Modifying the size distribution of microbubble contrast agents for high-frequency subharmonic imaging

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Purpose: Subharmonic imaging is of interest for high frequency (>10 MHz) nonlinear imaging, because it can specifically detect the response of ultrasound contrast agents (UCA). However, conventional UCA produce a weak subharmonic response at high frequencies, which limits the sensitivity of subharmonic imaging. We hypothesized that modifying the size distribution of the agent can enhance its high-frequency subharmonic response. The overall goal of this study was to investigate size-manipulated populations of the agent to determine the range of sizes that produce the strongest subharmonic response at high frequencies (in this case, 20 MHz). A secondary goal was to assess whether the number or the volume-weighted size distribution better represents the efficacy of the agent for high-frequency subharmonic imaging.

Methods: The authors created six distinct agent size distributions from the native distribution of a commercially available UCA (Targestar-P[®]). The median (number-weighted) diameter of the native agent was $1.63\text{ }\mu\text{m}$, while the median diameters of the size-manipulated populations ranged from 1.35 to $2.99\text{ }\mu\text{m}$. The authors conducted acoustic measurements with native and size-manipulated agent populations to assess their subharmonic response to 20 MHz excitation (pulse duration $1.5\text{ }\mu\text{s}$, pressure amplitudes $100\text{--}398\text{ kPa}$).

Results: The results showed a considerable difference between the subharmonic response of the agent populations that were investigated. The subharmonic response peaked for the agent population with a median diameter of $2.15\text{ }\mu\text{m}$, which demonstrated a subharmonic signal that was 8 dB higher than the native agent. Comparing the subharmonic response of different UCA populations indicated that microbubbles with diameters between 1.3 and $3\text{ }\mu\text{m}$ are the dominant contributors to the subharmonic response at 20 MHz. Additionally, a better correlation was observed between the subharmonic response of the agent and the number-weighted size-distribution ($R^2 = 0.98$) than with the volume-weighted size distribution ($R^2 = 0.53$).

Conclusions: Modifying the size distribution of the agent appears to be a viable strategy to improve the sensitivity of high-frequency subharmonic imaging. In addition, when the size distribution of the UCA has not been suitably modified, the number-weighted size distribution is a useful parameter to accurately describe the efficacy of the agent for high-frequency subharmonic imaging. © 2013 American Association of Physicists in Medicine. [<http://dx.doi.org/10.1118/1.4813017>]

Key words: nonlinear ultrasound imaging, subharmonic imaging, microbubble contrast agents, *vasa vasorum* imaging, atherosclerosis

1. INTRODUCTION

The rupture of atherosclerotic plaques may trigger life-threatening events such as myocardial infarction and stroke.¹ An imaging modality capable of predicting the likelihood of plaque rupture events could be used to objectively assess cardiovascular risk.² There is increasing evidence that the abnormal proliferation of the *vasa vasorum* is linked to plaque rupture.^{3,4} Therefore, the assessment of *vasa vasorum* density by imaging could help cardiologists identify life-threatening plaques.²

High-frequency (>10 MHz) contrast-enhanced-ultrasound imaging methods have been proposed to visualize the adventitial *vasa vasorum*.^{5,6} Nonlinear modes such as harmonic and subharmonic imaging could prove useful in this application, as they improve the detection of the agent.⁷ Goertz and colleagues have demonstrated the feasibility of visualizing the adventitial *vasa vasorum* in atherosclerotic rabbits with subharmonic intravascular ultrasound imaging.⁸ While their results are encouraging, the future clinical translation of this technique will depend on its ability to detect UCA response with high sensitivity and agent-to-tissue contrast.²

Phospholipid-encapsulated agents are being used for applications such as *vasa vasorum* imaging, because their high-frequency (HF) subharmonic response is reported to be considerably stronger than polymer-encapsulated agents.^{7,9} However, commercially available phospholipid-shelled UCA have a broad size distribution—only a small percentage of agent microbubbles may be responsible for the overall subharmonic response.¹⁰ Modifying the size distribution of UCA by enriching the number of “acoustically active” microbubbles could be a simple approach toward improving the sensitivity of high-frequency imaging.¹⁰

The goal of the work reported in this paper was to investigate size manipulated populations of the UCA to determine the range of microbubble sizes that produce the strongest subharmonic response at high-frequencies (in this case, 20 MHz). A secondary goal was to assess whether the number or the volume-weighted size distribution should be used to describe the efficacy of the agent for HF subharmonic imaging (SHI). We hypothesized that modifying the size distribution of the agent will enhance its subharmonic response at high frequencies. To corroborate this hypothesis, we measured the subharmonic response of six agent size distributions to 20-MHz excitation. These agent populations were created from Targestar-P® (Targeson Inc, San Diego, CA), a lipid-encapsulated decafluorobutane microbubble contrast agent, with a shell composed primarily of phospholipid and polyethylene glycol (PEG)-stearate.¹¹

