale field effects. While several papers mention microbubble oscillation and displacement as a means of inducing sonoporation, they are limited by optical microscopy, which, despite having high spatiotemporal resolution in one plane, does not consider the 3D geometry of microbubble movement or overall acoustic environment (Fan et al. 2014; van Rooij et al. 2016). To the best of our knowledge, there have been no existing studies on how microbubble presence affects sound propagation in a 3D volume during the sonoporation process.

Moreover, there are three distinct timescales important in sonoporation: 1) the time of bubble oscillation, equal to the duration of the sound pulse (microseconds), 2) the timescale of bubble destruction (accompanied by gas diffusion) and radiation force (milliseconds), and 3) the timescale of cell membrane permeation (seconds to minutes). For the first timeframe, super high frame rate microscopy with a Brandaris camera has been used (de Jong et al. 2009; van Rooij et al. 2016), and for the third, *in vitro* studies of propidium iodide uptake into cells and membrane resealing mechanics have been performed (Helfield et al. 2016; Hu et al. 2018). We concentrate on the second timescale and hypothesize that examining the temporal window after application of an ultrasound pulse may further elucidate this process.

The objective of this work was to evaluate the effect of various acoustic parameters and microbubble concentrations on the resulting microbubble activity during sonoporation in cell enclosures. To this end, we have performed quantitative representations of microbubble concentrations in a 3D volume, acoustic attenuation measurements through increasing microbubble concentrations, and high-resolution and ultrafast ultrasound imaging of bubble behavior during and after ultrasound exposures. We evaluated conditions similar to those found in the literature: moderate pressures (125 kPa–1 MPa), cycles (20–1000), and concentrations (10k – 10M MBs/mL) using both single pulses and repeated pulses with a single element focused 1 MHz transducer. Studying microbubble behavior in a tightly controlled experimental design may help explain the conflicting results in published literature and may lead to reproducible sonoporation.

MATERIALS AND METHODS

Microbubble Preparation

Microbubbles 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]) (Avanti Polar Lipids Inc, Alabaster, AL) in a 95:5 molar ratio were prepared as described previously (De Cock et al. 2015) and used for imaging studies to mimic prior Opticell sonoporation work (De Cock et al. 2015; Helfield et al. 2016; van Rooij et al. 2016). Sonovue (Bracco Suisse SA, Geneva, Switzerland) was additionally used in attenuation studies for comparison with a clinically relevant microbubble. Average bubble diameter by volume was measured using a Multisizer 3 (Beckman Coulter, Brea, CA) and determined to be approximately 3 μm for the custom formulation and 4.5 μm for Sonovue. To understand how microbubble concentration in a 3D volume impacts bubble-to-cell ratio, theoretical calculations were made to estimate the effective concentration that appears next to cells. A Clinicell (MABIO, Tourcoing, France) was chosen as the representative cell enclosure, and has dimensions of 0.5 cm $\times$ 6.2 cm $\times$ 3.8 cm (Fig. 1). The average distance between microbubbles (d_{avg}) was calculated as shown in Equation 1, where V is the volume of the container (m^3) and N_{MB} is the total number of microbubbles in the container.

$$d_{avg} = \sqrt[3]{\frac{V}{N_{MB}}} \quad (1)$$

Distance between microbubbles was tracked at varying concentrations (10k to 100M microbubbles/mL). Shown in Figure 1 are the assumptions made in determining this metric, where 3 μm $\times$ 3 μm cubes represent microbubbles, an approximation given the 3 μm diameter custom microbubble. This was compared against the standard length of endothelial cells (15 μm). Void fraction (ϕ), or the fraction of the solution that exists in the gas phase (microbubbles) versus the liquid phase (solvent) was also calculated at increasing concentrations as shown in Equation 2 as a secondary measure of microbubble dispersity, where V_{MB} is the volume of a single microbubble (m^3), N_{MB} is the total number of microbubbles in the container, and V is the total volume of the container (m^3).

$$\phi = \frac{V_{MB} \times N_{MB}}{V} \quad (2)$$

Acoustic Attenuation

All experiments were conducted using a custom-made single element focused transducer (diameter = 20 mm, focal distance = 80 mm, frequency = 1 MHz). 20 cycle sound pulses were sent using a Tektronix AFG 3102 Arbitrary Waveform Generator (Tektronix, Inc., Beaverton, OR), amplified by an ENI 2200L RF Amplifier (ENI, Rochester, NY), and transmitted by the focused transducer. Transmit pressure, defined here as peak negative pressure, was scaled up 10% per pressure reduction through the Clinicell when measured relative to the free-field. Transmit parameters can be seen in Table 1. The sound pressure of a pulse transmitted by the 1 MHz transducer reaching a 0.4 mm membrane hydrophone (Precision Acoustics Ltd., Dorchester, UK) after passage through a Clinicell filled with increasing concentrations of microbubbles was measured. Microbubble concentration was measured using the Multisizer 3. The Clinicell was placed 4 cm away from the focused transducer, as beam characterization measurements showed that this distance had the largest pressure amplitude. The hydrophone was placed 2 cm away from the enclosure to measure the acoustic pressure after transmission through the Clinicell upon exiting. The cell enclosure was placed in the vertical orientation for this experiment, as shown in Fig. 2a, to avoid the effects of sound reflection at the water-air interface. Bubble rising effects due to buoyancy were minimized by quick measurement and refilling each time and were not seen in imaging. The signals were acquired using a DPO7054C Oscilloscope (Tektronix, Inc., Beaverton, OR) and analyzed in MATLAB (The MathWorks, Inc., Natick, MA, USA). Received peak negative pressure was measured for each microbubble concentration.

