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Characterization of the dynamic activities of a population of microbubbles driven by pulsed ultrasound exposures in sonoporation

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Abstract

Ultrasound driven microbubble activities have been exploited to transiently disrupt the cell membrane (sonoporation) for non-viral intracellular drug delivery and gene transfection both *in vivo* and *in vitro*. In this study, we investigated the dynamic behaviors of a population of microbubbles subjected to pulsed ultrasound exposures and their impact on adherent cells in terms of intracellular delivery and cell viability. By systematically analyzing the bubble activities at time scales relevant to pulsed ultrasound exposures, we identified two quantification parameters that categorized the diverse bubble activities subjected to various ultrasound conditions into three characteristic behaviors, i.e., stable cavitation/aggregation (Type I), growth/coalescence and translation (Type II), and localized inertial cavitation/collapse (Type III). Correlation of the bubble activities with sonoporation outcome suggested that Type III behavior resulted in intracellular delivery, while Type II behavior caused death of a large number of cells. These results provide useful insights for rational selection of ultrasound parameters to optimize outcomes of sonoporation and other applications that exploit the use of ultrasound-driven bubble activities.

Keywords

Sonoporation; Ultrasound; Microbubbles; Cavitation; Intracellular delivery; Acoustic radiation force; High speed videomicroscopy

INTRODUCTION

Due to the large acoustic impedance difference between the gas inside bubbles and the surrounding aqueous medium, a rich variety of microbubble phenomena can be generated by ultrasound application including cavitation (Deng and Lizzi 2002; Wu and Nyborg 2008). In stable cavitation, microbubbles expand and contract at the incident ultrasound frequency

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with small amplitudes around their equilibrium radius. In transient or inertial cavitation, ultrasound induces large expansion of bubble radius (to more than twice of the initial value) and rapid contraction or collapse dominated by inertia of the surrounding fluid, generating fluid microjets and shock waves during the destruction of bubbles (Chomas et al. 2001; Lauterborn and Ohl 1997; Miller and Thomas 1995; Wu and Nyborg 2008). Transfer of momentum from the pulsating or collapsing bubbles to surrounding medium also generates microstreaming of fluid around the bubbles (Collis et al. 2010; Elder 1959; Marmottant and Hilgenfeldt 2003; Tho et al. 2007).

Numerous studies have demonstrated that ultrasound application in the presence of microbubbles enhances intracellular uptake of membrane-impermeable therapeutic compounds (Delalande et al. 2010; Li et al. 2009; Tsunoda et al. 2005), through the membrane disruption generated by ultrasound-induced bubble activities (sonoporation) (Fan et al. 2010; Fan et al. 2012; Kudo et al. 2009; Prentice et al. 2005; Schlicher et al. 2006). Sonoporation has been exploited as a new strategy for non-viral intracellular drug delivery and gene transfection *in vivo* and *in vitro*.

The critical roles of ultrasound-induced microbubble activities in sonoporation have been recognized. Ultrafast optical imaging at frame rate above millions frames per second (Mfps) has revealed details regarding single bubble behaviors driven by a short (a few μ s) ultrasound pulse and their impacts on nearby cells, including fluid microjet penetrating cell membrane (Prentice et al. 2005) and compression on cells (van Wamel et al. 2006).

However, in practical sonoporation experiments (Juffermans et al. 2006; Juffermans et al. 2009; Meijering et al. 2007; Suzuki et al. 2008; van Wamel et al. 2006; Vandenbroucke et al. 2008), a large number of microbubbles and longer ultrasound exposures (e.g. 1 – 60 s) are usually used. Besides cavitation, the effect of the primary and secondary acoustic radiation forces on microbubbles can be significant under these conditions. The primary radiation force is a force associated with the incident ultrasound field (Dayton et al. 2002; Macedo and Yang 1973). The secondary acoustic radiation force (or the secondary Bjerknes force) (Crum 1975; Doinikov 2002) is the attracting or repelling force among bubbles associated with the scattered field of the primary ultrasound wave by the bubbles (Dayton et al. 1999a; Dayton et al. 1997; Feuillade 2001). The acoustic radiation forces can displace bubbles (Gessner et al. 2012; Rychak et al. 2007; Zhou et al. 2012) and lead to their aggregation (Dayton et al. 1999a; Dayton et al. 1997; Dayton et al. 1999b; Postema et al. 2004).

The behaviors of a population of microbubbles under the influence of pulsed ultrasound exposures have not been fully elicited or explored. Microbubbles not only respond to the incident ultrasound field but also interact with each other due to multiple scattering, leading to a variety of highly dynamic and complex activities that are difficult to measure and analyze. Consequently, optimization of sonoporation relies on relating post-sonoporation outcome with the ultrasound parameters, such as acoustic pressure, duty cycle, pulse repetition frequency (PRF), and the total exposure time (Meijering et al. 2007; Rahim et al. 2006), without the detailed knowledge and direct correlation of the ultrasound-induced activities of multiple microbubbles and their impact on cells. Due to the differences of

geometrical, physical and other factors associated with individual experiments, the observed specific association between ultrasound parameters and delivery outcome can be experiment-specific, thus may not be readily translatable, making comparisons between experiments difficult if not entirely impossible.

