Removal of ligand-bound liposomes from cell surfaces by microbubbles exposed to ultrasound

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Abstract Gas-filled microbubbles attached to cell surfaces can interact with focused ultrasound to create microstreaming of nearby fluid. We directly observed the ultrasound/microbubble interaction and documented that under certain conditions fluorescent particles that were attached to the surface of live cells could be removed. Fluorescently labeled liposomes that were larger than 500 nm in diameter were attached to the surface of endothelial cells using cRGD targeting to $\alpha v \beta 3$ integrin. Microbubbles were attached to the surface of the cells through electrostatic interactions. Images taken before and after the ultrasound exposure were compared to document the effects on the liposomes. When exposed to ultrasound with peak negative pressure of 0.8 MPa, single microbubbles and groups of isolated microbubbles were observed to remove targeted liposomes from the cell surface. Liposomes were removed from a region on the cell surface that averaged 33.1 μm in diameter. The maximum distance between a single microbubble and a detached liposome was 34.5 μm . Single microbubbles were shown to be able to remove liposomes from over half the surface of a cell. The distance over which liposomes were removed was significantly dependent on the resting diameter of the microbubble. Clusters of adjoining microbubbles were not seen to remove liposomes. These

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observations demonstrate that the fluid shear forces generated by the ultrasound/microbubble interaction can remove liposomes from the surfaces of cells over distances that are greater than the diameter of the microbubble.

Keywords Microbubbles · Cavitation · Shear stress · Microstreaming · Biochemical targeting · Cell membrane · Endothelial cells · HUVEC

1 Introduction

Most of what is known about the effects of ultrasound/microbubble interactions on the surfaces of cells comes from studies of sonoporation. Sonoporation uses microbubbles and ultrasound to physically create small transient ruptures in cell membranes to enhance drug delivery into the cell [1, 2]. The ultrasound exposure causes the diameter of the microbubble to shrink and expand with the compression and rarefaction portions of the ultrasound pulse [3, 4]. This oscillation behavior can cause microstreaming of the fluid around the microbubble edges [5, 6]. Microstreaming creates shear force over the cell surface, which can cause membrane disruption [5, 7, 8]. The larger the oscillation, the greater the shear forces. The magnitude of this oscillation depends on several factors that influence the resonance frequency of the microbubble, one of the most influential of which is the diameter of the microbubble [9]. An increase in diameter of just 1.5 μm can cause the oscillation power of the microbubble to drop from 0.6 W to almost 0 W [9]. Microbubble diameter has been shown to have a significant influence on the biological effects of the microbubble–ultrasound interaction [10].

When the ultrasound peak negative pressure is high enough, the microbubble can undergo inertial cavitation, a violent implosion event that can send out a radial shockwave [11] or a directed fluid jet resulting from asymmetric microbubble collapse [12, 13]. Both the shockwave and jet formation modes of inertial cavitation are intense enough to open transient holes in cell membranes [14–16]. These microbubble–ultrasound interactions have also been shown to cause microcapillary damage and rupture in addition to sonoporation [17, 18].

It is not known how the interaction between the microbubble and the ultrasound might affect particles attached to the surface of the cells. It is known that ultrasound–microbubble interactions near a ridged surface can create a fluid flow pattern, which radiates away from the microbubble across the surface [19]. We hypothesized that the fluid flow from the ultrasound–microbubble interaction would radiate out over a cell surface and affect attached liposomes over an area that is greater than just the poration site.

This study used a specially designed system that combined ultrasound and optical microscopy to directly observe the interaction between ultrasound driven microbubbles and non-echogenic fluorescent liposomes that were attached to the surface of human umbilical vein endothelial cells (HUVEC). These cells were grown in monolayers on glass cover slips as shown in Fig. 1. The microbubbles used DSTAP lipid to give them a positive surface charge [20] allowing them to attach to the negatively charged cell surfaces through electrostatic interactions [21]. The liposomes were attached to the cells using cRGD ligand binding [22] to $\alpha\text{v}\beta 3$ integrin that was naturally expressed on the HUVEC surfaces [23]. The expression of $\alpha\text{v}\beta 3$ integrin has a characteristic spatial pattern around the periphery of the cell where the focal adhesion points are located [24, 25]. The cells used in these studies also have this

characteristic distribution in their binding patterns, meaning that the cell surface binding is primarily between the $\alpha v\beta 3$ integrin on the surface of the cells and the cRGD on the surface of the liposomes. Nonspecific binding of the liposomes to the cells would not be able to produce this sort of pattern and would instead result in liposomes randomly distributed over the surface of every cell. Under certain conditions, the $\alpha v\beta 3$ integrin becomes dissociated from the focal adhesion points and becomes more randomly distributed around the surface of the cell. This is also consistent with some of our cells and does not indicate that non-specific binding is the main type of observed binding occurring in these cases. The extent of adhered liposome removal from the surface of the cell due to nearby ultrasound–microbubble interaction was measured using fluorescence microscopy.