High-Resolution Imaging of the Sonoporation Process

A Philips iU22 (Philips Medical Systems, Bothell, WA) was used to visualize the bubble activity, destruction, and movement induced by the 1 MHz transducer. An L15-7io probe operating at 12 MHz, provided high-resolution at a moderately high frame rate of 51 Hz B-mode fundamental imaging. The imaging mechanical index (MI) was set to 0.05 to avoid bubble destruction. The imaging system inherently applies a time gain compensation, which was set to the default value for tissue. The same transmit setup was used as in the attenuation studies, except that the Clinicell was placed in the horizontal orientation, seen in Fig. 2b. This position was chosen to accommodate imaging. Exposures were performed immediately after introduction in the tank environment to limit bubble rising. Images were acquired while the 1 MHz transducer was firing at 1 Hz or 51 Hz PRF. Since the experiments were in water and there were no other ultrasound scatterers in the medium, it was adequate to use fundamental B-mode imaging as opposed to contrast-specific imaging modes for visualizing microbubbles. Images loops were analyzed in QLAB (Philips Medical Systems, Bothell, WA). For the 1 Hz PRF condition, images were acquired in real-time (51 Hz in this case) after multiple exposures from the focused transducer. For the 51 Hz PRF condition, images were acquired over a five second exposure. The conditions tested can be seen in Table 1, and were chosen to represent parameters used in published *in vitro* sonoporation studies.

High Frame Rate Imaging of the Sonoporation Process

High frame rate imaging was performed with a Vantage 128 (Verasonics, Kirkland, WA) system in order to image and resolve the bubble activity during and after ultrasound excitation with a single element 1 MHz transducer. A linear array operating at 15 MHz (Vermon, France) was used for imaging. The transmitted imaging pulse was 2 cycles long and the MI was 0.05 to avoid bubble destruction. 8000 frames at an 8 kHz frame rate were captured while imaging in the orientation shown in Fig. 2b. A single pulse at 1 MPa and with duration 20, 200, or 1000 cycles was fired by the single element 1 MHz transducer. One second image loops were collected with the imaging system to study bubble destruction and gas diffusion at this greatly improved temporal resolution (compared to conventional scanners).

RESULTS

Microbubble Concentration Measurements

Average distance between microbubbles and void fraction at increasing microbubble concentrations can be seen in Fig. 3a and b. Distance between microbubbles decreases with increasing concentration (Fig. 3a), while void fraction increases (Fig. 3b). Even at the highest concentrations used (10 million microbubbles/mL), the distance between microbubbles is still only about 50 μm . Void fraction remains quite low (10^{-7} – 10^{-4}), indicating that despite the high microbubble concentrations, still the actual microbubble volume is minimal. Also shown in Fig. 3c are the pictorial representations of how these microbubble concentrations may manifest near cell monolayers. Concentrations used in prior works range from 100,000 microbubbles/mL to 10 million microbubbles/mL (Hu et al. 2013; van Rooij et al. 2016). However, only when the microbubble concentration reaches 100 million microbubbles/mL, a concentration much greater than what was used in the above publications, does the “bubble-to-cell” ratio near cells reach 1:1.

Acoustic Attenuation

Despite low void fractions, with increasing concentration of microbubbles, the attenuation increases and the resultant signal decreases (Fig. 4). Figure 4a shows the propagated peak negative pressure for the custom microbubble formulation and Fig. 4b for Sonovue. For both formulations, higher pressure pulses experienced more attenuation. When using the custom microbubbles at a concentration of 1,000,000 microbubbles/mL, the pressure received after transmitting with 500 kPa had already decreased by over 50%, while it had only decreased about 20% when transmitting with 125 kPa. Sonovue experienced slightly more attenuation than the custom formulation; however, the overall trends seen with the custom formulation and Sonovue remain mostly consistent.

High-Resolution Imaging of the Sonoporation Process

Shown in Figures 5 and 6 are images acquired with a Philips iU22 of bubbles in the Clinicell during sonoporation-type excitations from a 1 MHz transducer. Fig. 5 shows the effect of repeated 20 cycle pulses, while Fig. 6 shows the effect of repeated 200 cycle pulses. The

effect of changing other parameters (pressure, concentration, and PRF) is also shown in both figures.