Another major challenge in studying the detailed ultrasound-induced microbubble activities is the uncertainty associated with spatial locations of microbubbles with respect to cells due to the intrinsic nature of bubbles, which can also lead to large variation in sonoporation outcome (Zhou et al. 2012). Although optical tweezer (Prentice et al. 2005) and a combined optical/acoustic technique (Zhou et al. 2012) have been used to control single bubble location, few techniques exist to control the spatial placement and cavitation of a large number of microbubbles in relation to cells. As shown in our previous studies (Fan et al. 2013; Fan et al. 2012), the use of targeted microbubbles that can be attached to adherent cell surfaces via specific ligand-receptor binding provides a way to control at least the initial positions of the bubbles with respect to cells. While the use of targeted bubbles has many advantages and utilities in sonoporation and ultrasound molecular imaging (Klibanov 2009), such approach involves expensive antibodies and reagents along with specialized microbubbles, and is not always attainable in practical applications.

In this study, we used an experimental configuration where non-targeted microbubbles were in close contact with cells in a monolayer without the use of antibodies or specialized modification of bubbles. We employed high speed videomicroscopy at a frame rate of 20,000 frames/s (fps) to capture the microbubble activities during pulsed ultrasound application. We quantitatively characterized the microbubble activities at time scales relevant to the pulsed ultrasound exposures and correlated these activities with ultrasound-mediated intracellular uptake and cell viability.

MATERIALS AND METHODS

Cell culture and microbubbles

Human umbilical vein endothelial cells (HUVECs) were cultured in flasks in complete human endothelial growth medium (Lonza CC-3124, Walkersville, MD) in a humidified incubator at 37°C and 5% CO₂. HUVEC were plated on the inside of one membrane of an OptiCell™ (Nunc, Rochester, NY), which consists of two 75 µm thick polystyrene membranes separated by 2 mm, and reached approximately 90% confluency at the time of experiment.

Definity® microbubbles (Lantheus Medical Imaging, Billerica, MA) are C3F8 gas bubbles each encapsulated by a phospholipid shell with a mean diameter in the range of 1.1 – 3.3 µm. After activation following the manufacturer's protocol, the microbubbles were mixed in complete culture medium at a final concentration of ~ 10⁷ bubble/ml. The culture medium in the OptiCell™ with HUVECs was replaced by the microbubble mixture right before experiments.

Experimental setup and ultrasound exposure

The Opticell™ containing HUVECs and bubbles was positioned on the microscope stage with the membrane with seeded cells on the top facing downward to allow microbubbles in solution to float upward to the adherent cells. A plastic fence-like structure was then securely attached on top of the Opticell™ to form a well to hold water for acoustic coupling (Figure 1A). A 1.25 MHz non-focused ultrasound transducer was positioned at a 45° angle with its active element immersed in water about 7 mm away from the cells on the membrane (Figure 1A). The transducer was driven by a function generator (Agilent Technologies 33250A, Palo Alto, CA) and a 75 W power amplifier (Amplifier Research 75A250, Souderton, PA) and was characterized in free field with a calibrated needle hydrophone with an active element of 40 μm (Precision Acoustics HPM04/1, UK). Ultrasound pulses with various parameters were used in the experiments including acoustic pressure from 0.06 MPa to 0.6 MPa, PRF from 10 Hz to 1 kHz, Duty cycle (DC) from 0.016% to 20%. The total duration of ultrasound application was 1 s.

Videomicroscopy of ultrasound induced dynamic activities of microbubbles

An ultrafast camera (Specialised Imaging Multi-Channel Framing Camera SIM02, Herfordshire, UK) at a frame rate of 2,000,000 fps and exposure time of 100 ns was used to image the expansion and contraction of individual microbubbles during an acoustic cycle of 1.25 MHz. To capture ultrasound induced microbubble activities during the whole duration of ultrasound application of 1 s in this study, a high-speed camera (Photron FASTCAM SA1, San Diego, CA) was used at a frame rate of 20,000 fps and exposure time of 50 μs.

From the high speed videomicroscopy images, we quantified the percentage change in the number of microbubbles as function of time, defined as the number of bubbles in each image frame divided by the number of bubbles prior to ultrasound application, to assess microbubble destruction induced by ultrasound application. We determined the translational movement of microbubbles by the distance of they moved from one image frame to the next.

We also examined the change in radius of a bubble after an ultrasound pulse (during the pulse-off period between pulses) as function of time to ascertain the change of microbubble property (existence of shell and gas dissolution) due to ultrasound application. The radius-time curve of a bubble was determined using a semi-automatic custom program based MATLAB (Mathworks, Natick, MA) to measure the bubble radius frame by frame consecutively. We fitted the radius-time curve using a gas dissolution model of an encapsulated bubble (Ferrara et al. 2007):

$$-\frac{dr}{dt} = \frac{L}{\frac{r}{D_\omega} + R_{shell}} \left(\frac{1 + \frac{2\sigma_{shell}}{P_a r} - f}{1 + \frac{3\sigma_{shell}}{4P_a r}} \right), \quad (1)$$

Where $r(t)$ is the bubble radius, L the Ostwald's coefficient, D_ω the gas diffusivity in water, R_{shell} the resistance of the shell to gas permeation, σ_{shell} the surface tension of the shell, P_a the hydrostatic pressure outside the bubble, and f the ratio of the gas concentration in the bulk medium versus that at saturation. The goodness of the fitting was evaluated using

$$R^2 = 1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i (y_i - \bar{y})^2}, \text{ where } y_i \text{ is the observed value, } f_i \text{ the associated modeled value, and } \bar{y} \text{ the mean value.}$$

Fluorescent imaging and sonoporation-mediated delivery outcome

Fluorescence imaging of cells was performed using a monochromator (DeltaRAMX™ PTI, Birmingham, NJ) to assess sonoporation delivery outcome. The emission was filtered by a dichroic light filter (Chroma 52002, Rockingham, VT) with passbands of green and red. The fluorescent images were acquired with a cooled CCD camera (Photometrics QuantEM, Tuscon, AZ).