Receptor-mediated endocytosis of vehicles by HUVECs can be triggered by attachment to $\alpha v\beta 3$ integrin, but is limited to particles that are smaller than 350 to 500 nm in diameter [26]. The liposomes used here were mostly greater than 500 nm in diameter and remained on the surface of the cell.

Three different configurations of microbubbles attached to cells were studied. The first configuration was looking at cells that had a single microbubble attached. The second configuration looked at cells with multiple microbubbles attached that were separated from one another. The third configuration was large clusters of adjoining microbubbles surrounding large portions of individual cells.

2 Materials

L- α -phosphatidylcholine (EPC) from chicken eggs, distearoyl phosphatidylcholine (DSPC), 1, 2-distearoyl-3-trimethylammonium propane (DSTAP), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Distearoyl phosphatidylethanolamine-methyl poly(ethylene glycol) MW5000 (mPEG-DSPE), and DSPE-PEG-maleimide were purchased from Laysan Bio, Inc. (Arab, AL, USA). 1,2-propanediol, glycerol, ethanol, polyoxyethylene 40 stearate, and perfluorohexane were purchased from Sigma-Aldrich (St. Louis, MO, USA). All water was purified using the Milli-Q Plus System (Millipore Corporation, Bedford, MA, USA). DiO was purchased from Biotium, Inc. (Hayward, CA, USA). The PBS was purchased from Hyclone Laboratories Inc. (Logan, UT, USA). Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). EBM-2 media was purchased from Lonza Inc. (Basel, Switzerland). The trypsin (.25% T/2.21 mM EDTA) was purchased from Mediatech Inc. (Manassas, VA, USA). The penicillin-streptomycin used in the EBM-2 media was purchased from Gibco (Invitrogen, Grand Island, NY, USA). The fetal bovine serum used in the EBM-2 media solution was purchased from Hyclone (Logan, UT, USA). The cRGD was purchased from Anaspec Incorporated (Fremont, CA, USA).

3 Methods

3.1 cRGD lipid conjugation

The fluorescent liposomes were targeted to the surface of the HUVEC cells using cRGD ligand that was covalently bound to the lipids used to make the liposome membrane.

One milligram of cyclic RGD (cRGD) and 7.8 mg of DSPE-PEG-maleimide (MW = 3400) were dissolved separately in chloroform/methanol (1:1) (v,v). The DSPE-PEG-cRGD was synthesized by dropping the cRGD solution into the DSPE-PEG-maleimide solution (molar ratio = 1:1) and stirring for 2 h at room temperature. Chloroform was added to adjust the final volume to achieve a 4 mM concentration.

3.2 HUVEC culture

The HUVEC cells were cultured with EBM-2 media with pen/strep from Hyclone and 10% FBS from Invitrogen. At around 80% confluency, the adherent cells were detached from the expansion flask using Trypsin (0.25% T/2.21 mM EDTA); 10,000 cells were then injected into a Rose Chamber [27, 28] along with 400 μ l of EBM-2 media as shown in Fig. 1. The Rose Chamber as described in Ibsen et al. [28] was chosen as the sample holder for this experiment because it uses a rubber gasket in between two thin glass coverslips to create a closed cell culture chamber. The closed configuration also isolated the cells and the cell culture media from the outside environment allowing the Rose Chamber to be partially submerged in the water tank that coupled the ultrasound energy from the transducer to the cells as described by Ibsen et al. [28]. The glass cover slips were only 0.13 mm thick and did not impede the transmission of ultrasound from the water tank into the cell culture media and ultimately to the cells themselves. The sealed environment of the Rose Chamber also reduced the effects of removing the cells from the cell incubator for the ultrasound exposure. The cells injected into the Rose Chamber were allowed to adhere to the bottom glass cover slip of the Rose Chamber overnight. The Rose Chamber was inverted after this so the adhered cells were now at the top, allowing microbubbles in the cell culture media to rise by buoyancy-driven forces to naturally interact with and adhere to the cells.

