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Optical Verification of Microbubble Response to Acoustic Radiation Force in Large Vessels with *In Vivo* Results

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Abstract

Objectives—To optically verify the dynamic behaviors of adherent microbubbles in large blood vessel environments in response to a new ultrasound technique using modulated acoustic radiation force.

Materials and Methods—Polydimethylsiloxane (PDMS) flow channels coated with streptavidin were used in targeted groups to mimic large blood vessels. The custom modulated acoustic radiation force beam sequence was programmed on a Verasonics research scanner. *In vitro* experiments were performed by injecting a biotinylated lipid-perfluorobutane microbubble dispersion through flow channels. The dynamic response of adherent microbubbles was detected acoustically and simultaneously visualized using a video camera connected to a microscope. *In vivo* verification was performed in a large abdominal blood vessel of a murine model for inflammation with injection of biotinylated microbubbles conjugated with P-selectin antibody.

Results—Aggregates of adherent microbubbles were observed optically under the influence of acoustic radiation force. Large microbubble aggregates were observed solely in control groups without targeted adhesion. Additionally, the dispersion of microbubble aggregates were demonstrated to lead to a transient acoustic signal enhancement in control groups (a new

phenomenon we refer to as “control peak”). In agreement with *in vitro* results, the “control peak” phenomenon was observed *in vivo* in a murine model.

Conclusions—This study provides the first optical observation of microbubble binding dynamics in large blood vessel environments with application of a modulated acoustic radiation force beam sequence. With targeted adhesion, secondary radiation forces were unable to produce large aggregates of adherent microbubbles. Additionally, the new phenomenon called “control peak” was observed both *in vitro* and *in vivo* in a murine model for the first time. The findings in this study provide us with a better understanding of microbubble behaviors in large blood vessel environments with application of acoustic radiation force, and could potentially guide future beam sequence designs or signal processing routines for enhanced ultrasound molecular imaging.

Keywords

Acoustic response; microbubbles; modulated acoustic radiation force; secondary radiation force; targeted adhesion

1. ABSTRACT

Ultrasound molecular imaging has been used for pre-clinical applications including detection of cancer and cardiovascular inflammation. Our group recently developed a modulated acoustic radiation force-based pulse sequence for detection of targeted adhesion using short imaging protocols and without necessitating control measurements. Many previous studies have performed optical verification of microbubble behavior in order to better understand microbubble binding dynamics. However, optical verification studies to date have been limited to small blood vessel environments or with application of constant acoustic radiation force. In this study, the first optical observation of adherent microbubble dynamic behaviors in large blood vessels under the influence of secondary radiation force and molecular forces was achieved. The formation of adherent microbubble aggregates was closely related to different types of binding. Large microbubble aggregates were observed solely in control groups without targeted adhesion. Additionally, the dispersion of microbubble aggregates were demonstrated to lead to a transient acoustic signal enhancement in control groups (a new phenomenon we refer to as “control peak”). *In vivo* verification of the *in vitro* results was performed in a large abdominal blood vessel of mouse model for inflammation. In agreement with *in vitro* results, the “control peak” phenomenon was observed only with control microbubbles immediately after cessation of acoustic radiation force. Findings in this study could potentially guide design and/or development of beam sequences and signal processing methods for enhanced ultrasound molecular imaging.

2. INTRODUCTION

Microbubbles are the most widely studied ultrasound contrast agent with demonstrated pre-clinical efficacy for anatomic/molecular imaging and drug/gene delivery [1]–[6]. In ultrasound molecular imaging, targeted microbubbles are conjugated with ligands that bind to molecular markers associated with diseases of interest. Because microbubbles are gas-filled and exhibit a highly nonlinear response to impinging ultrasound, they give rise to a strong, harmonic rich, echo signal that provides clear differentiation of regions containing

microbubbles (*e.g.* blood vessels and tumors) and surrounding tissue. Consequently, molecularly targeted microbubbles are designed to attach to vascular endothelium through specific ligand-receptor bonds and identify regions of tissue associated with disease biomarkers [7]–[10]. Pre-clinical success of ultrasound molecular imaging has been demonstrated in small blood vessel environments for various diseases including breast [11]–[13], prostate [14], [15] and pancreatic [16] cancers, atherosclerosis [17]–[19], and inflammatory diseases of the bowel [20]. In these studies, various imaging strategies have been used to address the challenge of differentiating the signal arising uniquely from bound adherent microbubbles from the signals attributable to freely circulating microbubbles and tissue. First, in all instances, some form of nonlinear microbubble detection such as pulse inversion (PI) or “contrast pulse sequences” (CPS) is used [21], [22]. These techniques work by suppressing (primarily via cancellation) signals from surrounding tissue that exhibit a lower harmonic response compared to microbubbles [21], [22]. However, these methods alone do not isolate bound microbubble signals from freely circulating microbubble signals. In order to isolate the bound microbubble signal, several strategies are used in combination with nonlinear microbubble detection, such as waiting periods (8 – 12 min) for free microbubble clearance [8], [23] or the use of acoustic destruction-replenishment [24], [25]. More recently, several real-time signal processing methods have been developed for real-time (*i.e.* no waiting period) measurement of bound microbubbles. For instance, a dwell-time based fast method was introduced for application of cancer imaging *in vivo* [26], [27]. Other techniques involve slow-time inter-frame frequency based filtering methods [28]–[30].