2. PREVIOUS RELATED WORK

Harmonic and subharmonic imaging are contrast agent specific modalities that have been extensively researched for the conventional diagnostic frequency range (1–7 MHz).^{12–15} Harmonic imaging (HI) is limited at high frequencies because significant harmonic signal is also generated in tissues at diagnostic pressures, which degrades the agent-to-tissue contrast.⁷ SHI can achieve high agent-to-tissue contrast, because a measurable subharmonic response is not observed in tissues at diagnostic pressures.⁷ However, the primary limitation of HF-SHI is its poor sensitivity, which is caused by the (1) high pressure thresholds required to incite subharmonic response,¹⁶ and (2) weak subharmonic backscatter, due to high damping and smaller scattering cross section of the contributing microbubbles. Several groups, including ours, are developing techniques to improve the sensitivity of SHI at high transmit frequencies (>10 MHz).^{17–21}

Although alternate pulsing and detection schemes^{19,20,22,23} can improve imaging performance, SHI is limited by the lack of microbubble contrast agents that are optimized for HF applications.⁹ The size distribution of the agent is a major determinant of its acoustic activity.²⁴ Techniques such as decantation, centrifugation, and pore-filtration have been previously reported to isolate microbubble sizes from the native agent^{16,25} for adapting it for the frequency range of interest.

Streeter and colleagues conducted a study at 40 MHz to compare the (linear) imaging performance of essentially monodisperse contrast agents with diameters of 3.3 and 0.9 μm .²⁶ They demonstrated that the agent with larger diam-

eter produced better imaging performance, which is contrary to the predictions of classical scattering theory. This discrepancy can be explained by the observation that the linear frequency dependent backscatter of phospholipid-encapsulated UCA does not demonstrate sharp peaks.¹⁸ In the absence of strong resonance from smaller bubbles, the stronger acoustic signal from larger bubbles is expected, owing to their greater scattering cross section.¹⁸

The range of microbubbles sizes that contribute to the subharmonic signal can be different in size from those which contribute to the linear signal or other nonlinear imaging modes.^{18,24} Attenuation measurements have been previously reported to characterize the size dependent acoustic response of the agent.^{10,27} Goertz and colleagues showed that reducing the mean size of the agent shifts the peaks in their attenuation spectra shifted toward higher frequencies. These results suggested that smaller microbubbles are acoustically active at higher frequencies. Attenuation measurements can give insight into the resonant frequency of the agent, which may be useful to indicate the desirable UCA size for subharmonic imaging. However, recent studies have demonstrated that the subharmonic response may not peak exactly at the resonance or twice the resonance frequency of the agent.²⁸ Further, attenuation measurements are generally conducted in the linear regime at low pressures; the resonance frequency of microbubbles may change with the incident pressure.²⁹ Therefore, it is not clear how the size distribution of the agent affects its subharmonic response.

It had been previously assumed that microbubbles with diameters between 1 and 2 μm are the primary contributors to the subharmonic signal for transmit frequencies above 10 MHz.^{30–32} Consequently, an experimental UCA reportedly composed of micrometer to submicrometer sized microbubbles investigated for subharmonic imaging at 20–30 MHz frequencies.³² While this experimental UCA demonstrated noticeable subharmonic activity, it was not established that their size distribution was suitable for the range of frequencies used. Moreover, the exact size distribution of this agent was not reported for proprietary reasons.

Very few studies have attempted to experimentally compare the high-frequency subharmonic response of different agent sizes. Helfield and colleagues investigated the subharmonic response of individual microbubbles of five different lipid-shelled agents at 25 MHz.¹⁸ Their study has improved our understanding of microbubble dynamics and underscored the size dependence of the agent subharmonic response. However, nonuniformity of bubble shell properties and the presence of a boundary near the investigated microbubbles can make it difficult to infer the acoustic response of bulk UCA suspensions from studies conducted with single microbubbles.³³ The present study investigated the subharmonic response of bulk suspensions of microbubbles, which may be a better indicator of the imaging performance of UCA.