Propidium iodide (PI) (Sigma Aldrich, St. Louis, MO), a cell membrane impermeable nucleic acid intercalating agent, was used to assess intracellular delivery by sonoporation. As PI only fluoresces at these wavelengths when inside the cell, it provides a good real-time marker for delivery via membrane disruption using fluorescence imaging. While pronounced increase in PI fluorescence is often used to indicate cell death, here transient increases in PI fluorescence were used as a marker for sonoporation and intracellular delivery. Dissolved in extracellular medium at a final concentration of 100 μ M, PI entering the sonoporated cells was detected within the cells by fluorescence emission at 610 nm with excitation at 539 nm.

Calcein AM assay was used to assess cell viability. Calcein AM, a cell-permeable, non-fluorescent compound, was dissolved in extracellular medium (final concentration 1 μ M) before ultrasound. Calcein AM is converted to green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases, and detected at 520 nm with excitation at 488 nm. After ultrasound exposure, sonoporation of the cells resulted in increase of PI fluorescence (red) and leakage of small amount of calcein before the resealing of the membrane disruption. Since the duration and size of the membrane disruption or pore is transient and small, the calcein fluorescence signal in cells will exhibit hardly any change. Thus co-localization of PI and calcein fluorescence indicate viable cells with delivery. In contrast, if a cell was permanently damaged due to irreversible/significant membrane disruption, complete leakage of calcein would occur with pronounced PI fluorescence after 3 min after ultrasound application. The duration of 3 minutes was set empirically based on the observation that both red and green fluorescence intensities became stable.

Cell viability is defined as the percentage of calcein positive cells after ultrasound application, calculated as the number of calcein positive cells after ultrasound application divided the total number of cells in an image. The percentage of both PI and calcein positive cells (PI uptake and surviving cells) after ultrasound application relative to the total number of cells was defined as the delivery efficiency.

From our real time imaging at high frame rate of cells and microbubbles during the entire process of ultrasound application and post ultrasound assessment, we tracked all the cells in the field of view to account for them in the calculation of delivery and viability.

RESULTS

The use of Opticell™ in our experiment configuration (Figure 1A) ensured that the microbubbles were in the same 2D plane with the cells during ultrasound application. With a concentration of 10^7 bubbles/ml in the bulk solution, the stabilized bubble-cell ratio in 2D before ultrasound application was found to be 8.2 ± 1.8 bubbles/cell for all experiments ($n = 50$) in this study after the bubbles floated up and settled stably against the cell monolayer by buoyancy (Figure 1B).

We conducted experiments and systematically examined the responses of microbubbles to pulsed ultrasound exposures with various parameters (acoustic pressure from 0.06 MPa to 0.6 MPa, PRF from 10 Hz to 1 KHz, DC from 0.016% to 20%, total exposure 1 s). Despite the various ultrasound exposure conditions, we observed that by quantifying the bubble activities using bubble destruction and bubble translation, the seemingly diverse and highly dynamic bubble activities could be categorized into three characteristic behaviors with distinctly different key features.

Stable cavitation and aggregation of microbubbles (Type I) corresponding to high cell viability

Characteristic Type I microbubble behavior is the stable cavitation and aggregation of microbubbles with no or little bubble destruction, which commonly occurred when low acoustic pressures and long pulse durations were used. In these cases, as shown by the example in Figure 2 and supplemental video 1 (acoustic pressure of 0.06 MPa, duty cycle of 20%, PRF of 20 Hz corresponding to a pulse duration of 10 ms), microbubbles were not destructed during ultrasound pulses and cells remained intact (no PI uptake) and viable (green fluorescence signal of calcein).

In addition, the microbubbles often compressed on the nearby cells in a repeated fashion corresponding to the PRF of the pulsed ultrasound exposure (yellow arrow in Figure 2A), due to the influence of the primary acoustic radiation force on the bubbles (Zhou et al. 2012). The compression impact in these cases with low acoustic pressures was usually insufficient to generate sonoporation or PI uptake in the cells, which remained viable after ultrasound application, as indicated by calcein retention (Figure 2A).

Occurrence of stable cavitation of the bubbles was clearly indicated by the circulatory motion of the surrounding smaller bubbles, indicating the fluid flow (microstreaming) generated by the cavitation of the bubble of interest (Figure 2B and supplementary video 1). Similarly as in particle imaging of velocity (PIV), motion of the satellite bubbles was used to estimate the peak shear stress (14.61 ± 2.28 Pa, $n = 10$), which was insufficient to generate sonoporation in these cases as expected, although this estimated value may overestimated the microstreaming generated shear stress because of the contribution of the attractive secondary Bjerknes forces on the approaching velocity of the satellite bubbles toward the bubbles of interest.