Seeding the Rose Chamber with low densities of cells ensured they would be continuously expanding and not reaching confluency to avoid any suppression of $\alpha v\beta 3$ integrin expression. It also ensured that the cells would have enough space between one another so that the ultrasound/microbubble interaction events would affect only single cells. Care was taken to make sure no air bubbles were introduced into the Rose Chamber during the media filling process [28].

Fig. 1 Schematic of cells in the Rose Chamber Cell Culture setup. The cells were viewed from above by the microscope objective and ensonified from below by the transducer. The fluorescent liposomes targeted to the surface of the cells are shown along with the positively charged microbubbles that are attached to the negatively charged cells. The buoyancy of the microbubbles ensured that they would rise to bind to the cells

3.3 Liposome preparation

The liposomes used for these experiments were non-echogenic and were not influenced by the ultrasound because they contained only water. A 1.5-ml microcentrifuge tube was filled with 76 μl of EPC in chloroform (26 mM)(20 mg mL^{-1}), 10 μl of cholesterol in chloroform (100 mM)(387 mg mL^{-1}), 25 μl of DSPC in chloroform (51 mM) (40 mg mL^{-1}), and 20 μl mPEG5000-DSPE in chloroform (8.6 mM) (50 mg mL^{-1}). To visualize lipid membranes, 5 μl of 1 mM DiO in chloroform was added. To allow targeting to the $\alpha\text{v}\beta 3$ integrin 16 μl of the 4 mM cRGD lipid construct described above was added to constitute 40% of the final PEGylated lipid content in the liposome. The lipid mixture was dried under argon stream while vortexing to evaporate all the chloroform. The dried lipid film was resuspended by adding 1 ml of PBS and vortexing for 1 min. The microcentrifuge tube was then bath sonicated for 10 min and then probe sonicated for 10 s. The resulting liposomes were about 2 to 5 μm in diameter.

3.4 Microbubble preparation

The microbubbles were made separately from the liposomes and the two were administered separately to the cells. The microbubbles were stabilized with a lipid monolayer composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), polyoxyethylene 40 stearate, and the positively charged lipid 1,2-stearoyl-3-trimethylammonium-propane (DSTAP). The addition of the positively charged lipids to the microbubble membrane has been well documented to make the microbubbles positively charged, allowing them to bind to negatively charged DNA [29–31], and also to bind to the surface of negatively charged cells [20, 21] through electrostatic forces. This binding is shown schematically in Fig. 1. The microbubbles were formed by mixing 1.6 mmol of polyoxyethylene 40 stearate, 1.4 mmol of DSTAP, and 2.5 mmol of DSPC together in chloroform [20]. The microbubbles were not fluorescently labeled. A lipid film was created by vacuum evaporation of the chloroform, dried under argon, and rehydrated into 1 ml of PBS. The headspace of the vial was filled with an air and perfluorohexane (PFH) gas mixture. The correct ratio between air and PFC prevented the microbubbles from undergoing size changes that were osmotically driven. Filling the microbubble with pure air would quickly cause it to dissolve away and collapse. Using a concentration of PFH gas that was too high would cause the collapse of the microbubble as the PFH condensed into a liquid droplet. If too much air was used, then an osmotic driving force would move the nitrogen out, reducing the microbubble to its collapse radius [32, 33]. The correct ratio of air to PFH was obtained here by drawing 0.5 ml of PFH liquid into a 5-ml syringe and filling the rest of the 4.5-ml space with air. The PFH was encouraged to quickly vaporize and establish an equilibrium with the air by rotating the syringe to coat the walls with PFH and letting it sit for 5 min. This gas mixture was then injected into a parafilm-sealed glass vial containing the lipid water mixture. The syringe needle was bent at a 130° angle to prevent the injection of liquid PFH [34]. As the 4 ml of the PFH/air mixture was injected, it displaced the pure air that was originally in the vial.

The microbubbles were then created by probe sonication using a Fisher Scientific Model 100 Sonic Membrane Disruptor, which was operated at the liquid–gas interface in short 3-s pulses. The sonication power used was 25 W. Excess phospholipid fragments and deflated microbubbles were removed by three washes consisting of centrifugation at 1500 rpm for 30 s and replacement of the PBS. The resulting microbubbles ranged in diameter from 2 to 10 μm .