Figure 5(a-d) shows the images recorded after multiple firings from a 20 cycle, 500 kPa pulse at a 1 Hz PRF (i.e., 1 s between firings) through 500,000 microbubbles/mL. This PRF was chosen to analyze bubble activity after repeated single exposures at a slow rate (1 Hz) while acquiring images at a much higher rate (51 Hz). The first pulse was sent at time $t = 0$ s (Fig. 5a), the second pulse at $t = 1$ s, and so forth. As more pulses are sent, progressing from Fig. 5a to d, the amount of bubble elimination, defined here as the combination of bubble destruction and bubble motion, is increased. However, the amount of motion seen was minimal, implying that most bubble elimination that occurs using 20 cycle pulses is due to bubble destruction alone. Complete bubble elimination through the entire volume of the propagation path did not occur using this setting until 8 pulses had been sent (Fig. 5d).

Figure 5e examines the effect of increasing the sonication PRF (not to be confused with the imaging frame rate) to 51 Hz, as pulse repetition has been considered in various published works (De Cock et al. 2015; Fan et al. 2014). After 8 pulses were sent at 51 Hz (in 157 ms), the resultant image looks nearly the same as after 8 pulses sent at 1 Hz (in 7 s) (Fig. 5d), implying that when using this pulsing scheme, the only change is the rate of bubble elimination. Fig. 5f and g show the impact of changing sonication acoustic pressure. When a 200 kPa pulse was used (Fig. 5f), there was much less bubble elimination than when 500 kPa was used, even after 20 repeated pulses. Conversely, when a 1 MPa pulse was sent (Fig. 5g), there was much more bubble elimination. The width of bubble elimination also increased with increasing pressure. This is because at higher pressures a larger portion of the beam is above the bubble destruction threshold. Fig. 5h shows the effect of increasing microbubble concentration. At this higher concentration, the bubble elimination that reaches the top of the Clinicell due to the 1 MHz firings is reduced.

Figure 6(a-d) shows the images recorded after multiple firings from a 200 cycle, 1 MPa pulse at a 1 Hz PRF through 500,000 microbubbles/mL. Figs. 6a and c show the images acquired immediately after the end of the 1st and 2nd transmit pulses, respectively. Figs. 6b and d show the evolution of movement that occurred subsequent to these transmit pulses. While the initial pulse caused a small degree of bubble destruction, the time between pulse excitations allowed for visualization of resultant bubble motion, shown with yellow arrows. Indeed, the bubble elimination that eventually reaches the upper membrane of the Clinicell is a result of this movement, and not just destruction, as demonstrated in Fig. 6d.

Figure 6e shows the effect of using a 51 Hz sonication PRF. Unlike when using 20 cycles, increasing the pulse repetition rate had a significant impact on the bubble elimination profile. Specifically, the short time between repeated pulses reduced the amount of movement that could occur prior to the next pulse. Therefore, the unique movement patterns seen using a 1 Hz PRF and long cycles were not seen when using a higher PRF. Fig. 6f and g show the effect of using two lower pressures. Bubble elimination that occurs from these two transmit sequences is smaller than the 1 MPa transmit pulse, but motion still occurs, as indicated with yellow arrows. Finally, increasing the concentration increased the complex

motion shown in Fig. 6d. Pulses sent through a high concentration of microbubbles resulted in bubble swirling surrounding the direction of axial propagation (Fig. 6h).

In summary, increasing the number of cycles in the pulse increased the amount of bubble motion that occurred after the end of the transmit pulse. Increasing acoustic pressure increased the area of bubble elimination, both in the lateral and axial direction. Higher concentrations reduced the bubble elimination that reached the top of the Clinicell. Finally, using a higher pulse repetition frequency only changed the bubble elimination profile when movement was occurring.

High Frame Rate Imaging of the Sonoporation Process

High frame rate imaging using a Verasonics 128 was used to show the effect of a single 1 MPa pulse at high temporal resolution not possible with conventional ultrasound. Similar to the experiments using the Philips iU22, the results from high frame rate imaging can be broken into the individual variable effects of cycles and concentration. The two columns in Fig. 7, (a–d) and (e–h) show progressions from two different single pulses through different bubble concentrations. The time scale starts when the pulse is fired. Therefore, for the 200 cycle condition, the sonoporation pulse duration is 2 imaging frames, and for the 1000 cycle condition, the pulse duration is 8 frames.

Figure 7(a–d) shows the progression of a 1 MPa, 200 cycle pulse through a Clinicell filled with 500,000 microbubbles/mL. Shown in Fig. 7b is the frame taken 2.9 ms after the pulse begins. The bubble elimination shown with a red arrow is theorized to be due to bubble destruction from the high-pressure excitation as it is present immediately after the pulse ends. The void areas shown in Fig. 7c and d that occur after the initial bubble destruction are theorized to be due to bubble movement from acoustic streaming and gas diffusion.

Figure 7(e–h) shows the progression of a 1 MPa pulse with an increased number of cycles through a higher microbubble concentration. Fig. 7f, a frame taken shortly after the end of the pulse, shows that there is considerably more initial bubble destruction than in the 200 cycle pulse condition. Similarly, as shown in subsequent frames, there is more dramatic movement that occurs, highlighted with the yellow arrow in Fig. 7g. Finally, as in Fig. 6h where a high microbubble concentration was also used, a similar swirling pattern develops in Fig. 7h.