The ultrasound pulses also caused the initially separated microbubbles to aggregate when they attracted and moved toward each other to form islands of bubble aggregates (yellow

and blue circles and arrows in Figure 2C) due to the secondary Bjerknes forces among the bubbles. The aggregates further attracted each other as ultrasound application continued, forming even larger aggregates (red and white arrows in Figure 2C) due to the increased attracting forces.

In summary, the Type I microbubble activities generated by ultrasound pulses with low pressure amplitudes caused no or little destruction of microbubbles (Figure 2D) and led to no PI uptake or cell death.

Growth/coalescence and translation of microbubbles (Type II) resulting in cell death

When higher acoustic pressure amplitudes were used (e.g. 0.43 MPa) while keeping the duty cycle (20%) and PRF (20 Hz) the same as the previous example in Figure 1, a distinctively different bubble behavior emerged, which was dominated by significant growth/coalescence of microbubbles and large translational movement of the coalesced bubbles, regarded as Type II behavior (Figure 3A and supplementary video 2).

In these cases, bubbles exhibited large scale aggregation rapidly and coalesced almost immediately after the application of the ultrasound pulses, growing and forming large bubbles during the first pulse (10 ms) (Figure 3B). The coalesced bubbles continued to grow by attracting/fusing with more bubbles and by rectified diffusion, reaching a diameter up to 30 μm (Figure 3C). In the meantime, the large bubbles rapidly moved large distances, destroying the cells in their paths. Not surprisingly, the number of bubbles rapidly decreased in these cases immediately after ultrasound application, and almost all bubbles were destroyed after the first pulse (~ 10 ms) in these cases (Figure 3D).

Localized collapse of microbubbles (Type III) leading to intracellular delivery

Compared to the example in Figure 3, ultrasound exposures with the same high acoustic pressure (e.g. 0.43 MPa) but a reduced duty cycle (or pulse duration) to just several microsecond led to another different characteristic type of bubble behavior. As shown in Figure 4 and supplemental video 3, with high acoustic pressure and short pulse duration (e.g. 0.43 MPa, PRF 20 Hz, duty cycle 0.016% corresponding to pulse duration of 8 μs), the number of bubbles and the size of bubbles rapidly reduced with minimal translational movement (Figure 4, supplementary video 3). During the short pulse duration (8 μs), the bubbles did not gain sufficient momentum to move before collapse, exhibiting a bubble behavior characterized by localized collapse (inertial cavitation) with limited translational movement, regarded as Type III behavior, which led to intracellular PI delivery with high cell viability (Figure 4A). In these cases, inertial cavitation due to the high acoustic pressure was indicated by the clear decreases of the number and size of the bubbles in correspondence with the application of each ultrasound pulses (Figures 4B and 4C).

Ultrasound induced change in the encapsulated microbubbles

As encapsulated microbubbles were used in this study, we examined how these microbubbles changed after pulsed ultrasound application by analyzing the bubbles exhibiting Type III behavior in more detail.

As shown in Figure 5A, inertial cavitation of the microbubbles in Type III behavior was confirmed by ultrafast imaging at 2 Mfps, where the microbubble indeed expanded to more than twice of its equilibrium radius and contracted significantly during the acoustic cycle of 1.25 MHz. The decrease of bubble equilibrium radius during the whole duration of the pulsed ultrasound application was captured by high speed imaging at 20 kfps (Figures 5B and 5C). A marked decrease in bubble radius ($0.46 \pm 0.13 \mu\text{m}$, $n = 10$) was generated immediately after the short ultrasound pulse, due to inertial cavitation (arrow around 8 μs in Figure 5C). The bubble continued on to shrink gradually during the pulse-off period before the next ultrasound pulse. Fitting of the radius-time curve during the pulse-off period after the first pulse ($0^+ \text{ s} - 0.05 \text{ s}$ in Figure 5C) with the bubble dissolution model in Eq. (1) indicated that the bubble dissolution behavior after the first pulse resembled that of perfluorocarbon gas bubbles with an intact lipid shell. The second ultrasound pulse caused another steep drop in bubble radius ($0.80 \pm 0.10 \mu\text{m}$, $n = 10$) (arrow at 0.05 s in Figure 5C) before undergoing additional shrinkage during the pulse-off period. The rate of bubble radius reduction was higher, and model fitting of the radius-time curve (from 0.05 – 0.1 s in Figure 5C) suggested that the bubble dissolution was represented by bubbles composed of $90 \pm 5\%$ perfluorocarbon mixed with $10 \pm 5\%$ air without a shell ($R^2 = 0.85 \pm 0.07$).

The faster rate of shrinkage indicated a disruption of the encapsulating shells of the bubbles. After the lipid shell was destroyed or no longer existed, bubble dissolution increased due to the effect of surface tension and the presence of the more diffusive air in the bubble. Eventually, the bubbles reduced significantly in size and application of additional ultrasound pulses was no longer able to initiate cavitation of these smaller bubbles due to higher cavitation threshold (Holland and Apfel 1990), rendering them ineffective for sonoporation.