Recently, ultrasound molecular imaging in large blood vessel environments has received attention [19], [31]. In this case, obtaining high binding efficiency is a challenge due to the higher flow rates and less microbubble-vessel wall contact. Two primary strategies have been developed for improving binding efficiency in large blood vessel applications. First, increased binding efficiency can be achieved by incorporating multiple ligands into the targeted microbubble shell, for example, for detecting inflammation, microbubbles targeting both P-selectin and VCAM-1 [32]; and microbubbles incorporated with both P- and E-selectin antibodies have been used [20]. Secondly, the application of acoustic radiation force (ARF) pulses within the microbubble imaging sequence has been studied as a means to force the microbubbles in closer proximity to the vessel wall and increase the attachment to potential binding sites [33]–[37]. The approach has been demonstrated *ex vivo* in large vessel environments where nonlinear detection and slow-time inter-frame filtering was used to detect adherent microbubbles [38], [39]. In other approaches, such as singular spectrum-based targeted molecular (SiSTM) imaging [39]–[41], a statistical signature specific to the adherent microbubble signal in large blood vessels was also demonstrated to achieve good results via ARF.

Recently, our group developed a new modulated ARF-based imaging sequence [42]. In this technique, ARF pulses were turned on for a given period; and acoustic data was collected continuously both during the application and after cessation of ARF. A parameter related to the normalized amount of residual adherent microbubbles was extracted from this sequence. *In vitro* studies have demonstrated several advantages of the modulated ARF approach,

primarily including the ability to quantitatively measure molecular marker concentration along the large vessel wall [43], [44] using a short imaging sequence that does not require a separate control injection to normalize for non-specific binding [27], [45]. The ability to quantitatively measure the molecular marker concentration has potential clinical significance as it could enable better interventional decisions such as by establishing more reliable and quantitative thresholds. In these early *in vitro* studies, several unexplained phenomena were observed in the microbubble echo signal. In particular, we observed the presence of a transient echo signal enhancement in control microbubble populations (hereafter referred to as the “control peak”) [42]. Furthermore, to the best of our knowledge, to date no optical verification studies have been performed that observe the interactions between microbubble dynamics, ARF, secondary ARF, and targeted adhesion forces in large blood vessel environments.

In this paper, we perform the first observation of microbubble dynamics in large blood vessel environments with synchronized optical microscopy and ultrasound imaging. The effects of ARF and targeted adhesion were examined and the physical mechanism responsible for the transient ultrasound signal spike (control peak) was elucidated. We verified the hypothesis that the dispersion of microbubble aggregates gives rise to the control peak. Finally, acoustic responses of adherent microbubbles observed in *in vitro* setups were verified *in vivo* in a large abdominal blood vessel in a murine model for inflammation.

3. MATERIALS AND METHODS

3.1. Microbubble preparation

Biotinylated lipid-perfluorobutane microbubbles were synthesized in-house using existing methods [46]. The stock concentration of microbubbles was approximately $9 \times 10^9 \text{ mL}^{-1}$ with an average diameter of 2.2 μm (standard deviation of diameter = 1.5 μm , median diameter = 1.9 μm , resonance frequency $\approx 4.4 \text{ MHz}$) [47].

For the *in vitro* experiments with vessel-mimicking flow channels, the microbubbles were diluted in saline (0.9% sodium chloride) solution (Baxter Healthcare Corporation, Deerfield, IL, USA), to a concentration of $5 \times 10^5 \text{ mL}^{-1}$. To minimize non-specific adhesion, 0.5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) was added to the microbubble dispersion [42], [48].

For the *in vivo* experiments with mice, microbubbles targeted to the inflammation marker P-selectin were prepared. The biotinylated microbubbles were conjugated to biotinylated monoclonal P-selectin antibody (RB40.34, BD Biosciences, CA, USA) as previously described [8]. Streptavidin was used to couple the antibody to the microbubble surface. The antibody was added at a concentration of 1.5 μg per 10^7 microbubbles to obtain maximal surface coverage [8]. Biotinylated microbubbles, without antibodies, were used as controls.

3.2. Modulated ARF pulse sequence

The modulated ARF pulse sequence was programmed on a Verasonics programmable ultrasound scanner (Vantage System, Verasonics, Redmond, WA, USA). The details of this

custom pulse sequence were described in previous studies [42]–[44]. Briefly, the sequence collected raw radiofrequency (RF) data continuously over 180 s at a frame rate of 5 Hz. The sequence was separated into three sections: 10 s of imaging, 70 s of interspersed imaging and ARF, and 100 s of imaging – displaying the baseline, rise, and decay of adherent microbubble concentration along the bottom channel wall (Fig. 1). A L12-5 38mm linear array and a CL15-7 compact linear array transducer (Philips Healthcare, Andover, MA, USA) were used for the *in vitro* and *in vivo* experiments, respectively. The acoustic parameters of the imaging and ARF pulses from both transducers are listed in Table I. Acoustic pressures of the pulses were confirmed by a calibrated PVDF hydrophone (GL-0200, Onda, Sunnyvale, CA, USA). In order to minimize the possible effects of imaging pulses on microbubble binding dynamics, the voltage applied on the ultrasound transducer for the imaging pulses were maintained at the lowest limit of the scanner (1.6 V), resulting in a mechanical index (MI) of 0.02 for L12-5 38mm transducer and 0.003 for CL15-7 transducer. The maximum pressure of ARF pulses was measured as 189.3 kPa (MI = 0.09) for L12-5 38mm transducer and 126.9 kPa (MI = 0.06) for CL15-7 transducer, which are both well below the limit above which bursting may occur [49].