Figure 8 shows the effect of a 20 cycle pulse through 500,000 microbubbles/mL. In Fig. 8a, the image taken 2.5 ms after the pulse was fired, there was no amount of initial bubble elimination at these ultrasound settings; however, after 100 ms, a sizable void area has developed (Fig. 8b) without observing any type of bubble motion in the images that preceded. This is reasoned to be due to gas diffusion after bubble shell disruption, known to occur within durations on the order of 100 ms after the collapse of a gas volume of this size (Epstein and Plesset 1950). No bubble movement was seen using 20 cycles, confirming the observations taken with the iU22 that little radiation force occurs when using short cycle lengths.

High frame rate imaging with the Verasonics extended the findings from imaging with a diagnostic scanner; specifically, that secondary microbubble acoustic radiation force is dependent on the duration of the sound pulse as well as the microbubble concentration. Despite the high pressure excitation, total bubble elimination was minimal. In fact, only the 1000 cycle pulse shown in Fig. 7(e-h) demonstrated complete bubble elimination through the length of the Clinicell for the acquisition duration studied. Even in this case, it was only after the resultant diffusion and bubble motion that the microbubbles were eliminated from the top membrane. These results imply that the bubble activity near the top membrane (where cells would normally be) is due to a complex combination of multiple phenomena and not just simply microbubble oscillations.

DISCUSSION

The propagation of sound through microbubble volumes in a typical *in vitro* sonoporation experimental setup was investigated with high-resolution conventional imaging and high frame rate plane wave imaging. Through this work, several important findings were observed. First, even high (for clinical standards) microbubble concentrations in a 3D volume still result in low bubble-to-cell ratios. At the highest microbubble concentration tested (10 million microbubbles/mL), the distance between bubbles is only about 50 μm . Compared with endothelial cell diameters of approximately 15 μm , these concentrations do not reach 1:1 bubble-to-cell ratios. That said, this is only an approximate calculation. If extrapolated to include bubble expansion beyond the initial radius, this ratio would be more favorable (Qin et al. 2009). Regardless, the common usage of bubble-to-cell ratio as a ratio of the total number of microbubbles to total number of cells does not account for experimental geometry, rendering it a misleading statistic. Indeed, when the term was originally used, it was for cell suspensions and not monolayers (Guzmán et al. 2003; Ward et al. 2000). Additionally, void fraction was calculated and presented for the concentrations used in this study. Given the dispersity of microbubbles, the void fraction is very low (typically less than 10^{-4}) even at high concentrations. These two metrics imply that high bubble concentrations still yield sparse microbubble placement at typical literature parameters between 1 and 10 million microbubbles per mL (De Cock et al. 2015; van Rooij et al. 2016).

Despite low void fractions, high microbubble concentrations create large acoustic attenuation. The most striking result of the attenuation experiments is the extreme loss of pressure that occurs with increasing microbubble concentration. Even at low concentrations, between 100k and 1M microbubbles/mL, there can be up to a 50% loss in acoustic pressure. This result implies that for single pulsed sonoporation experiments through a volume of microbubbles, the *in situ* pressures meant to be delivered to microbubbles next to cells are not the pressures that would exist in the medium when bubbles are not present. Further, these observations remained similar between the custom formulation and Sonovue. It was observed that Sonovue created slightly more attenuation, likely because of its larger average size. This is consistent with prior work showing that larger bubbles have a more pronounced effect on sound attenuation (de Jong et al. 1992).

From the attenuation experiments, two additional observations can be made: first, that the effect of concentration-dependent attenuation is more pronounced at higher pressures, and second, that higher microbubble concentrations yield stronger harmonic signal generation. As seen in Fig. 4, for both Sonovue and the custom microbubble, the received signal decreases at a higher rate when using 500 kPa than when using 125 kPa. This seems counterintuitive, as it would make sense that a higher pressure propagates further while destroying more bubbles. However, higher pressure pulses result in a more dramatic microbubble radius increase (Lentacker et al. 2014; Qin et al. 2009) and therefore more attenuation, while smaller bubble oscillations at lower pressures are less attenuative to the overall beam.

Increasing the microbubble concentration also increased the amount of harmonic signal generation. This is shown in Fig. 9d, where at 10M microbubbles/mL, the strength and number of harmonic peaks generated from a 125 kPa transmit pulse is increased due to resonant scattering. Additionally, nonlinear propagation of sound in the medium (water) results in harmonic generation and thus alteration of the original frequency content, shown by the second harmonic peak in Fig. 9b, before microbubbles had been added. Therefore, not only does the presence of microbubbles affect the delivered pressure, but it also alters the frequency content of the ultrasound pulse that reaches the microbubbles adjacent to the cells of the monolayer.