Correlation of dynamic bubble activities with sonoporation outcome

We systematically analyzed that the ultrasound-induced microbubble activities and found that two quantification parameters, i.e., the percentage of bubble destruction and the averaged initial displacement of bubbles (evaluated from the first and second images, recorded at 20 kfps, after the onset of ultrasound application), categorized the ultrasound-induced dynamic activities of a population of microbubbles. Although a variety of ultrasound parameters were applied and a large population of bubbles were involved (e.g. around 300 bubbles in an image), the percentage of destruction and displacement of bubbles separated the bubble behaviors into three separate groups. As indicated by the three boxes in Figure 6A, the experiments in which bubbles exhibited stable cavitation (Type I) clustered in Box 1, with little changes in the number of bubbles and small displacements. Box 2 enclosed experiments where bubbles exhibited Type II behavior featuring large translational movement and significant decrease in the number of bubbles due to bubble coalescence. Box 3 included experiments where bubbles exhibited Type III behavior with localized destruction of microbubbles with minimal translational movement.

The corresponding sonoporation delivery outcome (delivery efficiency and cell viability) resulted from these experiments also clustered into three separate groups indicated by the Boxes in Figure 6B. Corresponding to the experiments in Box 3 in Figure 6A (Type III behavior), for which more than 85% bubbles were destructed, a delivery efficiency of $32.0 \pm$

9.5% was generated with a $84.9 \pm 6.8\%$ viability ($n = 19$, Box 3 in Figure 6B). The bubbles that exhibited significant coalescence/growth and large initial translational movement ($28.9 \pm 9.3 \mu\text{m}$, $n = 18$) (Box 2 in Figures 6A) (Type II behavior) caused cell death in more than 70% of cells (Box 2 in Figure 6B). Comparison of the delivery outcomes indicates that Type III behavior with rapid destruction of microbubbles with limited translational movement was most preferable for intracellular delivery.

DISCUSSION

The significance of acoustic cavitation and ultrasound driven bubble activities in a wide range of applications has stimulated extensive studies of these remarkable phenomena. In this study, we investigated the dynamic behaviors of a population of non-targeted microbubbles under the influence of pulsed ultrasound exposures, a situation that is more representative of those in practical sonoporation experiments. We identified two quantification parameters that categorized the bubble activities into three types of characteristic behaviors, and by correlating with sonoporation delivery outcome, determined the preferable microbubble activities for sonoporation mediated delivery. These results provide a systematic description of the dynamic responses of non-targeted microbubbles in a population driven by pulsed ultrasound application that may have important implications for sonoporation and other applications involving ultrasound microbubble interaction. Below we discuss the findings and limitations of this study.

Imaging ultrasound-induced activities of bubbles in a large population over a long duration

Practical sonoporation studies for drug and gene delivery often involve many microbubbles under the influence of ultrasound exposure lasting for multiple seconds or even minutes. In these cases, the dynamic activities of multiple bubbles will be compounded by both the features of individual bubble dynamics and the interactions among the bubbles, resulting in a variety of highly dynamic and complex behaviors with multiple time scales spanning several orders of magnitude. Consequently, investigating of such bubble dynamic behaviors is challenging.

Ultrafast imaging with frame rate above several Mfps provides a necessary means for direct observation of the dynamics of isolated single bubbles at nanosecond time scale during several μs (Postema et al. 2004; Prentice et al. 2005), but with limited number of frames it is unable to capture the activities of many bubbles during longer ultrasound exposures. In this study, we employed high speed videomicroscopy at 20 kfps to record the activities of hundreds of bubbles during 1 s of pulsed ultrasound exposure. Although imaging at this frame rate did not resolve the actual bubble expansion/contraction or collapse during an acoustic cycle at 1.25 MHz, it permitted recording of the activities of multiple bubbles during a longer time duration. Frame rate of 20 kfps provided sufficient temporal resolution for imaging microbubble activities with a time scale in the same order of the pulse repetition periods (1/PRF) (10 – 100 ms) used in this study. Although longer durations (e.g. >10 s) have been used in sonoporation applications, recording of a duration of 1 s sufficiently

captured the key dynamic features of microbubble activities induced by pulsed ultrasound exposures with PRFs relevant for typical sonoporation studies.

Experimental setup for *in vitro* sonoporation studies

Typical *in vitro* sonoporation studies were conducted in standard cell culture dishes where adherent cells on the dish bottom or suspended cells were mixed with microbubbles in the bulk solution (Karshafian et al. 2010; Mehier-Humbert et al. 2005; Schlicher et al. 2006). In such situations, free floating bubbles in the solution are not consistently in close contact with cells during ultrasound exposures. The uncertainty due to the inherent nature of gaseous bubbles makes it difficult for studying bubble dynamic activities and obtaining consistent outcome.

In this study, we used Opticell™ to achieve an experimental environment where non-targeted microbubbles stayed in close contact with the adherent cells in a 2D monolayer without use of ligands or antibodies or other modification of microbubbles. By placing the Opticell™ with seeded cells on top facing downward, the bubbles were in stable contact with the cells on the inside of the membrane above. Similarly, due to buoyancy of the bubbles, their movements were essentially confined within the 2D plane with the cells on the membrane. The restriction in bubble movement in the vertical direction made it possible for microscopic imaging of both cells and microbubble activities during the whole duration of ultrasound application, enabling correlation of microbubble activities with sonoporation delivery outcome of a population of bubbles and cells.