3.3. PDMS flow channels

Due to its good optical transparency and simple, robust fabrication process [50], [51], polydimethyl-siloxane (PDMS) was used to fabricate the flow channels mimicking large blood vessels. PDMS (Sylgard 184, Dow Corning, Midland, MI, USA) with 10:1.1 pre-polymer to curing agent ratio was mixed and placed in a vacuum chamber to degas for 30 min. The PDMS was then poured into clean petri dishes with 4 mm-diameter borosilicate glass rods (McMaster-Carr, Robbinsville, NJ, USA) placed horizontally to mold the bottom part of the flow channels (Fig. 2a, Step 1). For the top cover of the flow channels, 1 mm-thick PDMS layer was poured into a petri dish. After the PDMS was cured at 80 °C for 30 min, the glass rods were carefully cut out in a dust-free environment (Fig. 2a, Step 2). The top and bottom PDMS layers were plasma treated using a laboratory corona treater (BD-20, Electro-Technic Products, Chicago, IL, USA) [52] and then bonded together (Fig. 2a, Step 3). Finally, connection tubing was glued to the two ends of the PDMS device. Targeted flow channels were incubated with 50 µg/mL streptavidin (AnaSpec, Fremont, CA, USA) solution for 12 h while control flow channels were not incubated. Before the experiments, both targeted and control flow channels were flushed with 1% (w/v) BSA (Sigma-Aldrich, St. Louis, MO, USA) solution to prevent non-specific adhesion [38], [42].

3.4. In vitro experiment setups

The response of microbubbles to modulated ARF pulse sequence in the PDMS flow channels was detected acoustically and visualized optically. The acoustic system consisted of a Verasonics scanner equipped with a L12-5 38 mm transducer. The ultrasound transducer was placed perpendicular to the flow channel, 10 mm away from the top surface of the PDMS device (Fig. 2b). Fresh microbubble dispersion was prepared every 10 min during the experiments and the concentration was measured using a Coulter counter (Coulter Multisizer 3, Beckman Coulter, Brea, CA, USA). A syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) was used to inject the microbubble dispersion into the flow channels at a flow rate of 45.2 mL/min (6.0 cm/s).

For optical visualization, the PDMS device and the ultrasound transducer were fixed on the stage of an inverted microscope (IX71, Olympus, Center Valley, PA, USA). The objective lens (M-Plan 40X ELWD Brightfield Objective, numerical aperture = 0.5, working distance = 10.10 mm, Nikon, Japan) was focused at the bottom channel wall, while the ultrasound transducer was vertically aligned to the axis of the objective lens. The depth of field of the objective lens was calculated to be approximately 3 μm , which is suitable to resolve only one layer of adherent microbubbles. The optical resolution was calculated to be approximately 0.6 μm . A fiber optic illuminator (NOVAFLEX Fiber Optic Illuminator, World Precision Instruments, Sarasota, FL, USA) was used as the light source for the microscope. The optical light guide was placed adjacent to the ultrasound transducer and angled at approximately 25° with respect to the axis of the objective lens. Consequently, the most directly transmitted light from the light guide missed the lens and thus produced an almost black background; and most scattered light from the transparent microbubbles could still enter the lens and thus form the bright microbubble image. A digital single-lens reflex camera (EOS Rebel T3i, Canon, Japan) was connected to the inverted microscope to record images at a frame rate of 60 Hz. The microscope field of view (FOV, 314.8 $\mu\text{m} \times 176.8 \mu\text{m}$) was large enough to include a significant number (typically greater than 50) of microbubbles for statistical analysis.

3.5. In vivo experimental setups

All animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee at the University of Virginia. C57/BL6 female mice (The Jackson Laboratory, ME, USA) were used for the study. The inflammation marker P-selectin was selected for microbubble targeting [8], [53]. Upregulation of P-selectin was achieved by intraperitoneal injection (IP) of tumor necrosis factor- α (TNF- α , Sigma-Aldrich, St. Louis, MO, USA) approximately 2 – 3 hours before the start of imaging (0.5 μg per mouse). The mouse was initially anesthetized in the induction chamber using 2.5% isoflurane in air. It was then transferred to a heated imaging table (TM150, Indus Instruments, Houston, TX, USA), laid prone, and kept under anesthesia (2% isoflurane) throughout the course of the experiment (Fig. 3a). A tail-vein catheter was affixed to administer the microbubbles. The inferior vena cava was imaged using a CL15-7 compact linear array transducer at a frequency of 15 MHz (Fig. 3b). Due to the proof-of-concept nature of the work, only one animal per experimental condition was used.

