The influence of distance between microbubbles on the fluid flow produced during ultrasound exposure

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The collapse dynamics of lipid monolayer-coated microbubbles in the clinically-relevant size range under $6\text{ }\mu\text{m}$ in diameter have not been studied directly due to their small size obscuring the collapse visualization. This study investigates the influence of inter-microbubble distance on the shape of lipid debris clouds created by the collapse of the microbubble destroying the microbubble lipid monolayer. The shape was highly influenced by the fluid motion that occurred as the microbubbles collapsed. It was observed that at inter-microbubble distances smaller than $37\text{ }\mu\text{m}$ the microbubbles began to interact with one another resulting in distorted and ellipsoid-shaped debris clouds. At inter-microbubble distances less than $10\text{ }\mu\text{m}$, significantly elongated debris clouds were observed that extended out from the original microbubble location in a single direction. These distortions show a significant distance-dependent interaction between microbubbles. It was observed that microbubbles in physical contact with one another behaved in the same manner as separate microbubbles less than $10\text{ }\mu\text{m}$ apart creating significantly elongated debris clouds. It can be hypothesized that small inter-microbubble distances influence the microbubble to collapse asymmetrically resulting in the creation of fluid jets that contribute to the formation of debris fields that are elongated in a single direction. © 2014 Acoustical Society of America.

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I. INTRODUCTION

The ability of lipid-coated microbubbles to undergo inertial cavitation when exposed to focused ultrasound has made them important particles for use in a variety of therapeutic medical applications including drug delivery (Ibsen *et al.*, 2013b; Kheirrolomoom *et al.*, 2007; Klibanov, 2006; Lentacker *et al.*, 2006; Liu *et al.*, 2006; Unger *et al.*, 1998), histotripsy and high intensity focused ultrasound (Roberts *et al.*, 2006; Yu *et al.*, 2008), and enhancing drug extravasation from blood vessels by facilitating microcapillary damage (Bohmer *et al.*, 2010; Caskey *et al.*, 2011; Skyba *et al.*, 1998; and Stieger *et al.*, 2007). Studying how the ultrasound-induced collapse of microbubbles is influenced by nearby surfaces and adjacent microbubbles is important in order to understand these therapeutic mechanisms. The collapse of microbubbles under static pressure was first described by Lord Rayleigh (Rayleigh, 1917). Under the influence of sufficiently high peak negative pressure ultrasound the microbubbles can undergo expansion and then adiabatic collapse, allowing the microbubbles to concentrate the acoustic energy (Apfel, 1997; Kodama and Tomita, 2000; Neppiras and Noltingk, 1951; Noltingk and Neppiras, 1950). This process is known as inertial cavitation and is

different from non-inertial cavitation where the microbubble oscillates in diameter with the compression and rarefaction phases of the ultrasound pulse (Flynn, 1964). Non-inertial cavitation can result in microstreaming creating fluid motion around the microbubble (Collis *et al.*, 2010; Elder, 1959; Tho *et al.*, 2007).

There are two main types of microbubble collapse during inertial cavitation. The first is a symmetric collapse of the microbubble with a resulting radial expansion of the shockwave (Kodama and Tomita, 2000). The second is an asymmetric collapse of the microbubble resulting in a jet of fluid (Miller *et al.*, 1996; Postema *et al.*, 2005). The asymmetric collapse of microbubbles concentrates the energy into a smaller volume and can project that energy for longer distances (Brujan *et al.*, 2005).

Much of the previous work done to visualize how microbubble collapse is influenced by other nearby microbubbles has been done with bubbles that are either uncoated, or too large to pass into the microcirculation. Microbubbles that can pass through the majority of the microcirculation in medical applications need to be smaller than $6\text{ }\mu\text{m}$ in diameter (Hogg, 1987). The average size of microbubbles used medically as ultrasound contrast agents is $2.5\text{ }\mu\text{m}$ in diameter (Schneider, 1999). The microbubbles also need to be stabilized with a lipid coating because uncoated microbubbles have extremely short half-lives, making them less attractive for clinical use (Ferrara *et al.*, 2007). These coatings can

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affect how the microbubbles respond to the ultrasound (van der Meer *et al.*, 2007).