As stated before, sonoporation efficacy is dependent on microbubble concentration. It has been shown that there is more biological effect (be it sonoporation or cytotoxicity) at high microbubble concentrations (Guzmán et al. 2003; van Rooij et al. 2016). Paradoxically, achieving maximal bubble-to-cell ratios also substantially decreases available pressure to induce bubble oscillation near cell monolayers. Several groups have mitigated this issue by inverting the setup, such that ultrasound goes through cells first before insonifying microbubbles (Park et al. 2011; Qin et al. 2016). While this does limit microbubble-dependent acoustic attenuation, it may still induce microbubble collapse and movement away from cells at destructive pressures, which could impact working microbubble concentration for maximum sonoporation effect. Nevertheless, the vast majority of published work to date used the monolayer/transducer orientation investigated with the present work (De Cock et al. 2015; Helfield et al. 2016; van Rooij et al. 2016).

Bubble destruction, gas diffusion, and movement were shown to be dependent on acoustic parameters and bubble concentration. The results from imaging the sonoporation process confirm and extend the findings from acoustic attenuation. The presence of microbubbles in a volume container affects both the pressure received as well as the acoustic environment itself. Pressure increase caused an increase in destruction area, which is intuitive given that a larger portion of a high pressure beam can still be above the microbubble destruction threshold. What is more surprising is that pressures as high as 1 MPa (MI of 1 at 1 MHz) at moderate microbubble concentrations still do not eliminate all microbubbles in the axial propagation path due to bubble-induced acoustic shadowing. This validates the observation shown in the attenuation experiments that high pressures still result in high attenuation. Pulse length (number of cycles) was also shown to impact the amount of bubble radiation force as well as bubble elimination that occurs following an ultrasound pulse. This can be

seen in both the high-resolution imaging with the iU22, where the 1 MPa pulse at 200 cycles results in an intense movement phenomenon (Fig. 6[a-d]), and in the high frame rate imaging where 1000 cycles causes a swirling motion to occur (Fig. 7[e-h]). This is consistent with prior findings that cycle lengths above 200 are more likely to push microbubbles (Mannaris and Averkiou 2012). Additionally, as shown in Fig. 7, a larger number of cycles resulted in a larger region of bubble elimination. This is explained by the fact that microbubble shell integrity is compromised in just a few cycles but gas diffusion and complete elimination of the microbubble lasts on the order of milliseconds. Therefore, the difference between smaller and larger cycles may be attributed to microbubble fragmentation during long cycle oscillations and thus accelerated gas diffusion. Finally, increasing the microbubble concentration not only increases the difficulty for sound to propagate through the volume, but also plays a role in bubble swirling and motion effects.

One additional observation seen from high frame rate imaging was the delineation between bubble destruction, gas diffusion, and bubble movement. As shown in Fig. 7, the initial bubble destruction occurs immediately, and is dependent on concentration, cycles, and pressure. However, this initial oscillation and destruction does not happen next to cells. It is clear from this result that bubble oscillation as the dominant form of cell permeation is not valid in this type of experimental setup. Rather, it is the subsequent bubble activity (diffusion and motion) due to a combination of a pressure gradient and inertial force that occurs within 100 ms of the dissolution of the microbubble shell (de Jong et al. 2009; Epstein and Plesset 1950) that reaches cells in some cases.

Finally, the effect of repeated pulses was examined. As shown in the high frame rate images, a single pulse results in microbubble destruction, gas diffusion, and propagation. When this is repeated, as in the scenario using repeated pulses (or even long cycles), this effect is enhanced, and the complex gas diffusion and motion results in a continually changing microbubble concentration through the axial propagation direction. Therefore, not only does the pressure received through a volume of microbubbles change, but so too does the microbubble concentration next to cells. The microbubble concentrations that were assumed to be the “effective” concentrations will be inaccurate when examined after a series of repeated ultrasound pulses. Interestingly, it was observed that the rate at which pulse repetition occurs also has an effect on overall microbubble movement when using transmit parameters that induce bubble movement (such as long pulses). If the pulse repetition frequency is high enough such that the medium does not have time to return to steady state, then sending repeated pulses limits the amount of bubble movement that occurs for each pulse.

Although consideration was taken to accurately emulate common *in vitro* experimental designs, given the wide parameter space, testing all possible ultrasound combinations remains an impossible task. An additional limitation to this study was that the orientation of the Clinicell parallel to the water-air interface for optimal imaging induced standing waves that were not fully representative of a single source excitation. Orienting the setup at an angle would have mediated this issue. That said, despite the limitations of parameter space and experimental setup, the outcomes and observations should remain universal: high microbubble concentrations are preferred for maximum probabilistic sonoporation efficacy,

but the presence of microbubbles creates sound attenuation and a wide variety of post-excitation events, which work to confound the overall acoustic environment. Perhaps the only truly accurate way to understand sonoporation mechanism is through single cell-single bubble interactions (Fan et al. 2012), as opposed to using macroscopic experiments to understand microscopic events. Additionally, while these experiments were not conducted on cell monolayers, the goal of this work was not to evaluate *in vitro* sonoporation, but rather to understand and hopefully elucidate why sonoporation on cell monolayers has been inconclusive despite wide research in the area.