The acoustic transparent membranes of the Opticell™ and our experimental configuration with a water coupling chamber and a 45° incident angle of ultrasound application (Figure 1A) avoided reflections and standing wave formation from boundaries of a typical plastic cell culture dish, thus simplified the ultrasound field experienced by the bubbles and cells. Unlike cells on the bottom of a cell culture dish, the cells and bubbles in this study were not located on a highly reflective boundary. While our experimental setup may serve as a good configuration for adherent cells *in vitro* and even represent some *in vivo* settings, *e.g.*, microbubbles in vessels close to endothelial cell layers, effects of boundaries may not be completely eliminated due to the presence of air/liquid boundaries enclosing a finite volume of liquid.

Parameters of pulsed ultrasound exposures

There exists a large parameter space associated with pulsed ultrasound exposures with multiple parameters including acoustic pressure, PRF, pulse duration (or duty cycle). Furthermore, the initial concentration of bubbles or the bubble cell ratio is also expected to play important roles in the dynamic interplay of parameters of pulsed ultrasound exposures in governing the behaviors of the bubble population (Figure 7). As the spatial distances among bubbles will significantly affect the interactions associated with the secondary Bjerknes force (Crum 1975; Doinikov 2002), which increases inversely with the square of the distance, increase in the bubble concentration will reduce the acoustic pressure amplitude for bubble coalescence/growth (Type II) and vice versa.

Although it might be desirable to conduct experiments to cover the complete parameter space, it is not practically achievable because quantitative analysis of the microbubble activities (with sufficient temporal resolution for many bubbles during a long duration) is enormously time-consuming. In this study, we used representative parameters for pulsed ultrasound exposures that are in the similar ranges of those used in many reported sonoporation studies. By systematically analyzing the bubble activities, we identified three characteristic bubble behaviors (Type I, Type II, and Type III) based on two quantification parameters (bubble destruction and translation movement) that described the key features and roles of microbubble behaviors in sonoporation driven by pulsed ultrasound exposures. The percentage of bubble destruction is clearly the consequence of inertial cavitation, typically occurring at high enough acoustic pressure above the cavitation threshold, generating intracellular delivery. The significant translational movement of bubbles, especially the large bubbles formed by growth/coalescence after aggregation, a main factors that caused cell death, is the result of the work done by the acoustic radiation forces, which are dependent on the acoustic pressure and the size of the bubbles. While the dynamic interplay of multiple ultrasound parameters resulted in complex phenomena (Figure 7), the two quantification parameters we identified represent key aspects of the fundamental physical principles of ultrasound interaction with multiple bubbles. As such, they served well as two orthogonal metrics for characterizing ultrasound induced behaviors of a population of microbubbles in this study.

Localized collapse of microbubbles (Type III) led to intracellular delivery and cell survival

Conventional approach for optimizing ultrasound parameters for sonoporation relies on relating ultrasound parameters with post-ultrasound assay of outcome, usually without information of the detailed activities of microbubbles and their impact on cells. Our results from this study revealed explicitly, by correlating the detailed microbubble behaviors with sonoporation outcome, that localized inertial cavitation, typically generated by short ultrasound pulses with high pressures, generated the highest delivery efficiency and cell viability, compared to other two types of behaviors, although no effort was placed in this study to further improve the delivery efficiency.

Rahim et al (Rahim et al. 2006) found that the optimal ultrasound parameters for gene transfection were peak negative pressure of 0.25 MPa, PRF of 1 kHz, and pulse duration of 40 μ s (40 cycles/pulse at 1 MHz). Phillips et al (Phillips et al. 2010) also reported that ultrasound pulses with 0.3 MPa acoustic pressure, 100 Hz PRF, and pulse duration of 50 μ s (50 cycles/pulse at 1 MHz) performed the best for gene transfection. Based on our findings in this study, it may be postulated that it is highly likely that the above two studies, which used short pulse durations and high acoustic pressures, involved microbubble activities that were inertial cavitation in nature (Type III). Therefore results of this study may provide useful guidance for study design to improve sonoporation outcome and for comparison of experimental results and observations.

Dynamic interplay of pulsed ultrasound parameters in microbubble behaviors

We show in this study that microbubble activities driven by pulsed ultrasound exposures can be described by three characteristic types of behaviors. For example, as shown in Figure 6A,

at a fixed PRF of 20 Hz, very low acoustic pressures (e.g. 0.06 – 0.12 MPa) generated primarily Type I behavior (stable cavitation) of microbubbles regardless of duty cycle (e.g. 0.016% – 20%). However, at high acoustic pressures (e.g. > 0.42 MPa), microbubble behaviors are sensitive to duty cycles. For example, microbubbles exhibited Type III behavior at low duty cycle of 0.016%, but with increased duty cycle to 20%, Type II behavior emerged. At low duty cycle of 0.016%, low acoustic pressure pulses (e.g. < 0.12 MPa) generated Type I behavior while high acoustic pressures (e.g. 0.4 MPa) generated Type II behavior at the same PRF of 20 Hz.

Although experiments in this study were performed with selected ultrasound parameters, our experimental results nevertheless revealed the general trends regarding how combination of ultrasound pulse parameters determined microbubble behaviors, as summarized in Figure 7. Three zones of duty cycle and acoustic pressure of pulsed ultrasound (at fixed PRF) are indicated in the parameter map where Type I, II, and III behaviors are likely to occur. For example, very low acoustic pressures will primarily generate Type I behavior for a relatively large range of duty cycle. Transition of the bubble behavior from Type I to Type II occurs with increasing acoustic pressures at high duty cycles. On the other hand, at low duty cycle, low acoustic pressures will induce Type I behavior while high acoustic pressure will induce Type III behavior. Type III behaviors occurs at high acoustic pressures and low duty cycles, and increasing the duty cycle will transition the bubble behavior from Type III to Type II.