Laser pulses are often used to create uncoated microbubbles that have diameters on the millimeter scale to visualize their collapse dynamics. Two millimeter scale uncoated bubbles created in close proximity with this method were shown to asymmetrically collapse forming jets pointed toward each other (Lauterborn and Kurz, 2010). Uncoated microbubbles that are 500 μm in diameter have been observed to change their oscillation behavior as they approach one another, producing chaotic-type oscillations (Leighton, 1995). A few studies have looked at lipid-coated microbubbles in the 10–20 μm diameter range and have observed them to undergo asymmetric collapse with jet formation when exposed to ultrasound (Chen *et al.*, 2011a; Chen *et al.*, 2011b; Postema *et al.*, 2005).

Most of the work done to characterize how the microbubbles interact with surfaces has also been done with large uncoated microbubbles. When laser-created bubbles in the 2–3 mm diameter range come into direct contact with a rigid surface they undergo an asymmetric collapse pointed directly at the surface. This dissipates the energy of the jet in a radial pattern across the surface causing the bubble to appear to flatten out over the rigid surface (Lauterborn and Kurz, 2010; Vogel *et al.*, 1989). Observations of microbubble jet formation near a rigid surface have been conducted with uncoated laser-generated microbubbles as small as 100 μm in diameter (Brujan *et al.*, 2005; Crum, 1979). These types of microbubbles have also been created near the air–water interface and observed to undergo jet formation (Wang *et al.*, 1996). When these laser-created bubbles come into direct contact with a flexible surface, such as a gel or a vessel wall, they also have the ability to create jets that are oriented in different directions depending on the orientation of the bubble and surface (Chen *et al.*, 2011a; Gracewski *et al.*, 2005; Kodama and Tomita, 2000).

These types of collapse phenomena have not been directly observed with microbubbles less than 6 μm in diameter because this size range is too small to allow for reliable direct observation of an asymmetric collapse. The optical distortions created by the index of refraction difference between the gas and the surrounding water as well as the microbubbles' small size obscure the actual dynamics of collapse and involution that is the hallmark of jet formation. This makes it a challenge to directly observe the collapse dynamics and determine the fluid jet direction in a statistically significant number of microbubbles less than 6 μm in diameter.

Fluorescence imaging was used to study the fluid flow that occurred as a result of the microbubble interaction with ultrasound to overcome the limitations of direct observation at this clinically relevant size scale. A fluorescent dye was incorporated into the lipid monolayer that surrounded each microbubble. The ultrasound-induced collapse of the microbubbles fragmented the lipid monolayer into a fine debris cloud that was larger than the original microbubble and did not suffer from any optical distortions from the gas. The shape of the fluorescent debris cloud revealed details about the direction of the fluid flow. The microbubbles studied here were positioned against a glass cover slip which served

as a rigid surface. Studying the shape of the debris field created by groups of microbubbles at varying separation distances can yield information about how the microbubbles influenced one another as they collapsed.

II. MATERIALS AND METHODS

A. Materials

Distearoyl phosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and distearoyl phosphatidylethanolamine-methyl poly(ethylene glycol) MW5000 (mPEG-DSPE 5k) was purchased from Laysan Bio, Inc. (Arab, AL). Dulbecco's phosphate buffered saline (DPBS) was purchased from Hyclone Laboratories Inc. (Logan, UT). 3,3'-diiodododecylcarbocyanine, perchlorate (DiO) was purchased from Biotium, Inc. (Hayward, CA). Perfluorohexane (PFH) was purchased from Sigma-Aldrich (St. Louis, MO).

B. Microbubble fabrication

To fabricate lipid-coated microbubbles, stock solutions of lipid and lipophilic dye were prepared in chloroform at 20 mg/ml DSPC, 50 mg/ml mPEG-DSPE 5k, and 1 mM DiO. Fifty μl of the DSPC solution, 20 μl of the mPEG-DSPE 5k solution, and 30 μl of the DiO were successively added to a 4 ml glass vial while vortexing. The amount of DiO in the combined solution was 2 mol % of the total lipid and dye content. The chloroform was evaporated under an argon gas stream while the solution was under vortex. This created a lipid film along the inner surface of the vial. Five hundred μl of DPBS was added to the film and the lipids and dye were resuspended by vortexing the vial for 15 s followed by heating at 75 °C for 1 min. The cycle of vortexing and heating was repeated until the lipids were well suspended and no lipid residue was left on the vial walls. The sample was left to cool to ambient temperature.