3.5 Liposome and microbubble administration to cells

The cells were exposed to the targeted liposomes by adding 20 μl of the liposome solution to 400 μl of media and replacing the existing media in the Rose Chamber with this mixture. The cells were allowed to incubate with the liposomes for 30 min, at which point the media was replaced with 400 μl of fresh media to leave only the liposomes that were attached to the cells. Approximately 30 μl of the microbubble solution was then drawn into a 1-ml syringe and slowly injected into the Rose Chamber. The Rose Chamber was flipped from its orientation during the cell adherence process so the microbubbles would be driven up by buoyancy to be next to the cells as shown in Fig. 1. The positively charged microbubbles naturally attached to the negatively charged surfaces of the cells. The chamber was rotated at an angle to make sure the cells got an even coating of microbubbles as the microbubbles shifted across the top of the Rose Chamber. The chamber was then held at an angle for 2 min to allow all the non-adhered microbubbles to float up to one corner where they would stay out of the way once the chamber was returned to a level orientation during the ensonification experiment.

3.6 Imaging and ultrasound ensonification

The cells at the top of the Rose Chamber were imaged and ensonified using a custom built system, a full description of which is given in Ibsen et al. [28, 35]. The system was shown to successfully collect fluorescent images of a cell before and after an attached microbubble was exposed to ultrasound. The data presented here includes a full description and analysis of nine different cells studied under three different experimental conditions. The system used a water tank to couple the ultrasound to the cell samples. Ultrasound was generated with a submersible 2.25-MHz transducer (V305-Su, 0.75" element diameter, 1" spherical-focal length, Panametrics, Waltham, MA, USA) using a waterproof connector cable (BCU -58 - 6 W, Panametrics, Waltham, MA, USA). A needle hydrophone (HNP-0400 Broadband Needle Hydrophone AH - 2020-100 with hydrophone pre amp, 50 kHz–100 MHz, 0 + 20 dB, Onda Corporation, Sunnyvale, CA, USA) was used to measure the sound field. The hydrophone was mounted on an XYZ micromanipulator stage and the hydrophone was positioned so that the measured peak pressure from the transducer was the highest. This was defined as the focal zone of the transducer. An arbitrary waveform generator (PCI 5412, National Instruments, Austin, TX, USA) was used to create different waveforms and was controlled using a custom designed program (LabVIEW 8.2, National Instruments, Austin, TX, USA). A 300-W amplifier (VTC2057574, Vox Technologies, Richardson, TX, USA) was used to create an ultrasound pulse with a negative peak pressure level of 0.8 MPa, a peak-to-peak pressure difference of 1.6 MPa, and a mechanical index of 0.53, as measured in an underwater free-field setting. A high speed camera (FASTCAM 1024 PCI, Photron, San Diego CA, USA) acquired the image sequences.

A 100 $\times$ oil immersion objective (Nikon, Melville, NY, USA) was used for fluorescent imaging. The Rose Chamber was oriented at the air–water interface of the water tank [28]. The ultrasound transducer was aligned to focus at the top of the Rose Chamber where the cells were adhered. The bottom cover slip of the Rose Chamber was 0.13 mm thick and so had very little effect on the ultrasound focusing through it.

Focused ultrasound reached the level of the cells and destroyed microbubbles without causing disturbance to non-echogenic liposome membranes [34] or cell membranes. The oil used with the oil immersion lens prevented the surface of the glass, where the cells were adhered, from reflecting ultrasound, as it would have if there was only air on the side opposite to the cell culture media.

The Rose Chamber was visually inspected using the microscope optics to find cells that had both microbubbles and fluorescent liposomes attached to the surface. Once identified, the cells were then ensonified with a single 2.25-MHz, 10-ms, 1.6-MPa sine wave pulse that had a mechanical index of 0.53. The pulse length was chosen to make sure the microbubbles were exposed to enough cycles to get the maximum amount of effect.

3.7 Image analysis

White light illuminated pictures were taken to determine the location of microbubbles on the cells. The air inside the microbubbles created a large index of refraction break with the surrounding water which resulted in ring distortions, bright spots, and dark spots as the microbubble was raised through the focal zone of the objective. These changing distortions were unique to the microbubbles and allowed them to be identified from other dust, debris, and cell vacuoles as pointed out in the images. The microbubbles were not fluorescently labeled and so did not show up on the fluorescent images. The microbubbles have been pointed out by white arrows in the images. Movies were taken at 60 frames per second to allow the cells to be monitored. Frames taken before and after the ultrasound exposure were extracted from the movies and then analyzed to determine the changes that occurred to the cell surface. The living cells were capable of moving across the surface of the glass and over several hours could move up to 5 μm . The before-and-after ultrasound pictures were taken 17 ms apart. All of the observable differences in the two pictures were due to physical changes caused by the microbubble interaction with the ultrasound. The cells themselves were not capable of making observable changes over that short time period.