3.6. Experimental framework

The first set of *in vitro* experiments was designed to demonstrate the acoustic response of adherent microbubbles with application of the modulated ARF pulse sequence (Fig. 1a). Microbubbles at a concentration of $5 \times 10^5 \text{ mL}^{-1}$ in saline were perfused continuously through the flow channels at a flow rate of 45.2 mL/min (average flow velocity of 6.0 cm/s). The ultrasound scanner (equipped with L12-5 38mm transducer) and the video microscopy camera recorded simultaneously and collected data continuously for 180 s. Ten trials each were performed on targeted and control flow channels. The trials were performed on multiple PDMS devices and on different days to eliminate the measurement bias from device-to-device and microbubble batch-to-batch variations, respectively.

The second set of *in vitro* experiments was designed to confirm the hypothesis that the dispersion of microbubble aggregates leads to the transient ultrasound signal enhancement (control peak [42]) (Fig. 1b). The same modulated ARF pulse sequence was applied to the control flow channels. Microbubble dispersion at the same concentration ($5 \times 10^5 \text{ mL}^{-1}$) and flow rate (45.2 mL/min) as the previous set of experiments was injected through flow channels for the first 70 s. The syringe pump was then turned off and the flow channel was sealed by switching off the stopcocks at the two ends of the flow channel. Ten trials of control flow channels were performed.

The *in vivo* experiments were designed to verify the *in vivo* presence of a control peak, which occurs only with control microbubbles immediately after cessation of acoustic radiation force. For each mouse, a bolus injection of 25×10^6 microbubbles (100 μL at a concentration of $250 \times 10^6 \text{ mL}^{-1}$) was administered through a tail vein catheter. Simultaneously, the modulated ARF pulse sequence (from CL15-7 transducer) was applied. The experiments were designed to have the following mouse and microbubble combinations: (1) control mouse with control microbubbles ($C_{\text{Mouse}} + C_{\text{MB}}$); (2) targeted mouse with control microbubbles ($T_{\text{Mouse}} + C_{\text{MB}}$); and (3) targeted mouse with targeted microbubbles ($T_{\text{Mouse}} + T_{\text{MB}}$).

3.7. Data analysis

For the *in vitro* experiments, raw RF data exported from the Verasonics system was beamformed and then analyzed to extract the ultrasound signal magnitude curves within the region of interest (ROI). The width of the ROI was chosen as 2 mm so that ten consecutive laterally adjacent A-lines could be averaged together to increase the signal-to-noise ratio (SNR). The depth of the ROI was chosen as 0.2 mm to include just the entire bottom vessel wall. The location of the ROI was determined by a custom designed program that detected the bottom vessel wall based on its high signal magnitude. For each trial, the average signal magnitude of the focused echo data within the ROI was calculated over time (180 s) from the 900 consecutive image frames [42].

Video data exported from the camera was used to analyze the spatial and temporal properties of the adherent microbubbles. For each frame of the video, the color image was converted to a binary image with proper thresholds to differentiate the adherent microbubbles (bright) from background (dark). Two parameters were used to characterize the spatial distribution of adherent microbubbles based on each frame of binary images. Normalized microbubble area (A_{MB}) was defined as the ratio of the combined area of all adherent microbubbles to the area of microscope FOV:

$$A_{\text{MB}} \left(\frac{N_{\text{MB}}}{N_{\text{FOV}}} \right) \times 100\% \quad (1)$$

where N_{MB} is the sum of all white pixels of the image, and N_{FOV} is the number of pixels of the image. Normalized area of the maximum aggregate ($A_{\text{aggregate}}$) was defined as the ratio of the area of the largest microbubble aggregate to the area of microscope FOV:

$$A_{aggregate} = \left(\frac{N_{aggregate}}{N_{FOV}} \right) \times 100\% \quad (2)$$

where $N_{aggregate}$ is the sum of white pixels of the largest microbubble aggregate.

For analysis of the *in vivo* experiments, the signal magnitude curves of adherent microbubbles were obtained by averaging signal magnitudes of all pixels within the ROI. Due to the irregularity of the blood vessel shape and effects of physiological motions, a different method was used to define the ROI of the bottom vessel wall (Fig. 4). First, the focused RF data of all 900 frames was summed together to form the “hot” mapping illustrated in Fig. 4b. Then, a larger rectangular region (4.0 mm × 1.4 mm, green window in Fig. 4b) placed on the bottom vessel wall was used to establish the ROI. The area within this region with signal magnitudes higher than half of the maximum signal magnitude of this region was defined as the ROI of adherent microbubbles (Fig. 4c). This ROI-definition method was used consistently to eliminate bias when selecting the ROI and reduce the effects of physiological motions on the location of the ROI.

The data analysis was performed in the MATLAB (Mathworks, Natick, MA, USA) environment. For Student’s *t*-tests, the differences were considered statistically significant for calculated *p*-values less than 0.05.