To our knowledge, only one study, by Cheung and colleagues has investigated the subharmonic response of size-manipulated agent populations. However, the major focus of their study was to assess whether microbubbles excited at resonance or twice the resonance frequency were involved in

subharmonic oscillations. Therefore, they did not attempt to suitably modify the size distribution of the agent; their investigation was limited to three agent size populations that were created by filtering Definity® (Lantheus Medical Imaging, North Billerica, MA). Further, all the populations investigated were smaller than the native agent. The present study investigated six UCA size distributions that were created using different protocols than those outlined by Cheung and colleagues. Also, a wider size range of microbubble populations was assessed—both larger and smaller than the native agent, to assess the desirable size range of microbubbles with greater accuracy.

In previous studies, Sontum *et al.* and Gorce *et al.* recommended that volume-weighted averages should be used instead of number-weighted averages as assay/dosage parameters for describing the efficacy of ultrasound contrast agents.^{24,34} They noted that microbubbles smaller than 3 μm did not contribute significantly to the acoustic signal for (linear) imaging at diagnostic frequencies (1–7 MHz), even though such microbubbles were most abundant in the agent formulation. However, volume distributions are weighted to the third power of the microbubble diameter, therefore they were representative of larger microbubble sizes, which were acoustically active. Consequently, they reported that the echogenicity of the agent was better correlated with its volume concentration, and proposed that the volume-weighted size distribution should be employed as the assay/dosage parameter. It is an important question to assess whether the volume weighted size distribution of the agent is also indicative of the efficacy of the agent for subharmonic imaging at high-frequencies. With this view in mind, we evaluated volume and number-weighted size distributions in this study as predictors of the subharmonic response of the agent.

3. METHODS

3.A. Agent handling and sizing

The agent size was measured with a Casy™ cell counter (Model TTC, Roche Innovatis AG., Switzerland). The Casy™ counter measured sizes from 0.7 to 20 μm , which were acquired using 1024 size channels, each having a width of 0.02 μm . The device reported the number of microbubble counts against the average size of the channel. The number/volume-weighted size distribution and the concentration of the agent were estimated from three independent measurements based on the principle of electrozone sensing.³⁵

3.B. Creating distinct agent size distributions

Contrast agents with median diameters between 1.35 and 2.99 μm were created from the native population. The microbubble diameters contained in these agent populations ranged from submicrometer to a few micrometers. Note that a large majority microbubbles in commercial UCA are <5 μm in diameter, because larger microbubbles may be filtered by the lung microcirculation.

The time-evolution of microbubble size was used to create contrast agents with lower median sizes than the native agent.

Microbubbles shrink in size over time due to gas diffusion through the shell,^{25,36} at a rate which is inversely proportional to their concentration. The agent provided by the manufacturer is sealed with perfluorocarbon headspace to reduce gas diffusion and to improve the shelf life of the agent. We extracted the agent from the vial, diluted it in phosphate buffered saline (PBS, pH = 7.4, containing 10.9 mM phosphates, 137 mM sodium chloride, and 2.7 mM potassium chloride) to a concentration of 1.15×10^9 microbubbles/ml, and allowed it to rest under air headspace at 1 atm and 5 °C to accelerate shrinking. Agent samples were periodically extracted, and their size distributions/concentrations were measured. Agent samples with mean diameters 1.44 and 1.35 μm were obtained after a period of 3 and 5 days, respectively. Although the concentration of microbubbles progressively decreased as the median size was lowered; up to 40% yields were obtained even for the smallest agent size created.

A gravity separation method similar to the technique reported by Sontum and colleagues³⁷ was used to create contrast agents with larger median diameters. Glass vials (2 ml in volume) were inverted, and the largest microbubbles were allowed to float to the top of the vial. The suspension in the lower half of the vial was then discarded, retaining the larger microbubbles. The resulting agent was diluted by adding 1 ml of PBS, and the process was repeated, to progressively enrich the population with larger microbubbles. In the current study, larger microbubbles—1.89 and 2.15 μm median sizes were prepared by one and two rounds of static floatation (30 min each). We found that floatation techniques were not efficient in creating sizes larger than the 2.15 μm agent. Therefore, the agent with 2.99 μm median diameter microbubbles was prepared by diluting Targestar-P 1:10 in PBS and centrifuging for 2 min at 200XG for five rounds, following the method of Feshitan and colleagues.²⁵