4 Results

4.1 Effects from single microbubbles on adhered liposomes

The effect of ultrasound interacting with a single microbubble on the membrane of single cells is shown in Figs. 2 and 3. The top row shows the bright field images of the cells, which serve mainly to determine the number and location of the attached microbubbles because the microbubbles were not visible under fluorescence imaging. The microbubbles are indicated by the white arrows. The outline of the cell is highlighted by the gray line. The “before ultrasound” row shows fluorescent images of the HUVEC cells with the fluorescent cRGD targeted non-echogenic liposomes adhered to the surface highlighting the outline of the cell. The “after ultrasound” row shows the effect of the ultrasound–microbubble interaction on adhered fluorescent liposomes. The “difference between before and after ultrasound” row highlights in red the liposomes that were removed by the ultrasound–microbubble interaction.

Fig. 2 Effects from ultrasound interactions with single microbubbles where adhered liposomes were removed. These liposomes have been targeted to the cell using cRGD to attach to $\alpha v \beta 3$ integrin expressed on the cell surface. The single microbubble is pointed out by the *white arrows*. The white light illumination serves mainly to identify the size, location, and number of microbubbles because they were not visible under fluorescent imaging. The cell outline is highlighted by the *gray lines*. Cell 1: The single microbubble in this bright field image is 0.9 μm in diameter. The fluorescence before ultrasound image shows that the cell surface has many fluorescent liposomes on it, five of which were removed after the ultrasound–microbubble interaction event. The 34.5-μm diameter oval outline shows the region that includes the microbubble and removed liposomes. Cell 2: This cell has a single 1-μm diameter microbubble on the surface. The other circular structures were cellular vacuoles that were non-echogenic and were not destroyed with the ultrasound pulse. The oval outline shows the region of liposome removal, which is 10 μm in diameter. Cell 3: The microbubble attached to the cell surface had a diameter of 2 μm. The before ultrasound image shows the fluorescent liposomes outlining the edge of the adherent cell. The after ultrasound image shows the removal of these adhered liposomes from the right-hand surface of the cell due to the ultrasound–microbubble interaction. The oval outline shows the area over which liposomes were removed and is 51.2 μm in diameter

4.1.1 Figure 2 - cell 1

A single 0.9- μm microbubble was attached to the surface of the cell. The ultrasound–microbubble interaction removed liposomes from across the length of the cell. The furthest distance between the microbubble and a removed liposome was 34.5 μm . The cell was partially detached from the surface of the glass causing a rotational translation parallel to the glass surface and remained in focus. The after ultrasound image was reregistered to correct for this change.

4.1.2 Figure 2 - cell 2

A single 1.0- μm microbubble was attached to the surface of the cell. Only the top section of the cell was affected by the ultrasound–microbubble interaction removing liposomes 5.1 μm away from the microbubble. Even though the cell was not detached from the glass substrate, there was a small translational shift in the location of those liposomes on the left-hand region of the cell from their original location in the before ultrasound image. This slight translational difference between the before and after ultrasound images caused the highlighted regions to appear on the left portion of the cell in the difference image. These highlighted regions indicate only the translational difference in the attached liposomes and not their removal from the surface, which is why they were not included in the oval.

4.1.3 Figure 2 - cell 3

A single 2.0- μm microbubble was attached to the surface of the cell. The ultrasound–microbubble interaction removed liposomes from the right half of the cell surface, creating a debris field of fluorescent lipid particles floating around the cell. The cell membrane was not destroyed because the left-hand side of the cell is still outlined by liposomes and there is still a faint visible outline of the residual fluorescent material left on the right-hand side of the cell. The ultrasound–microbubble interaction was powerful enough to partially detach this cell from the glass substrate causing a rotational translation. The after ultrasound image was reregistered to align the cell with its original orientation by rotating and translating the after ultrasound image to match the before ultrasound image. In this case, the cell remained flat against the glass substrate, which avoided the need to account for any out-of-plane motion. The cell was also undistorted after ultrasound exposure so no distortion adjustments had to be made. The effects of the ultrasound–microbubble interaction extended out from the microbubble itself to cover an area of 51.2 μm in diameter with 27.5 μm being the furthest distance between the microbubble and a removed liposome.