Even though this work was done purely as a characterization of an *in vitro* system, there are important points that can be applied to *in vivo* ultrasound-mediated therapy as well. The phenomenon of excess microbubble attenuation and acoustic shadowing at high microbubble concentrations is still relevant in highly perfused areas, and could cause unwanted pressure reduction at areas of interest for therapy (Song et al. 2008). Further, while microbubble streaming, the dominant microbubble phenomenon seen in our work, is probably more associated with *in vitro* experiments, complex microbubble post-excitation activity may still happen in large vessels *in vivo*. Finally, as shown in this work, achieving maximal “bubble-to-cell” ratios is a complicated task; however, these concentrations would similarly never be reachable *in vivo*. It is possible that the true mechanism behind ultrasound-mediated drug delivery *in vivo* is less about probabilistic encounters of bubbles near cells but more about other, more difficult to emulate phenomena, such as vascular damage, hyperthermia, or immune modulation (Goertz et al. 2012; Holt and Roy 2001; Liu et al. 2012).

The true complexity of trying to evaluate sonoporation by considering single variables (such as concentration or pressure or cycles) is that in a 3D volume, all variables impact each other. Increasing pressure increases the amount of cavitation (stable/inertial), which increases attenuation. Higher numbers of cycles result in more destruction and movement. High concentrations result in attenuation, and maintaining a constant concentration can be difficult when using repeated pulses. Further, since sonoporation is dependent on the distance between microbubbles and cells, trying to elucidate mechanism in such a disperse and varied environment (both acoustically and physically) is an oversimplification of a complex process.

CONCLUSIONS

Through this work, we have characterized a setup commonly used to evaluate *in vitro* sonoporation. We have shown that the confounding microbubble activity during sonoporation in cell enclosures is quite complex and is a result of spatially and temporally varying acoustic fields and bubble concentrations. Specifically, bubble elimination in cell enclosures is impacted by pressure, pulse duration, bubble concentration, and pulse repetition, all of which are common parameters that have been explored in previous works. This complex environment and transient spatiotemporal bubble and pressure variations make it impossible to study sonoporation in cell enclosures in a standardized and reproducible way. It is fair to say that the actual pressure and concentration values in some of the previously reported works were quite possibly not what were originally intended. Perhaps the most important conclusion from this work is that unless studies look explicitly at single

bubble-single cell sonoporation, it is impossible to separate the wide variety of parameters into single variables. Pressure and concentration affect microbubble behavior, while microbubble behavior affects *in situ* pressure and concentration. Trying to form conclusions about macroscale sonoporation outcome will therefore be both varied and confounding.

ACKNOWLEDGEMENTS

This work was supported by a grant from the United States Department of Defense (CA160415/PRCRP). SBK was supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number TL1 TR002318. We gratefully acknowledge Bracco Suisse SA for the Sonovue used in this study.

REFERENCES

- Bekeredjian R, Chen S, Grayburn PA, Shohet RV. Augmentation of cardiac protein delivery using ultrasound targeted microbubble destruction. *Ultrasound Med Biol Elsevier*, 2005;31:687–91.
- De Cock I, Zagato E, Braeckmans K, Luan Y, de Jong N, De Smedt SC, Lentacker I. Ultrasound and microbubble mediated drug delivery: acoustic pressure as determinant for uptake via membrane pores or endocytosis. *J Control Release Elsevier B.V*, 2015;197:20–28.
- de Jong N, Emmer M, van Wamel A, Versluis M. Ultrasonic characterization of ultrasound contrast agents. *Med Biol Eng Comput Springer*, 2009;47:861–73.
- de Jong N, Hoff L, Skotland T, Bom N. Absorption and scatter of encapsulated gas filled microspheres: Theoretical considerations and some measurements. *Ultrasonics Elsevier*, 1992;30:95–103.
- Epstein PS, Plesset MS. On the Stability of Gas Bubbles in Liquid-Gas Solutions. *J Chem Phys* 1950;18:1505–1429.
- Fan Z, Chen D, Deng CX. Characterization of the dynamic activities of a population of microbubbles driven by pulsed ultrasound exposure in sonoporation. *Ultrasound Med Biol NIH Public Access*, 2014;40:1260–72.
- Fan Z, Liu H, Mayer M, Deng CX. Spatiotemporally controlled single cell sonoporation. *Proc Natl Acad Sci U S A National Academy of Sciences*, 2012;109:16486–91.
- Ferrara K, Pollard R, Borden M. Ultrasound Microbubble Contrast Agents: Fundamentals and Application to Gene and Drug Delivery. *Annu Rev Biomed Eng* 2007;9:415–47. [PubMed: 17651012]
- Goertz DE, Todorova M, Mortazavi O, Agache V, Chen B, Karshafian R, Hynynen K. Antitumor Effects of Combining Docetaxel (Taxotere) with the Antivascular Action of Ultrasound Stimulated Microbubbles. Woloschak GE, ed. *PLoS One Public Library of Science*, 2012;7:e52307.
- Guzmán HR, McNamara AJ, Nguyen DX, Prausnitz MR. Bioeffects caused by changes in acoustic cavitation bubble density and cell concentration: a unified explanation based on cell-to-bubble ratio and blast radius. *Ultrasound Med Biol Elsevier*, 2003;29:1211–1222.
- Helfield B, Chen X, Watkins SC, Villanueva FS. Biophysical insight into mechanisms of sonoporation. *Proc Natl Acad Sci* 2016;201606915.
- Holt RG, Roy RA. Measurements of bubble-enhanced heating from focused, mhz-frequency ultrasound in a tissue-mimicking material. *Ultrasound Med Biol Elsevier*, 2001;27:1399–1412.
- Hu Y, Wan JMF, Yu ACH. Membrane Perforation and Recovery Dynamics in Microbubble-Mediated Sonoporation. *Ultrasound Med Biol* 2013;39:2393–2405. [PubMed: 24063956]
- Hu Y, Wan JMF, Yu ACH. Membrane Perforation and Recovery Dynamics in Microbubble-Mediated Sonoporation. *Ultrasound Med Biol Elsevier*, 2018;39:2393–2405.
- Iwanaga K, Tominaga K, Yamamoto K, Habu M, Maeda H, Akifusa S, Tsujisawa T, Okinaga T, Fukuda J, Nishihara T. Local delivery system of cytotoxic agents to tumors by focused sonoporation. *Cancer Gene Ther Nature Publishing Group*, 2007;14:354–363.
- Karshafian R, Bevan PD, Williams R, Samac S, Burns PN. Sonoporation by Ultrasound-Activated Microbubble Contrast Agents: Effect of Acoustic Exposure Parameters on Cell Membrane Permeability and Cell Viability. *Ultrasound Med Biol Elsevier*, 2009;35:847–860.