To create the gas microbubbles, the top of the vial was first covered with parafilm to create a barrier between the gas in the vial and outside the vial. A 5 ml syringe equipped with a 22 gauge needle was used to draw up 1 ml of liquid PFH. With the plunger fully drawn back, the syringe was rotated to coat the walls with the liquid PFH and left to sit for at least 3 min to encourage vaporization of PFH into the air within the syringe creating an air/PFH vapor mixture. The syringe needle was then bent at a 130° angle into a hook shape and was inserted through the parafilm cover into the vial headspace. Next, the syringe was pumped 65 times into the vial headspace to inject the PFH/air mixture into the vial headspace. Care was taken to prevent any liquid PFH from entering the vial. An XL-2000 probe sonicator (QSonica LLC., Newtown, CT) tip was immediately inserted through the parafilm cover and positioned 1 mm below the gas/liquid interface. The probe sonicator was then operated at 25 W for 3 s to create microbubbles. The resulting bubbles were left to sit for at least 5 min before further processing.

Excess lipid and dye were removed from the microbubble sample by a washing procedure. The microbubbles were moved to a microcentrifuge tube and centrifuged at 1000 rpm

for 3 min which caused the bubbles to float to the top of the liquid. The supernatant was partially removed and replaced with additional DPBS. This process was repeated 1 to 3 additional times and helped to reduce fluorescence background in the images from dye that was not associated with bubbles.

C. Ultrasound exposure

The ultrasound experiments were carried out using a modified version of a custom-designed system that combined fluorescence imaging with ultrasound (Ibsen *et al.*, 2013a). The basic system is shown in Fig. 1. A ten gallon tank of water was used to allow coupling between the ultrasound transducer and the microbubble sample. The fluorescent microbubbles in MilliQ purified water were placed in 10 μ l samples on a glass microscope slide and then covered with a glass cover slip for imaging. The glass slide was then placed at the air–water interface such that just the bottom of the slide was in contact with the water. By preventing the water level from reaching the top of the glass slide, the microbubble sample was prevented from being washed into the tank water. Positioning the microbubble sample at the air–water interface allowed a 100 $\times$ oil immersion objective to collect the images. The use of a high numerical aperture oil immersion objective allowed more fluorescent light to be collected from the microbubbles in order to achieve higher frame rates and allow debris cloud resolution.

The contact between the bottom side of the glass slide and the water allowed the ultrasound to travel through the

water and hit the bottom of the glass slide. The ultrasound intensity was attenuated by the glass but not enough to prevent microbubble cavitation from occurring in the sample.

A 3 min time delay between sample preparation and exposure to ultrasound allowed all the microbubbles to settle up against the glass cover slip due to their buoyancy. This ensured that there were no microbubbles in different focal planes and allowed all the microbubbles to be visible to the optical system.

Ultrasound was generated with a submersible 2.25 MHz transducer (V305-Su, 0.75 in. element diameter, 1 in. spherical focal length, Panametrics, Waltham, MA) using a waterproof connector cable (BCU-58 - 6W, Panametrics, Waltham, MA). The ultrasound was focused to a 1 mm² focal cross-sectional area allowing microbubbles within this region to be affected by the ultrasound pulse. The samples were scanned for microbubble pairs at different distances from one another. Once a group was identified it was centered into the ultrasound focal zone and hit with an ultrasound pulse that consisted of a 10-ms long 2.25-MHz sine wave with a peak negative pressure of 0.8 MPa. Inertial cavitation of microbubbles has been shown to occur at ultrasound peak negative pressure amplitudes as low as 0.58 MPa (Miller and Thomas, 1995). The interaction was recorded at 60 frames per second and saved for subsequent analysis.

The 0.8 MPa peak negative pressure does account for the attenuation of the glass slide. This was determined by placing the glass slide between the transducer and a needle

FIG. 1. (Color online) Schematic diagram of the microscope system used to observe and record the interaction of the fluorescent microbubbles with the focused ultrasound (figure adapted from Ibsen *et al.*, 2013a).

hydrophone (HNP-0400 Broadband Needle Hydrophone AH - 2020–100 with 2 mm sensitive element and hydrophone pre amp, 50 kHz to 100 MHz, 0 + 20 dB, Onda Corporation, Sunnyvale, CA).