4.1.4 Figure 3 - cell 1

A single 3.4- μm microbubble was attached to the surface of the cell. No fluorescent liposomes were removed from the surface of the cell after ultrasound exposure. A free-floating fluorescent liposome was seen in the upper left as the large white spot and was pushed out of the field of view by the ultrasound–microbubble interaction.

4.1.5 Figure 3 - cell 2

The cell had a 3.8- μm microbubble attached to it. The ultrasound–microbubble interaction did not remove any liposomes from the surface.

Fig. 3 Effects from ultrasound interaction with single microbubbles where no adhered liposomes were removed. The microbubbles are designated by the *white arrows*. The cell outline is highlighted by the *gray lines*. Cell 1: The single cell shown here had a microbubble on its surface that was 3.4 μm in diameter. The *bright white spot* at the top left in the before ultrasound image was a large fluorescent liposome that was free floating and not attached to the cell. This free-floating liposome was gone in the after ultrasound image due to the ultrasound–microbubble interaction. None of the adhered liposomes were removed from the cell surface. Cell 2: The cell had a microbubble that was 3.8 μm in diameter. No liposomes were removed as shown by the lack of highlighted regions in the difference image

4.2 Effects from multiple separate microbubbles on adhered liposomes

4.2.1 Figure 4 - cell 1

This cell had five microbubbles on the surface. As discussed in the Supplemental section, when viewed under a mixture of white light and fluorescent light, the true size of the microbubble at the top of the frame was 2.3 μm in diameter. The ultrasound–microbubble interaction removed fluorescent liposomes from the upper third of the cell. The cell

Fig. 4 Effects from ultrasound interaction with multiple separate microbubbles on the cell surface. The microbubbles are pointed out by the *white arrows*. The cell outline is highlighted by the *gray lines*. Cell 1: A single cell is shown here with five microbubbles on the surface. The determination of the microbubble diameters from the white light image is discussed in the Supplement. The diameters of the microbubbles from left to right are 3.9, 7.9, 2.3, 5.5, and 3.9 μm . After exposure to ultrasound, all five microbubbles were destroyed. The *oval outline* shows the region of liposome removal, which is 52.6 μm in diameter. In this case, there is a large variability in the distance between the microbubbles and the nearest intact liposome after ultrasound exposure as summarized in Table 1. The 2.3- μm microbubble was the furthest away from a removed liposome with a distance of 30.9 μm . Cell 2: Here, three bubbles were on the surface of the cell. Their diameters from left to right were 4, 4.7, and 2 μm . After exposure to the ultrasound, all three microbubbles were destroyed. The oval outline has a diameter of 17.1 μm and shows the region that includes the microbubbles and the removed liposomes. The largest distance between a microbubble and a removed liposome was 13.2 μm and was between the 2- μm diameter microbubble and the leftmost removed liposome

remained adherent to the substrate and did not undergo any translations. The liposome removal region is outlined by the oval, which is 52.6 μm in diameter. The furthest distance between a microbubble and a removed liposome was 30.9 μm and was between the 2.3 μm diameter microbubble at the top of the cell and the lower left-hand affected liposome.

Fig. 5 Effects from ultrasound–microbubble interactions when clusters of adjoining microbubbles were present on the cell surface. The microbubble clusters are pointed out by the *white arrows*. The cell outline is highlighted by the *gray lines*. Cell 1: The whole cell was surrounded by a cluster of 45 microbubbles that ranged in diameter from 2 to 15 μm . The entire cluster underwent a change of orientation after exposure to ultrasound. Some of the microbubbles merged together so the final count was 36 microbubbles. A bright area of fluorescence appeared after ultrasound exposure at the bottom of the cell and was likely a free-floating liposome cluster that was brought into the field of view by the ultrasound–microbubble interaction. No liposomes were removed from the cell surface. Cell 2: Here the lower end of the single cell was surrounded by seven microbubbles that ranged in diameter from 3 to 15 μm . All of the microbubbles were gone in the after ultrasound picture, but no liposomes were removed from the cell surface

4.2.2 Figure 4 - cell 2

This cell had three microbubbles. The ultrasound–microbubble interaction was able to remove liposomes with the furthest distance between a microbubble and a removed liposome being 13.2 μm .

4.3 Effects from clusters of adjoining microbubbles on adhered liposomes

Here, clusters of microbubbles were studied. Microbubble clusters behave differently from individual microbubbles under the influence of ultrasound due to interactions between individual members of the cluster [36]. The clusters were observed to rearrange orientation and microbubbles were observed to merge with one another when exposed to ultrasound, as shown in Fig. 5. The clusters of microbubbles shown here were found around isolated cells and were not near other microbubbles. The effects observed on the cells resulted only from the visible microbubble clusters.