For illustration, Figure 7 only includes acoustic pressure and duty cycle (or pulse duration) at a given PRF to illustrate their interplay in governing the characteristics of microbubble activities. Change of PRF will alter the exact divisions of the parameter regions for different characteristic behaviors, but the basic principles of ultrasound interaction with multiple bubbles remain valid.

It should be noted that transitioning from one type of bubble behavior to another is not a sudden change. The shadowed dividing lines separating different types of bubble behaviors in Figure 7 are meant to indicate the existence of transition regions. Depending on ultrasound parameters, the bubble activities can be a mixture of two or three characteristic types. It should also be noted that there are no intrinsically defined quantitative values for the two parameters to separate the three characteristic types of bubble behaviors in the continuous 2D parameter space.

Spatial locations of microbubbles with respect to cells

The physical and geometrical features in different experimental configurations introduce variations in the actual ultrasound field experienced by bubbles and cells in experiments due to acoustic transmission and reflections from boundaries. Thus, the specific relationship of ultrasound parameters with microbubble behaviors may well be different even with the same input ultrasound parameters. Furthermore, differences in the spatial locations of microbubbles with respect to cells in different experimental setups may lead to additional variations in ultrasound-induced bubble activities and sonoporation outcome.

Similarly as in our previous study on targeted microbubbles that were bound to the cell membrane (Fan et al. 2013), here we identified three characteristic behaviors of non-targeted

bubbles in this study that share some common features with the initially cell-bound bubbles but also have important differences.

Firstly, due to the covalent binding of the targeted bubbles with cells, ultrasound-induced movement of targeted bubbles were restricted especially at low acoustic pressures. Unlike non-targeted bubbles in this study (Type I), no aggregation of targeted bubbles was observed at low acoustic pressures where stable cavitation dominated.

Secondly, compared to the non-targeted bubbles exhibiting Type II behaviors, coalescence/growth of the initially anchored bubbles occurred at much higher acoustic pressures after the bonds of bubbles to cells were broken by the acoustic radiation forces. The translational movement of these initially bond bubbles was also less, with the total displacement of bubbles during the whole ultrasound application duration (1 s) less than the translational movement of non-targeted bubbles during the first several ms. In addition, we found that different quantification parameters were need to better characterize the differences in microbubble behaviors of targeted and non-targeted microbubbles. In this study, we identified the percentage of bubble destruction and the initial translational movement that best characterized the non-targeted bubbles, compared to bubble radius reduction and the total (accumulative) translational displacement during the whole ultrasound application for characterizing targeted bubbles.

Thirdly, although localized inertial cavitation of targeted microbubbles and non-targeted microbubbles (Type III) generated similar delivery efficiency (~ 33%), targeted bubbles induced much higher rate of cell death of ~50%, while non-targeted bubbles induced < 10% cell death, maintaining cell viability above 90%. Furthermore, it was recognized that for targeted bubbles, only a few and even 1 short pulse were needed to induce inertial cavitation of the targeted bubbles to generate sonoporation without killing cells, exerting higher impact to cells than non-targeted bubbles, likely due to the closer positions with cells.

The specific relationship of ultrasound parameters with non-targeted microbubble activities obtained in this study may not exactly translate to different settings. Nevertheless, the observed characteristic microbubble behaviors should represent the general characteristics of the microbubble dynamic activities induced by pulsed ultrasound exposures, as they are ultimately governed by the physical principles of ultrasound interaction with multiple bubbles. The concept that microbubble behaviors are directly responsible for sonoporation outcome should be kept in mind when comparing experiments in addition to the input values of ultrasound parameters.

Applications of dynamic microbubble activities driven by pulsed ultrasound

Although some characteristic bubble activities, such as aggregation, stable cavitation and associated micro-streaming, as well as translational movement, are not necessarily productive in generating intracellular delivery, these activities may have utilities or implications for other biomedical ultrasound applications as briefly summarized below. Detailed understanding of ultrasound-bubble interaction obtained from this current study may be beneficial for these diverse applications exploiting ultrasound-induced bubble activities.

Microfoam formation in capillary due to bubble aggregation has been suggested to be useful for block the entire vessels for beneficial purposes (Kotopoulos and Postema 2010). The formation of aggregated bubbles, dependent on ultrasound condition and exposure time, has also been exploited as a strategy to increase local bubble concentration in blood flow for diagnostic ultrasound imaging (Koda et al. 2011). The primary radiation force has been used to move targeted microbubbles towards a vessel wall to increase their binding efficiency with cell surface receptors for ultrasonic molecular imaging (Gessner et al. 2012; Patil et al. 2011; Rychak et al. 2007). At very low acoustic pressure (e.g. 0.1 MPa), stable cavitation was demonstrated to increase cellular calcium ion permeability, intracellular H₂O₂ level, protein nitrosylation, and rearrangement of F-actin cytoskeleton for potential application in control of gene expression and triggering vascular angiogenesis (Juffermans et al. 2009). Microstreaming produced by microbubble cavitation was suggested to drive fibrinolytic agents into the structure of a thrombus, thus increasing the bioavailability of the thrombolytic drug (Tachibana and Tachibana 1995). Shear stress generated by microstreaming was proposed to be a mechanism involved in ultrasound mediated opening of the blood-brain barrier (McDannold et al. 2008).