The needle hydrophone was used to measure the sound field of the transducer and find the focal zone. The hydrophone was mounted on an XYZ micromanipulator stage and the hydrophone was positioned so that the measured peak pressure from the transducer was optimized for the highest value. This was defined as the focal zone of the transducer.

D. Video analysis

The videos were analyzed using ImageJ software (version 1.46r, National Institutes of Health, Bethesda, MD) to measure the starting edge-to-edge distance of each microbubble to its nearest neighbor microbubble (“inter-bubble distance”) before ultrasound exposure. Isolated microbubbles with no other microbubbles visible in the microscope field of view were assigned the distance between the bubble edge and the edge of the field of view. The ultrasound-induced collapse of the microbubble caused the fluorescent lipid monolayer on the microbubbles to fragment, leaving behind a debris cloud. The dimensions of the fluorescent debris cloud for each microbubble were then measured and assigned to one of three categories. The first category was radial, where a circular pattern of debris was seen, resulting from conditions where the fluid jet was pointed normal to and directly toward the cover glass surface with little or no horizontal directional component. Debris clouds where there was less than a 1.3-fold difference between length (the longer dimension) and width were assigned to the radial category. The second category was ellipsoidal where a debris cloud was produced that was between 1.3 to 2 times longer than its width, resulting from a detectable horizontal directional component in the fluid jet. The third category, denoted as “elongated,” was where the debris cloud took on a longer shape such that its length was greater than 2 times the width.

E. Passive inertial cavitation detection

A passive detection method similar to that described by [Chen *et al.* \(2003\)](#) was used here to detect the occurrence of microbubble inertial cavitation in this experimental setup. The microbubbles were held inside of a Rose chamber which consisted of a circular rubber septum compressed between two glass coverslips to create a fluid space that allowed the microbubbles to be monitored both optically and acoustically as they were exposed to ultrasound ([Ibsen *et al.*, 2014](#)). The tip of a submersible broadband needle hydrophone (HNP-0400) from Onda Corporation in connection with their AH - 2020–100 hydrophone pre amp (50 kHz to 100 MHz, 0 + 20 dB) was placed inside the Rose chamber through the bottom opening created by removing the lower cover slip. No microbubbles were present. The ultrasound pulse was the same as that used in the experiments described in Sec. II C and consisted of a 10 ms 2.25 MHz sine wave with peak negative pressure of 0.8 MPa. The transducer was run for 20 separate pulses. Each pulse was recorded by the

hydrophone. The frequency spectra of these 20 signals were averaged together for analysis.

A 50 μ l sample of microbubbles at a concentration of 3.8×10^7 microbubbles per ml was then introduced into the Rose chamber without making any change to the setup using a hypodermic needle through the bottom opening of the Rose chamber. The microbubbles floated to the upper glass cover slip which is the same position that the microbubbles are located in the experiments described in Sec. II C. The same ultrasound pulse sequence was run 20 more times with the microbubbles being replenished after each ultrasound exposure pulse. The frequency spectra from these 20 signals were averaged together for analysis.

III. RESULTS

A. Passive inertial cavitation detection

Ultrasound insonation of the sample holder in the presence of the microbubbles increased the recorded signal strength compared to insonation in the absence of microbubbles as shown by the differential signal strength in Fig. 2. This increase in broadband frequency content is due to the broadband signal production typical of microbubble inertial cavitation. This is similar to previously published data on the passive acoustic detection of microbubbles ([Chen *et al.*, 2003](#)) demonstrating that inertial cavitation was taking place under the experimental conditions of our study. The signal strength difference in Fig. 2 is shown to decrease at 4.5 and 6.75 MHz because the transducer produced harmonics at these frequencies with enough intensity to overwhelm the signal from the microbubble cavitation. The 2.25 MHz fundamental frequency produced by the transducer overwhelmed the microbubble cavitation signal out to 3 MHz.

B. Debris cloud observation

A series of still frames from the collected videos showing different microbubble debris cloud forms is shown in Fig. 3. Figures 3(A), 3(B), and 3(C) show two microbubbles at an edge-to-edge distance of 36 μ m. These microbubbles did not

FIG. 2. (Color online) Passive detection of inertial cavitation in the experimental setup. The graph shows the relative difference in signal strength between signals recorded with and without microbubbles in the ultrasound focal zone. The observed increase in broadband frequency content in the presence of microbubbles shows that the microbubbles are undergoing inertial cavitation creating a detectable broadband acoustic signal.