- Kooiman K, Vos HJ, Versluis M, De Jong N. Acoustic behavior of microbubbles and implications for drug delivery. *Adv Drug Deliv Rev Elsevier B.V.*, 2014;72:28–48.
- Lentacker I, De Cock I, Deckers R, De Smedt SC, Moonen CTW. Understanding ultrasound induced sonoporation: Definitions and underlying mechanisms. *Adv Drug Deliv Rev Elsevier B.V.*, 2014;72:49–64.
- Liu H-L, Hsieh H-Y, Lu L-A, Kang C-W, Wu M-F, Lin C-Y. Low-pressure pulsed focused ultrasound with microbubbles promotes an anticancer immunological response. *J Transl Med BioMed Central*, 2012;10:221.
- Mannaris C, Averkiou MA. Investigation of Microbubble Response to Long Pulses Used in Ultrasound-Enhanced Drug Delivery. *Ultrasound Med Biol Elsevier*, 2012;38:681–691.
- Mitragotri S Healing sound: the use of ultrasound in drug delivery and other therapeutic applications. *Nat Rev Drug Discov Nature Publishing Group*, 2005;4:255–260.
- Park J, Fan Z, Deng CX. Effects of shear stress cultivation on cell membrane disruption and intracellular calcium concentration in sonoporation of endothelial cells. *J Biomech NIH Public Access*, 2011;44:164–9.
- Qin P, Han T, Yu ACH, Xu L. Mechanistic understanding the bioeffects of ultrasound-driven microbubbles to enhance macromolecule delivery. *J Control Release Elsevier*, 2018;272:169–181.
- Qin P, Xu L, Han T, Du L, Yu ACH. Effect of non-acoustic parameters on heterogeneous sonoporation mediated by single-pulse ultrasound and microbubbles. *Ultrason Sonochem Elsevier B.V.*, 2016;31:107–115.
- Qin S, Caskey CF, Ferrara KW. Ultrasound contrast microbubbles in imaging and therapy: physical principles and engineering. *Phys Med Biol NIH Public Access*, 2009;54:R27–57.
- Shapiro G, Wong AW, Bez M, Yang F, Tam S, Even L, Sheyn D, Ben-David S, Tawackoli W, Pelled G, Ferrara KW, Gazit D. Multiparameter evaluation of in vivo gene delivery using ultrasound-guided, microbubble-enhanced sonoporation. *J Control Release* 2016;223:157–164. [PubMed: 26682505]
- Song J, Klibanov AL, Hossack JA, Price RJ. Acoustic Attenuation by Contrast Agent Microbubbles in Superficial Tissue Markedly Diminishes Petechiae Bioeffects in Deep Tissue. *Invest Radiol* 2008;43:322–329. [PubMed: 18424953]
- Suzuki R, Oda Y, Utoguchi N, Maruyama K. Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles. *J Control Release Elsevier*, 2011;149:36–41.
- van Rooij T, Skachkov I, Beekers I, Lattwein KR, Voorneveld JD, Kokhuis TJA, Bera D, Luan Y, van der Steen AFW, de Jong N, Kooiman K. Viability of endothelial cells after ultrasound-mediated sonoporation: Influence of targeting, oscillation, and displacement of microbubbles. *J Control Release Elsevier B.V.*, 2016;238:197–211.
- Wang M, Zhang Y, Cai C, Tu J, Guo X, Zhang D. Sonoporation-induced cell membrane permeabilization and cytoskeleton disassembly at varied acoustic and microbubble-cell parameters. *Sci Rep Nature Publishing Group*, 2018;8:3885.
- Ward M, Wu J, Chiu J-F. Experimental study of the effects of optison® concentration on sonoporation in vitro. *Ultrasound Med Biol Elsevier*, 2000;26:1169–1175.