Fig. 6 Control cells exposed to ultrasound without any nearby microbubbles. Images show the bright field view of HUVEC cells along with corresponding fluorescent images of the adhered liposomes before and after ultrasound exposure. No changes were observed in the location or adherence of the liposomes as shown in the difference images indicating that under these conditions ultrasound alone does not affect the liposomes

Table 1 Data from Figs. 2, 3, 4, 5, and 6

Figure	Microbubble diameter in μm	Distance to closest intact liposome in μm	Diameter of liposome removal area in μm	Max distance from a microbubble to a removed liposome in μm	Cell detachment
2 - Cell 1	0.9	10.6	34.5	34.5	Yes
2 - Cell 2	1.0	2.9	10.0	5.1	No
2 - Cell 3	2.0	18.0	51.2	27.5	Yes
3 - Cell 1	3.4	0.0	0.0	0.0	No
3 - Cell 2	3.8	1.3	0.0	0.0	No
4 - Cell 1	2.3	20.0	52.6	30.9	No
	3.9	2.5			
	3.9	2.6			
	5.5	5.6			
	7.9	5.4			
4 - Cell 2	2.0	7.6	17.1	13.2	No
	4.0	0.5			
	4.7	8.3			
5 - Cell 1	2 - 15	NA	0	0	No
5 - Cell 2	3 - 15	NA	0	0	No
6 - Cell 1	None	NA	0	0	No
6 - Cell 2	None	NA	0	0	No
6 - Cell 3	None	NA	0	0	No

4.3.1 Figure 5 - cell 1

A large cluster of 45 microbubbles ranging in size from 2 to 15 μm surrounded the cell. The cluster rearranged its orientation around the cell. Some of the microbubbles merged as well. No liposomes were removed from the surface of the cell.

4.3.2 Figure 5 - cell 2

A cluster of seven microbubbles that ranged in diameter from 3 to 15 μm surrounded the lower end of the cell. The ultrasound exposure caused the microbubbles to leave the field of view, but no liposomes were removed from the cell surface.

4.4 Effects of ultrasound on adhered liposomes without nearby microbubbles

Here, the effect of just ultrasound on the adhered liposomes was studied as a control without the presence of nearby microbubbles. The liposomes are filled with water and do not have a high enough density difference with the surrounding water or any compressible components

Table 2 Data for microbubble size categories

Microbubble diameter in μm	Mean distance from microbubble edge to closest intact liposome after ultrasound exposure	<i>N</i>	Standard deviation of distance
1–3	11.8	5	7.2
>3	3.3	8	2.9

that would allow them to react to an ultrasound pulse at the intensities used in this experiment. That makes these liposomes non-echogenic. Figure 6 shows that ultrasound alone did not remove any liposomes from the surface of three different cells. The ultrasound used here was the same intensity as the ultrasound used in the other figures.

5 Discussion

The ultrasound–microbubble interaction was observed to remove adhered liposomes from the surface of cells. The region of liposome removal averaged $33.1\ \mu\text{m}$ in diameter for those observations. The furthest distance from a microbubble to a removed liposome ranged from 5.1 to $34.5\ \mu\text{m}$ with an average of $22.2\ \mu\text{m}$. Figure 2 shows that a single microbubble exposed to ultrasound can remove liposomes from an area that covers approximately one-half of a single cell. Liposomes could be removed from two or three cells if the microbubble was located in between them. Cells that had multiple microbubbles on the surface that were separated from one another also showed liposome removal from the surface of the cell. Clusters of adjoining microbubbles were not seen to remove any liposomes from the cell surface.

The furthest distance between a removed liposome and the microbubble could be affected by the number of attachments between the liposome and the cell surface. The force required to disrupt the $\alpha\text{v}\beta 3$ -RGD bond is near the range of 32 – $42\ \text{pN}$ [37], and the force required to pull one DSPE molecule from a DSPC/cholesterol vesicle similar to the one used here is $41\ \text{pN}$ [38]. Liposomes removed from the surface of the cells were probably subjected to forces of at least this magnitude. Liposomes that were bound by more than one ligand attachment should require a greater force to cause their detachment.