FIG. 3. (Color online) Still frames from videos showing examples of the three categories of debris cloud expansion. (A) Two fluorescent microbubbles are shown before ultrasound exposure with an edge-to-edge distance of $36\text{ }\mu\text{m}$. (B) The two microbubbles did not appear to interact with each other during their collapse and the resulting debris clouds are both radial in shape as shown in (C). (D) Here the microbubbles have an edge-to-edge distance of $8.0\text{ }\mu\text{m}$ before ultrasound exposure. (E) The microbubbles appear to have interacted with one another and distorted the shape of the resulting two debris clouds to be ellipsoidal in shape as shown in (F). (G) Here the two microbubbles have an edge-to-edge distance of $9.3\text{ }\mu\text{m}$ before ultrasound exposure. (H) The microbubbles appear to have interacted with each other during collapse resulting in long distortions of both of the lipid debris clouds that extend in a single direction from the original microbubble locations. (I) The dimensions depicted indicate two elongated debris clouds with lengths greater than 2 times their heights.

appear to influence one another's collapse behavior and both created radial debris clouds. Figures 3(D), 3(E), and 3(F) show microbubbles at a starting distance of $8.0\text{ }\mu\text{m}$. Some interaction appears to have occurred between the bubbles and distorted their fluorescent debris clouds into ellipsoidal shapes. Figures 3(G), 3(H), and 3(I) show microbubbles at a starting distance of $9.3\text{ }\mu\text{m}$. The microbubbles appear to have interacted with each other to distort both of their debris clouds into highly elongated forms that extend well beyond the original locations of the microbubbles. It is also important to note that the debris fields extend in a single straight-line direction from the original microbubble locations.

Figure 4 shows three microbubbles that are in physical contact with one another before exposure to ultrasound. The resulting debris cloud took an elongated shape that extended out from the original microbubble location in a single straight-line direction.

C. Analysis of debris cloud shape dependence on inter-microbubble distance and microbubble size

A total of 76 microbubbles were analyzed for debris cloud shape, microbubble size, and inter-bubble distance. The data are summarized in Table I.

Box plots of the inter-microbubble distances for the three different debris cloud shape categories are shown in Fig. 5. The data were not normally distributed so a two-tailed nonparametric one-way analysis of variance by ranks Kruskal-Wallis Test was performed at the 0.05 significance level. A significant difference was found in inter-microbubble distance between the three debris cloud shape categories ($p < 0.001$). *Post hoc* comparisons using the

Dunn-Sidak adjustment to counteract the problem of multiple comparisons showed a significant difference between the inter-microbubble distances that resulted in radial debris clouds and those that resulted in elongated debris clouds ($p < 0.001$). There was also a significant difference between the distances that resulted in ellipsoidal debris clouds and elongated debris clouds ($p = 0.041$). The difference between the microbubble distances that resulted in radial debris clouds and those that resulted in ellipsoidal debris clouds was not significantly different ($p = 0.067$). All statistical

FIG. 4. Three microbubbles are in physical contact with one another before ultrasound exposure. Exposure to ultrasound showed a resulting elongated lipid debris cloud that extends in a single direction from the original microbubble location. All other microbubble pairs or triplets in physical contact prior to ultrasound exposure were also observed to form elongated debris clouds.

TABLE I. Summary of microbubble data acquired from video analysis.

		N	Mean	Std. deviation	Std. error	95% Confidence interval for the mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Distance between microbubbles in μm	Radial	25	55.6	41.1	8.2	38.6	72.5	17.9	169.2
	Ellipsoidal	11	22.1	9.7	2.9	15.6	28.6	9.4	38.5
	Elongated	40	10.7	9.7	1.5	7.6	13.7	0.0	35.9
	Total	76	27.1	31.9	3.7	19.8	34.4	0.0	169.2
Microbubble diameter in μm	Radial	25	2.6	0.7	0.1	2.3	2.9	1.4	4.9
	Ellipsoidal	11	2.7	1.0	0.3	2.0	3.4	1.9	5.7
	Elongated	40	4.2	2.0	0.3	3.6	4.9	1.4	11.4
	Total	76	3.5	1.7	0.2	3.1	3.9	1.4	11.4

calculations were performed using IBM® SPSS® Statistics Version 21 software (Armonk, New York).