4. RESULTS

4.1. Acoustic responses in control and targeted channels

Signal magnitude curves in response to the modulated ARF pulse sequence are demonstrated in Fig. 5. For both control and targeted experiments, the curves represent the average from 10 trials. During the first 10 s when ARF pulses were not transmitted, the background signal magnitude of the bottom channel wall without any adherent microbubbles was shown to be approximately 1.5×10^3 (a.u.). During the next 70 s period with application of ARF, the signal magnitude in the control flow channels approached a steady-state value of approximate 2.5×10^3 (a.u.), which was 1.7-fold greater than the background. In the targeted (streptavidin-coated) flow channels, the signal magnitude increased in an exponential manner to a maximum magnitude of approximately 3.5×10^3 (a.u.), which was 2.3-fold greater than the background signal. The maximum increase of signal magnitude in the targeted flow channels has doubled compared to the control flow channels. During the last 100 s period after cessation of ARF, the signal magnitude in control flow channels dropped back to the background level ($p > 0.2$, $n = 10$). However, in the targeted flow channels a significantly higher residual signal remained ($p < 0.001$, $n = 10$). In the control flow channels a transient signal magnitude spike (control peak) [42] was observed immediately after cessation of ARF (Fig. 5a, d*, $t = 81.8$ s).

For further illustration of the trends observed in the signal magnitude curves, microscopy images (from one trial) from both control and targeted flow channels are provided in Fig. 5 (c to h). In the control flow channels, with application of ARF, adherent microbubbles were found to aggregate, ranging from several to hundreds of microbubbles (Fig. 5c, white

arrows). At the control peak ($t = 81.8$ s), large aggregates of adherent microbubbles were spread out (Fig. 5d). Few microbubbles remained adherent on the bottom channel wall after the cessation of ARF (Fig. 5e). In targeted flow channels, under ARF application, no large aggregates (size > 10 microbubbles) of adherent microbubbles were observed. After cessation of ARF, most of the adherent microbubbles (over 90% based on microbubble area) remained bound to the bottom channel wall. Furthermore, most bound microbubbles remained attached to the wall without any discernible motion over time ($t = 70.0, 81.8$, and 170.0 s).

4.2. Secondary radiation force led to generation of aggregates

In the control channels, where targeted adhesion was absent, the aggregation of adherent microbubbles occurred due to secondary radiation force [54]. Representative time-series images (Fig. 6) demonstrate the merging of two adherent microbubble aggregates with the formation of larger aggregate under the influence of ARF. On the other hand, the cessation of acoustic transmission resulted in immediate disruption of the microbubble aggregates. A representative example of aggregate dispersion after cessation of ARF is shown in Fig. 7 (ARF transmission stopped at $t = 80.0$ s). The aggregates of adherent microbubbles would usually break up and become sparsely distributed within approximately one second.

Quantification of the generation of microbubble aggregates in control flow channels is illustrated in Fig. 8. Normalized microbubble area (A_{MB}) was used to quantify the adherent microbubble fraction in the microscope FOV. Average A_{MB} gradually increased to approximately 1.5% from $t = 10.0$ to 80.0 s (Fig. 8a, black curve). After the cessation of ARF, A_{MB} rapidly dropped to zero and continued to be negligible for the remaining 100 s. In addition, the area of the largest aggregate of adherent microbubbles was quantified by normalized area of the maximum aggregate ($A_{aggregate}$). Average $A_{aggregate}$ gradually increased to approximately 1% from $t = 10.0$ to 80.0 s (Fig. 8b, black curve). After cessation of ARF, the average $A_{aggregate}$ also quickly decreased to zero for the remaining 100 s. In the control flow channels, the ratio of average $A_{aggregate}$ to average A_{MB} was approximately 0.5 during ARF application (from $t = 10.0$ to 80.0 s), demonstrating that the largest aggregate of adherent microbubbles consisted of half the total number of adherent microbubbles within the microscope FOV.

4.3. Targeted adhesion prevented generation of aggregates

Unlike secondary radiation force or flow shear forces that tend to displace microbubbles, the binding force of molecular bonds immobilizes microbubbles on the wall. In targeted flow channels with strong specific adhesion, it was more difficult for secondary radiation force or flow shear force to move molecularly bound microbubbles than that in control flow channels (Fig. 5). Quantification of the binding dynamics of adherent microbubbles in targeted flow channels is shown in Fig. 8. From $t = 10.0$ to 80.0 s, average A_{MB} linearly increased with time from approximately 0% to 1.7% (Fig. 8c, black curve, $R^2 = 0.99$). From $t = 80.0$ to 180.0 s, average A_{MB} remained at a constant level of 1.77%. Similarly, from $t = 10.0$ to 80.0 s, average $A_{aggregate}$ linearly increased with time from 0% to 0.15% (Fig. 8d, black curve, $R^2 = 0.84$). From $t = 80.0$ to 180.0 s, average $A_{aggregate}$ maintained a constant level of 0.15%. The maximum average $A_{aggregate}$ in targeted channels was approximately 0.15%, a 10-fold

reduction in the size of the largest aggregate of adherent microbubbles compared to the control channels (maximum average $A_{\text{aggregate}} = 1.4\%$). In addition, in the targeted flow channels, the ratio of average $A_{\text{aggregate}}$ to average A_{MB} remained approximately 0.08 after cessation of ARF (from $t = 80.0$ to 180.0 s).