Another possible source in the variation of liposome removal distance is the magnitude of the microbubble size oscillations when exposed to the ultrasound. The extent of the size oscillations are highly dependent on the resting size of the microbubble [9], the stiffness of the surrounding lipid monolayer, the viscosity of the surrounding fluid, and the ultrasound driving frequency [39]. Table 1 shows the distance between the original microbubble edge and the closest intact liposome left on the surface of the cell after ultrasound exposure. For microbubbles in the 1 – $3\ \mu\text{m}$ diameter range, the average of these distances was $11.8\ \mu\text{m}$ and for microbubbles greater than $3\ \mu\text{m}$ in diameter the average of these distances was $3.3\ \mu\text{m}$ (Table 2). The distances are significantly different between the two groups using a nonparametric Wilcoxon rank-sum test ($p = 0.019$). This shows that the microbubbles in the 1 – $3\ \mu\text{m}$ diameter range removed liposomes over a greater distance. The two cells that experienced enough force from the ultrasound–microbubble interaction to partially detach from the glass substrate had microbubbles in the 1 – $3\ \mu\text{m}$ range. Figure 4 (cell 1) shows multiple microbubbles of different sizes present on the cell. Liposome removal occurred at greater distances from the $2.3\text{-}\mu\text{m}$ diameter microbubble than the larger microbubbles.

It would appear that under these experimental conditions the microbubbles in the 1 – $3\ \mu\text{m}$ diameter range created the most intense fluid flow across the surface of the cell resulting in liposome removal over the greatest distances. One hypothesis to explain this observation is that under these experimental conditions microbubbles in the 1 – $3\ \mu\text{m}$ diameter range had oscillation behavior that resembled resonance type oscillations resulting in the largest size changes [9]. Larger size oscillations would create more intense fluid flow. If the 1 – $3\ \mu\text{m}$ diameter microbubbles were undergoing large

size oscillations, then it is possible that they could have undergone inertial cavitation. The ultrasound pulses used here had a peak negative pressure of 0.8 MPa and inertial cavitation has been shown to occur with microbubbles at ultrasound peak negative pressures as low as 0.6 MPa [40]. Passive acoustic detection has confirmed that microbubble inertial cavitation occurs in this experimental setup under these conditions [41]. If inertial cavitation did occur with the 1–3 μm diameter microbubbles, the resulting shockwaves and intense fluid flow could explain the observed large distances over which liposomes were removed and the partial cell detachments.

Microstreaming may have also taken place under these conditions. When exposed to ultrasound, it is possible for these microbubbles to oscillate with the ultrasound pressure waves creating microstreaming of the surrounding fluid [35, 42, 43]. Evidence for microstreaming in these experiments was observed in Fig. 3, cell 1, as seen by a large free-floating fluorescent liposome in the upper left that was pushed out of the field of view by the ultrasound interaction with the 3.4- μm diameter microbubble. The fluid flow was not strong enough to remove any liposomes from the cell surface. The same phenomenon was observed for the cluster of microbubbles shown in Fig. 5, cell 1. An unattached liposome cluster came into the field of view at the bottom of the cell after ultrasound exposure. Microbubbles in a cluster formation as a whole can create microstreaming [44], however, the microbubbles in the cluster can interact with one another effectively reducing the maximum oscillation magnitudes that can be achieved.

These results show that ultrasound–microbubble interaction events can remove ligand targeted liposome particles from cell surfaces. Future work will look at how this removal process might affect therapeutic applications where the use of targeted drug-delivery particles coincides with the use of ultrasound–microbubble interactions either for imaging purposes or for creating microcapillary damage to enhance particle extravasation into tissue [17, 18].

6 Conclusions

The data presented here shows that ultrasound–microbubble interactions can remove cRGD-targeted liposomes from endothelial cell surfaces. This was seen to occur over regions of the cell surface that averaged 33.1 μm in diameter. The maximum distance between a single microbubble and a detached liposome was 34.5 μm . This means that the microbubble–ultrasound interaction effect on the cell membrane extends beyond the region of attachment between the microbubble and the cell surface. A single microbubble can have an impact of removing liposomes from an area that approximately covers one-half of a single cell or on a few cells if it is located in between them. Microbubbles in the 1–3 μm diameter size range removed liposomes at a significantly further distance than the microbubbles greater than 3- μm in diameter. There is a possibility that inertial cavitation was occurring with microbubbles in the 1–3 μm diameter size range. Clusters of adjoining microbubbles did not remove any liposomes from the cell surface.

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Conflict of interest The authors declare that they have no conflicts of interest.

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