Box plots of the diameter of the microbubbles in the three different debris cloud expansion categories are shown in Fig. 6. There was no significant difference between the microbubble diameters of bubbles that produced radial and ellipsoidal debris clouds ($p = 1$) using the same statistical test described for the above comparisons of inter-bubble distance. There was a significant difference between the diameters of bubbles that produced radial debris clouds and those that resulted in elongated debris clouds ($p < 0.001$). This difference was likely a result of the larger microbubbles being generally closer to one another than the smaller bubbles in the study population, perhaps caused by how these bubbles settled at the liquid-coverslip interface. There was also a significant difference between the diameters of bubbles that produced ellipsoidal debris clouds and elongated debris

clouds ($p = 0.009$) which was likely also caused by the closer inter-bubble distance seen with larger microbubbles.

A scatter plot of the diameter of the microbubble versus the inter-microbubble distance is shown in Fig. 7 and is color-coded to the three different debris cloud categories. A clear pattern can be seen where radial debris cloud expansion occurs at the larger inter-microbubble distances and ellipsoidal and elongated debris clouds occur only at the smaller distances. Ellipsoid formation was observed to begin occurring at inter-microbubble distances of $37\ \mu\text{m}$. Below $10\ \mu\text{m}$ in inter-microbubble distance, bubbles were seen to exclusively produce elongated debris clouds.

IV. DISCUSSION

The morphology of the debris cloud was significantly influenced by the inter-microbubble distance as can be seen in Figs. 3 and 4. When the microbubbles were more than $37\ \mu\text{m}$ apart the resulting debris clouds were circular. The shape of the debris cloud began to distort at inter-microbubble distances shorter than $37\ \mu\text{m}$ resulting in ellipsoidal debris clouds. At an inter-microbubble distance of

FIG. 5. (Color online) Box plots of the distance between the microbubble and its nearest neighbor in microns for each of the three debris cloud expansion categories. Whiskers denote maxima and minima and the medians are represented as horizontal lines. The small circles denote data points that are statistical outliers in the data set which were defined as any points that were two standard deviations away from the mean. The radial and elongated debris cloud categories and the ellipsoidal and elongated categories were found to be statistically significantly different from each other ($p < 0.001$ and $p = 0.041$, respectively) with regard to inter-microbubble distance. The difference in inter-bubble distances resulting in radial and ellipsoidal debris clouds was not statistically significant ($p = 0.067$).

FIG. 6. (Color online) Box plots for the diameter of the microbubbles in each of the three debris cloud expansion categories. Whiskers denote maxima and minima and the medians are represented as horizontal lines. The small circles denote data points that are statistical outliers in the data set which were defined as any points that were two standard deviations away from the mean.

FIG. 7. (Color online) Scatter plot showing the diameters of the microbubbles plotted against distance between the microbubble and its nearest neighbor. Points are color-coded to the shape of the debris cloud. A trend is observed where radial expansion is seen at larger inter-microbubble distances and ellipsoidal and elongated debris cloud formation are seen at the shorter distances.

around $25\text{ }\mu\text{m}$ the debris clouds began to have elongated forms. Microbubbles that had less than $10\text{ }\mu\text{m}$ of separation or were in physical contact with one another prior to ultrasound exposure were always seen to result in elongated debris clouds. This supports the conclusion that the microbubbles interacted with other nearby bubbles to influence the fluid flow.

The formation of elongated and ellipsoidal debris clouds did not appear to be influenced by the size of the microbubble below $4\text{ }\mu\text{m}$ in diameter. Above $4\text{ }\mu\text{m}$ in diameter the microbubbles all had inter-microbubble distances below $30\text{ }\mu\text{m}$ except for one microbubble that was isolated and had a radial debris cloud. The population subset of microbubbles below $4\text{ }\mu\text{m}$ in diameter shows a statistically significant difference in inter-microbubble distance between those bubbles that formed elongated debris clouds and those that had radial debris clouds ($p < 0.001$) using the same statistical test as described above for comparisons of inter-microbubble distance. However, for this same population of bubbles below $4\text{ }\mu\text{m}$, there was no statistically significant difference in bubble size among all three groups ($p = 0.169$). This supports the conclusion that for microbubbles less than $4\text{ }\mu\text{m}$ in diameter, which is the relevant size range for clinically-used microbubbles, the dominant factor for the formation of elongated debris clouds was the inter-microbubble distance and not their size.