4.4. Further evidence of aggregate dispersion as the cause of the control peak phenomenon

Signal magnitude curves in response to modulated ARF pulse sequence with modulated flow (Fig. 1b) are displayed in Fig. 9a. The curve represents the average from 10 trials. For the first 10 s ($t = 0.0$ to 10.0 s) with flow but no ARF, the background signal magnitude was approximately 1.9×10^3 (a.u.). During the next 60 s ($t = 10.0$ to 70.0 s) with flow and application of ARF, the signal magnitude in control channels approached a steady-state magnitude of approximately 3.0×10^3 (a.u.). For the next 10 s ($t = 70.0$ to 80.0 s) with application of ARF but no flow, there was an immediate increase of approximately 0.3×10^3 (a.u.) in signal magnitude. This was due to less attenuation effect from freely circulating microbubbles, since no microbubbles were circulating. After cessation of ARF, a transient signal magnitude enhancement (control peak) of relatively low amplitude was observed ($t = 84.0$ s). The average curve from 80.0 to 86.0 s (red box in Fig. 9a) is shown in Fig. 9b. In this plot, the signal magnitude of each trial was normalized to its saturation magnitude at $t = 70.0$ s. The normalized signal magnitudes at $t = 84.0$ s (control peak) were significantly higher than those at $t = 80.0$ s (ARF just stopped) ($p < 0.05$, $n = 10$). After the control peak ($t = 84.0$ s), signal magnitude quickly dropped to a low level of about 2.0×10^3 (a.u.) at approximately $t = 100$ s. Since no ARF was applied, most adherent microbubbles dispersed from the lower wall due to buoyancy forces.

Representative B-mode images, reconstructed from raw RF data, corresponding to different time points are provided in Fig. 9 (c to f). At $t = 65.0$ s (steady-state), the lumen was filled with flowing microbubbles (Fig. 9c). At $t = 75.0$ s, 5 s after flow was stopped, the flow channel lumen was almost completely cleared, with microbubbles pushed downward to form a thin layer near the bottom channel wall (white arrows in Fig. 9d). At $t = 100.0$ s, microbubbles were gradually floating up, increasing the thickness of the microbubble layer (white arrows in Fig. 9e). At $t = 170.0$ s, the lumen was replenished with microbubbles (white arrows in Fig. 9f).

In addition, the microscopy images confirmed the dispersion of the aggregates due to buoyancy force after the cessation of ARF. Time-series images from one trial revealing the dispersion of an aggregate are shown in Fig. 10. Typically, aggregates with smaller sizes tended to disperse completely after ARF was turned off (white arrow in Fig. 10). It usually took approximately 20 s for the aggregates to disperse. On the other hand, aggregates with larger sizes tended to partially disperse after ARF was turned off (black arrow in Fig. 10). Blue arrows in Fig. 10 track a detached part of a partially dispersed aggregate. Even at $t = 180.0$ s, some large aggregates of adherent microbubbles remained on the bottom channel wall.

4.5. In vivo observation of the “control peak” in large blood vessels

The ultrasound signal magnitude curves of adherent microbubbles from *in vivo* mouse experiments are illustrated in Fig. 11. The magnitude curves obtained from control and targeted mice injected with control microbubbles were similar (Fig. 11a and Fig. 11b, respectively). Signal magnitudes dropped below the baseline to their minimum levels at approximately 10 s due to attenuation caused by a high concentration of freely circulating microbubbles. The signal magnitudes gradually increased to their maximum levels at approximately 60 s. The magnitudes increased sharply to form the control peaks (black arrows in Fig. 11a and Fig. 11b) immediately after the ARF was turned off at 80 s. In the targeted (inflamed vessel) mouse, injected with targeted microbubbles (Fig. 11c), the signal magnitude increased monotonically during ARF application ($t = 10.0$ to 80.0 s). It remained at approximately the same level for the rest of the sequence. A slight peak was detected immediately after the ARF was turned off (black arrow in Fig. 11c).

5. Discussion

In this paper, we performed the first optical verification of microbubble binding dynamics in response to modulated ARF in large blood vessel environments. The results observed in the PDMS-based flow channels were in excellent agreement with the results obtained from tissue-mimicking gelatin phantoms in previous studies [42]–[44]. A transient signal magnitude enhancement (control peak) was found immediately after ARF was turned off. The explanation for control peak was revealed by a series of experiments. In addition, for the first time, the control peak phenomenon was observed *in vivo* in a mouse model. The acoustic response of adherent microbubbles with application of modulated ARF sequence was also verified *in vivo*.

5.1. First optical verification of microbubble behaviors in large vessels

As revealed in previous studies [54], secondary radiation forces generate aggregation of adherent microbubbles. In control channels, where targeted adhesion was absent, the movement of adherent microbubbles was driven by secondary radiation force and/or flow shear force. Adherent microbubbles tended to aggregate with surrounding microbubbles and gradually grew into large aggregates (Fig. 6). After the cessation of ARF, those aggregates rapidly dispersed due to flow shear forces (Fig. 7). However, in targeted flow channels the movement of adherent microbubbles was strongly restricted by molecular binding forces. Consequently, no large aggregate formation was observed. Most adherent microbubbles also remained attached to the vessel wall after cessation of ARF (Fig. 8). In control flow channels, most adherent microbubbles possessed different kinds of motion, including sliding, rolling, aggregation, and dispersion. In the targeted flow channels, most adherent microbubbles remained firmly attached to their binding sites. Consequently, the differences of microbubble dynamics between targeted and control channels are significant, and could potentially be used to guide the development of new beam sequence or signal processing methods for better detection/isolation of adherent microbubbles.