Figure 1:

Model of concentration and dispersity of microbubbles in a 3D volume. Each grid (not to scale) represents a microbubble, which are approximated to be 3 μm cubes, as measured with a Multisizer 3.

Figure 2:

Setup orientations of attenuation and imaging experiments. (a) is the vertical orientation used for acoustic attenuation. (b) is the horizontal orientation used for microbubble imaging. (c) represents where cells would be located in reference to therapy and imaging transducers.

Figure 3: Representation of bubble-to-cell ratio using (a) distance between microbubbles, measured using Equation 1 and (b) void fraction, measured using Equation 2. (c) shows the visualization of these microbubble concentrations in comparison with 15 μm diameter endothelial cells.

Figure 4:

Microbubble attenuation at concentrations ranging from 10,000 to 10,000,000 microbubbles per mL. The transmit pressures used are 125, 250, and 500 kPa (peak negative pressure). Shown in (a) are the received peak negative pressure amplitudes of pulses through a volume of custom microbubbles after a single transmit pulse and shown in (b) are the peak negative pressure amplitudes through a volume of Sonovue microbubbles after a single transmit pulse.

Figure 5:

Representative images of 20 cycle pulse excitations with varied parameters. (a-d) show the effect of sending 500 kPa pulses through 500k MBs/mL at a repetition frequency of 1 Hz. The first pulse was sent at time $t = 0$ s, the second pulse at $t = 1$ s, and so forth. Images were acquired the frame immediately after the pulse was sent except for (a) which was acquired immediately before the pulse. It takes 8 pulses for complete bubble elimination through the Clinicell. (e-h) show the effect of changing different parameters, specifically, (e) increasing PRF, (f) decreasing pressure, (g) increasing pressure, and (h) increasing concentration. Increasing PRF only changes the rate of bubble elimination (seen in [e] versus [d]), decreasing or increasing pressure causes a decrease or increase in bubble elimination accordingly, and increasing concentration reduces bubble elimination.

Figure 6:

Representative images of 200 cycle pulse excitations with varied parameters. (a-d) show the effect of sending 1 MPa pulses through 500k MBs/mL at a repetition frequency of 1 Hz. The first pulse was sent at time $t = 0$ s. (a) and (c) were the frames acquired immediately after the 1st and 2nd pulses, respectively, and show their immediate effect, while (b) and (d) show the evolution of movement resulting from these pulses. Yellow arrows indicate areas where movement had occurred. (e-h) show the effect of changing different parameters, specifically, (e) increasing PRF, (f,g) decreasing pressure, and (h) increasing concentration. Increasing PRF reduces bubble elimination due to movement (seen in [e] versus [d]), decreasing pressure decreases bubble elimination, and increasing concentration reduces bubble elimination and causes swirling to occur.

Figure 7:

Images during high frame rate acquisitions with a Verasonics 128 of microbubble activity caused with a single pulse excitation. The time $t = 0$ denotes the firing of the pulse. (a-e) show the effect of a 1 MPa, 200 cycle pulse through 500k MBs/mL, and (e-h) show the effect of a 1 MPa, 1000 cycle pulse through 5M MBs/mL. For each condition, the red arrows represent the extent of initial bubble destruction immediately following the end of the ultrasound pulse, while the yellow arrows represent the subsequent microbubble behavior independent of bubble oscillation. These images show the delineation between immediate bubble destruction and subsequent bubble movement.

Figure 8:

Images from an 8kHz acquisition taken after a 1 MPa, 20 cycle pulse through 500k MBs/mL. The time $t = 0$ denotes the firing of the pulse. There is no bubble destruction seen in (a), while a sizable void has developed in (b), implying that bubble elimination occurred via gas diffusion since no motion was observed.

Figure 9:

Representative waveforms and power spectra of received pulses after a 125 kPa, 20 cycle pulse through either water or bubbles (10M MBs/mL). (a) and (c) show the time waveforms, and (b) and (d) show the spectra of those pulses, after cropping and windowing.

Table 1.

Overview of Acoustic Parameters Used.

	Pressure (kPa)	Cycles	Pulse Repetition Frequency	Concentration (microbubbles/mL)
Attenuation	125, 250, 500	20	Single Pulse	10k – 10M
High-Resolution Imaging	200, 500, 1000	20, 200	1 Hz, 51 Hz	500k, 5M
High FR Imaging	1000	20, 200, 1000	Single Pulse	500k, 5M