CONCLUSION

In this study, we investigated the detailed dynamic behaviors of microbubbles in a large population exposed to pulsed ultrasound exposures with parameters relevant to sonoporation. Systematic analysis of microbubble activities led to identification of two quantification parameters that characterized a variety of bubble activities into three characteristic types of behaviors, including stable cavitation and aggregation (Type I), large growth/coalescence and translational movement (Type II), and localized inertial cavitation or bubble destruction (Type III). Correlation of the characteristic behaviors with sonoporation outcome demonstrated localized inertial cavitation the most preferable for sonoporation mediated delivery, while large translation of coalesced bubbles caused cell death. These results provide useful insights for guiding sonoporation or other applications employing pulsed ultrasound exposures in the presence of multiple microbubbles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic illustration of experiment setup. HUVEC cells were cultured on one of the membrane of the Opticell, which was placed with cells facing downward to allow the microbubbles in the medium to float up to be near the cells. Ultrasound-induced bubble dynamic activities were captured by bright field videomicroscopy, while sonoporation delivery outcome was assessed by fluorescence imaging. (B) A typical bright field image showing the cells with the microbubbles before ultrasound application.

Scale bar = 50 μm .

Figure 2.

Type I behavior of bubbles featuring stable cavitation and aggregation of microbubbles. (A) Selective images showing stable cavitation of microbubble with some bubbles exerting repeated compression to the nearby cell (yellow arrow). No PI uptake was detected and cell viability was indicated by calcein retention. Application of the pulsed ultrasound exposure was shown above the images. The dashed lines indicate the times for the selected bright field images. (B) Microstreaming around a cavitating bubble was indicated by the motion of sounding smaller bubbles. (C) Microbubbles aggregated into bubble clusters (yellow and blue arrows) which continued to grow to form large clusters (red and white arrows). The large bubble aggregates exhibited significant translational movement across the cells (white arrow). (D) Normalized total number of bubbles as function of time ($n = 6$). The ultrasound pulses had acoustic pressure of 0.06 MPa, 20% duty cycle, 20Hz PRF, and 1 s of total duration.

Figure 3.

Type II of bubble dynamic behavior identified by significant growth, coalescence, and rapid translational movement. (A) Selective images showing that coalesced bubbles were formed and rapidly moved across over the cells, destroying large number of cells, as indicated by strong PI fluorescence signal and absence of calcein inside the cells. A schematic illustration of the pulsed ultrasound exposure was shown above the images. The dashed lines indicate the time points for selected bright field images. (B) Aggregation and coalescence of bubbles accompanied by translational movement. (C) Large bubbles formed by coalescence moved across over cells. (D) Normalized total number of bubbles as function of time ($n = 6$). The parameters for the pulsed ultrasound exposure were 0.43 MPa acoustic pressure, 20% duty cycle, 20Hz PRF, and 1 s total duration.

Figure 4.

Type III characteristic bubble dynamics dominated by localized inertial cavitation. (A) Selective images showing that bubble size reduced in size and number corresponding to each ultrasound pulse. Sonoporation was generated as indicated by PI uptake in cells. Cell viability was indicated by calcein retention. A schematic illustration of the pulsed ultrasound exposure was plotted above the image. The dash lines indicate the time points when the selected bright field images were acquired. (B) Normalized total number of bubbles as function of time ($n = 6$). (C) Averaged radius-time curve of selected bubbles with initial radius of $2.5 \pm 0.1 \mu\text{m}$ ($n = 22$). The ultrasound pulses applied had acoustic pressure of 0.43 MPa, PRF of 20 Hz, duty cycle of 0.016% corresponding to pulse duration of $8\mu\text{s}$, and total duration of 1 s.

Figure 5.

Change of microbubble size due to pulsed ultrasound application. (A) Ultrafast imaging of a bubble undergoing inertial cavitation. The scale bar is 10 μm . (B) Selected time-lapse bright field images (at 20,000 fps) of a bubble with an initial radius of 2.4 μm . (C) Radius of microbubble as function of time and fitting with a gas dissolution model. Ultrasound was applied at time 0, with acoustic pressure of 0.43 MPa, PRF of 20 Hz, duty cycle of 0.016% (or pulse duration of 8 μs), total duration of 1 s.

Figure 6.

Characteristic bubble behaviors induced by pulsed ultrasound exposures. (A) Scatter plot of 61 experiments clustered in three boxes (Box 1, 2 and 3) based on bubble activities characterized by quantification parameters of bubble destruction and initial translation of bubbles. Fifteen combination of different ultrasound parameters. Different color indicates different ultrasound parameter combinations. For each combination, 3 – 6 experiments were included. (B) Corresponding delivery outcomes of the experiments in (A) also clustered into three boxes (Box 1, 2, and 3). (C and D) Viability and delivery efficiency resulted from the three types of bubble behaviors (n = 24, 19 and 18 for each type) compared with controls.

Figure 7.

Schematic illustration of the interplay of ultrasound parameters (acoustic pressure and duty cycle) in determining microbubble dynamic behaviors. Transition of bubble behavior from one characteristic type to another dependent on ultrasound parameters.