5.2. Explanation for the “control peak”

From the results shown in Fig. 5 to Fig. 8, it is likely that the dispersion of microbubble aggregates in control channels caused the control peak. The results from Fig. 9 and Fig. 10 provide additional evidence for this conclusion. In these experiments, we controlled for the effects of flow shear forces. After ARF was turned off and without flow shear force, the only motion that microbubble aggregates possessed was dispersion caused by buoyant force. Consequently, the control peak in ultrasound signal was directly caused by the dispersion of microbubble aggregates. In the absence of flow shear force, only a certain portion of aggregates dispersed and flowed upward toward the transducer. As a result, the control peak observed in Fig. 9 had much smaller amplitude than that in Fig. 5.

5.3. First in vivo observation of the “control peak”

Although the control peak was observed consistently in previous studies under various acoustic and flow parameters, results were limited to *in vitro* experiments [42], [44]. Fig. 11 shows the first *in vivo* examples of control peaks. Interestingly, a tiny control peak was observed in the trial with the targeted (inflamed) mouse and targeted microbubbles (Fig. 11c, black arrow). The *in vitro* studies were conducted with biotinylated bubbles targeted to streptavidin. And biotin-streptavidin binding is the strongest known non-covalent interaction. Antibody-mediated microbubble binding to P-selectin is weaker than biotin-streptavidin interaction. This probably resulted in a small portion of targeted microbubbles exhibiting similar dynamics to control microbubbles. Overall, the results demonstrated that the control peak should be a universal phenomenon associated with the application of modulated ARF, and would be observed with high probability in future *in vivo* experiments. Our theory developed from *in vitro* phantom experiments could provide a good explanation for the control peaks observed *in vivo*. Furthermore, the control peak could possibly provide more information related to binding characteristics, which could be used to better detect/isolate targeted adhesion of microbubbles.

5.4. Limitations

The modulated ARF-based imaging sequence is designed for applications in large blood vessel environments. Signal magnitude of adherent microbubbles on the bottom vessel wall was analyzed using this sequence. The sequence is expected to be ineffective in small vessel environments (*e.g.* capillaries in tumor) where the bottom vessel wall cannot be resolved, and therefore, isolation of signals derived solely from adherent microbubbles is not possible. Additionally, the modulated ARF-based sequence relies on the relatively high flow shear forces in large blood vessels to disperse non-specifically bound MBs from the bottom vessel wall. Consequently, in small blood vessels with insufficient flow shear forces, the sequence is not expected to effectively differentiate targeted from non-specific microbubble adhesion.

One limitation of the current *in vivo* study was the use of biotinylated microbubbles as control microbubbles rather than non-specific isotype, which would have reduced the effects of non-specific binding. However, the low level of residual microbubbles observed in control microbubble experiments suggest that non-specific binding had only modest effects on our results and that our conclusions of feasibility demonstration is valid.

The *in vivo* experiments of this study were conducted in the inferior vena cava of mouse. Compare to large arteries, blood flow in the inferior vena cava exhibited lower flow velocity and pulsatility. Consequently, conclusions drawn from this *in vivo* study are limited to low-velocity and low-pulsatility flow, and may not be applicable for large arteries.

5.5. Conclusions

The significance of this study is related to microbubble binding dynamics in large blood vessel environments with application of modulated ARF, and future enhancements of ultrasound molecular imaging. The current studies on microbubble binding dynamics in large vessels are limited to their acoustic response. In this study, we not only studied the acoustic response, but also simultaneously observed the dynamics of adherent microbubbles optically. The formation of microbubble aggregates was observed to be closely related to different types of binding. In addition, we demonstrated that the dispersion of microbubble aggregates led to a transient acoustic signal enhancement (control peak). For the first time, we observed the control peak *in vivo* in a mouse model. Overall, the findings in this study provide us with better understanding of microbubble behavior in large blood vessel environments due to acoustic radiation force, and could guide future beam sequence design or developments of signal processing methods for enhanced ultrasound molecular imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematics of pulse sequences and corresponding flow status. The flow rate is 45.2 mL/min (6.0 cm/s) when flow status is ON. (a) Experiments with constant flow. (b) Experiments with modulated flow.

Figure 2.

Experimental setups used for real-time imaging of adherent microbubbles on the bottom channel wall. (a) Procedure for fabrication of PDMS flow device. The flow channels have a diameter of 4 mm. (b) Schematic of hardware components involved in the experimental platform. The ultrasound transducer (L12-5 38mm) was placed 10 mm above the PDMS device so that the distance between ultrasound transducer and bottom channel wall was 15 mm. The optic light guide was mounted at an angle of approximately 25° with respect to the transducer axis. Dimensions of the components are not to scale.

Figure 3.

(a) Experimental setup used for *in vivo* imaging of mouse. The mouse was in supine position. The location and orientation of the ultrasound transducer (CL15-7) was optimized to provide best view of inferior vena cava of the mouse. Dimensions of the components are not to scale. (b) A sample image of the inferior vena cava of the mouse.