Two types of interactions were likely occurring between microbubbles in this study when ensonified with ultrasound. The first was the influence of microstreaming fluid flow around each microbubble that occurred as the microbubbles started to oscillate just before collapse. The second were the secondary Bjerknes forces that caused microbubble attraction. This microbubble attraction is shown in Figs. 3(D) and 3(E). In Fig. 3(D) the microbubbles start at a center-to-center distance of $11.0\text{ }\mu\text{m}$ but the resulting debris clouds in

Fig. 3(E) have a center-to-center distance of $6.5\text{ }\mu\text{m}$ indicating that the microbubbles attracted each other before collapse. If the microbubbles were attracted to one another just before their collapse then the microstreaming occurring around each microbubble could also influence the individual microbubble oscillations. This influence would grow in intensity as the inter-microbubble distance decreased.

The elongated debris clouds were observed to project in a single direction from the original microbubble location. Work done by Tho *et al.* (2007) has shown the fluid flow patterns created by microstreaming in single and multi-bubble groups. Bubbles imaged in those experiments were not seen to create flow patterns that project in a single straight-line direction. The flow patterns were always balanced by a stream moving in the opposite direction or displayed a form that flared out at wide angles creating circular eddy current type formations (Tho *et al.*, 2007). For example, a stream moving away from the bubble to the right would be balanced by a stream moving away from the bubble to the left.

The observation that the debris fields created by the microbubbles in our experiments extend in a single direction from the original microbubble location without a balanced component in the opposite direction indicates that another contributing factor other than microstreaming was involved in shaping the debris cloud. One hypothesis is that this extra component could be created by an asymmetric collapse of the microbubble. Asymmetric collapse creates fluid jets that result in a single-direction straight line fluid flow that is not balanced by a diametrically opposed jet. This collapse behavior would be similar to what is seen in the literature with laser generated bubbles that were close to one another (Lauterborn and Kurz, 2010). The results of the passive acoustic detection showed that inertial cavitation of microbubbles was occurring in the experimental setup under these conditions which indicates that the microbubbles could be producing fluid jets.

Bubbles will jet toward a rigid surface, flattening out along the surface in a circular expansion (Lauterborn and Kurz, 2010; Vogel *et al.*, 1989). This could account for the circular debris fields created by the microbubbles when separated by greater than $37\text{ }\mu\text{m}$. As the inter-microbubble distance decreased, the microbubbles could have begun to influence one another, causing the asymmetric collapse to not just point at the glass slide, but also have a component that pointed parallel along the glass. This is similar to what has been observed previously with two laser-generated microbubbles in close proximity that are seen to influence each other and cause asymmetric collapse of both bubbles (Lauterborn and Kurz, 2010). In the case of our study, the jetting direction would be influenced by both the glass slide and by the neighboring microbubble creating horizontal components to what would usually be a perpendicular jet direction toward the rigid surface. Having these horizontal components in the direction of the asymmetric collapse could account for the elongated debris cloud expansion.

The therapeutic consequences of the inter-microbubble distance effect on fluid flow during microbubble collapse will be studied in future experiments. Of particular interest is understanding how this distance-dependent fluid flow

influences the adhesion of cells to glass cover slips, and how it influences microcapillary damage. The influence of this effect can also be studied for sonoporation (Bao *et al.*, 1997; Li *et al.*, 2011; Zarnitsyn *et al.*, 2008) and can also have implication for histotripsy and high intensity focused ultrasound tissue damage.

V. CONCLUSIONS

The starting distance between microbubbles prior to ultrasound exposure had a significant effect on the shape of the resulting lipid debris cloud. In our experimental configuration, inter-microbubble distances greater than 37 μm resulted in circular debris fields. This indicates that at this distance the microbubbles were not interacting with one another during collapse. At inter-microbubble distances less than 37 μm microbubble microstreaming and secondary Bjerknes forces likely began to influence and distort nearby microbubble oscillations resulting in ellipsoid-shaped debris clouds. At inter-microbubble distances less than 10 μm significant elongation of the debris clouds was observed in every instance, indicating a strong interaction with nearby bubbles. Microbubbles that were in physical contact with one another exclusively formed these elongated debris clouds. One hypothesis for the creation of the debris clouds that were elongated in a single direction is the asymmetric collapse of the microbubbles resulting in fluid jets.

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