Figure 4.

A depiction of data processing methods used for *in vivo* mouse experiments. (a) A stack of 900 B-mode images (5 Hz frame rate for 180 s) obtained using the modulated ARF pulse sequence showing the inferior vena cava of mouse. (b) The sum of all 900 frames of focused acoustic echo data (radiofrequency data). The green window (4.0 mm $\times$ 1.4 mm) shows a zoomed-in region on the bottom vessel wall used to establish the region of interest (ROI). The area within the green window with signal magnitude higher than half of its maximum magnitude was defined as ROI (c). The signal magnitude curve of adherent microbubbles was calculated by averaging signal magnitudes within the ROI.

Figure 5.

Averaged ultrasound signal magnitude curves of adherent microbubbles for control (a) and targeted (b) flow channels. Solid lines indicate the mean values from ten trials. Light color shadows indicate the corresponding error bars at the range of [mean $\pm$ standard deviation]. Images of adherent microbubbles (bright dots) within the microscope field of view (FOV) at different times: (c) control, $t = 70.0$ s, c^* in (a); (d) control, $t = 81.8$ s (control peak), d^* in (a); (e) control, $t = 170.0$ s, e^* in (a); (f) targeted, $t = 70.0$ s, f^* in (b); (g) targeted, $t = 81.8$ s, g^* in (b); (h) targeted, $t = 170.0$ s, h^* in (b). Microbubble dispersion flowed from left to right at a flow rate of 45.2 mL/min (6 cm/s) for the entire 180.0 s. Acoustic radiation force was applied from $t = 10.0$ to 80.0 s. White arrows (c) indicate the large aggregates of adherent microbubbles.

Figure 6.

Time-series images from a control flow channel revealing the formation of an aggregate of microbubbles (bright dots) merging from two smaller aggregates of microbubbles. Microbubble dispersion flowed from left to right at a flow rate of 45.2 mL/min (6 cm/s). Images were acquired at times separated by 33.3 ms.

Figure 7.

Time-series images from a control flow channel revealing the dispersion of an aggregate of microbubbles (bright dots) immediately after the cessation of ARF (ARF stopped at $t = 80.0$ s) due to flow shear force. Microbubbles flowed from left to right at a flow rate of 45.2 mL/min (6 cm/s). Images were acquired at times separated by 33.3 ms.

Figure 8.

Normalized microbubble area (A_{MB}) curves at control (a) and targeted (c) flow channels. Normalized area of the maximum aggregate ($A_{\text{aggregate}}$) curves at control (b) and targeted (d) flow channels. Crosses indicate the raw data from ten trials. Black solid lines indicate the mean values. Right y-axis labels show the actual microbubble area ($A_{MB}A_{FOV}$) and actual area of maximum aggregate ($A_{\text{aggregate}}A_{FOV}$) with the unit of μm^2 . A_{FOV} represents the area of microscope field of view ($A_{FOV} = 2.78 \times 10^3 \mu\text{m}^2$).

Figure 9.

(a) Averaged ultrasound signal magnitude curve of adherent microbubbles for control flow channels. Microbubbles flowed from left to right at a flow rate of 45.2 mL/min (6 cm/s) from $t = 0.0$ to 70.0 s. Consequently, the signal magnitude increase between 70.0 and 80.0 s was due to less attenuation effect from freely circulating microbubbles. Acoustic radiation force was applied from $t = 10.0$ to 80.0 s. Solid lines indicate the mean values from ten trials. Light color shadows indicate the corresponding error bars at the range of [mean $\pm$ standard deviation]. (b) Zoomed average curve with time ranging from 80.0 to 86.0 s (red box in (a)). The signal magnitude of each trial was normalized to its saturation magnitude at $t = 70.0$ s. The normalized signal magnitudes at $t = 84.0$ s (control peak) were significantly higher than those at $t = 80.0$ s (ARF just stopped) ($p < 0.05$, $n = 10$). B-mode images showing the flow channel lumen at different times: (c) $t = 65.0$ s, c* in (a); (d) $t = 75.0$ s, d* in (a); (e) $t = 100.0$ s, e* in (a); (f) $t = 170.0$ s, f* in (a). Yellow box in (c) shows the region of interest (ROI) on the bottom channel wall. White arrows indicate the floating microbubbles.

Figure 10.

Time-series images from a control flow channel revealing the dispersion of an aggregate of microbubbles (white arrow) after the cessation of ARF (ARF was turned off at $t = 80.0$ s) due to buoyancy force. Another aggregate of microbubbles (black arrow) partially dispersed. Blue arrows track the detached part of this aggregate of microbubbles. Microbubble dispersion was static. Images were acquired at 2 s intervals.

Figure 11.

Ultrasound signal magnitude curves of adherent microbubbles for *in vivo* mouse trials of (a) control mouse and control microbubble ($C_{\text{Mouse}} + C_{\text{MB}}$), (b) targeted mouse and control microbubble ($T_{\text{Mouse}} + C_{\text{MB}}$), and (c) targeted mouse and targeted microbubble ($T_{\text{Mouse}} + T_{\text{MB}}$). The bolus of 25×10^6 microbubbles was injected simultaneously with initiation of the modulated ARF sequence.