3.C. Characterizing subharmonic response of agent size distributions

The acoustic response of the six agent samples was characterized using the experimental setup shown in Fig. 1. We conducted transmission-mode^{17,38,39} RF measurements with two spherically focused transducers—a 20 MHz transmitting transducer (Model V317, Olympus NDT, Waltham, MA) and a 10 MHz receiving transducer (Model A312S, Olympus NDT). The measurements were conducted in a water tank with dimensions: 70 cm (length) $\times$ 50 cm (width) $\times$ 15 cm (height) at room temperature and atmospheric pressure. The transducers were aligned confocally using a computer controlled mechanical manipulating system (Bislide, Velmex Inc., Bloomfield, NY). The focal length and aperture diameters of both transducers were 51 mm and 6.35 mm, respectively. The frequency response of both transducers was assessed using a calibrated broadband membrane hydrophone (HGL-085, Onda Corporation, Sunnyvale, CA) as described previously.¹⁷

In each case, we diluted the agent distributions in Isoton™ (Beckman Coulter, CA) to a concentration of 1.5×10^6 microbubbles/ml, and kept them in suspension using a

FIG. 1. The experimental setup used for radio-frequency measurements conducted to characterize the subharmonic response of the agent sizes.

magnetic stirrer (Model 7X7-ALU, VWR International, Radnor, PA). The agent was held in a cylindrical plexi-glass chamber (50 mm diameter) with acoustically transparent windows (SaranTM wrap, S.C Johnson, WI) on diametrically opposite ends. An arbitrary waveform generator (Model 81150A, Agilent, Santa Clara, CA) was used to create rectangular gated sine-bursts (20 MHz center frequency, 30 cycles), which were amplified by a 40 dB linear power amplifier (Model 411LA, ENI, Rochester, NY) and fed to the transmitting transducer. A pulse repetition frequency of 1 kHz was used. To reduce the likelihood of capturing reflected signal from the water tank, we took two precautions: (1) a large tank (70 cm length) was used to attenuate potential wall reflections, and (2) the tank walls were angled relative to the acoustic axis of the transmitting transducer to prevent potential reflected waves from reaching the receiving transducer.

Low peak negative pressures (100–398 kPa) were used to reduce the likelihood of microbubble disruption.

The signals received by the 10 MHz transducer were amplified by 20 dB using a low noise amplifier (Model DPR, JSR Electronics, Pittsford, NY) and digitized to 12 bit precision at a sampling frequency of 500 MHz with a digital oscilloscope (Lecroy Wave Runner 44MXi, Lecroy Inc., Chester Ridge, NY). We averaged 40 traces in the frequency domain to improve the signal-to-noise ratio of the acquired spectra. Five groups of such spectra were acquired for each transmit setting and stored to disk for offline analysis in MATLABTM (The Mathworks Inc., Natick, MA). Baseline signals were acquired for each transmit condition, by replacing the agent chamber with an identical chamber which was filled only with IsotonTM solution. The received signal was processed carefully to remove (1) potential nonlinear propagation effects at the subharmonic frequency, and (2) spectral overlap of the fundamental and the subharmonic signal. Low-frequency nonlinear propagation artifacts can be generated by self-demodulation.²⁰ For rectangular gated signals such as those used in the study, the self-demodulation signal accumulates primarily at the beginning and the end of the excitation pulse.²⁰ We first eliminated the first and last two cycles of the received signal to eliminate self-demodulation signal generated by nonlinear propagation. Then, we employed a Chebyshev window to estimate the spectrum of the signal, which reduced the sidelobes of the transmitted signal by over 100 dB. We then computed the strength of the subharmonic signal relative to the baseline signal at 10 MHz. This processing methodology ensured that the subharmonic signal was contributed predominantly by microbubble scattering, and was unaffected by the directly transmitted signal.

4. RESULTS

Figures 2(a) and 2(b) show the estimated number and volume-weighted size distributions of (native) Targestar-P[®],

FIG. 2. (a) The number-weighted, and (b) volume-weighted size distribution of native Targestar-P[®], measured with a CasyTM counter. The legend shows the median diameter of the agent.

FIG. 3. The spectral response of (a) the 20 MHz transmitting transducer, and (b) the 10 MHz receiving transducer, that was measured with a calibrated broadband hydrophone.

which were acquired from Casy™ counter measurements. The number-weighted size distribution of Targestar-P® exhibited a skewed distribution, with a median diameter of approximately $1.63 \mu\text{m}$. The concentration of the agent was 2.3×10^9 microbubbles/ml. Note that for skewed distributions, the median is a more robust indicator of the central tendency than the mean.³⁴ Both volume and number-weighted agent size distributions reported in this study were normalized to the maximum for comparison. The volume-weighted size distribution emphasizes the contribution of the larger microbubbles in the agent population.

Figure 3 shows the frequency response of the transducers used in the experiments. Two transducers were used in this study, because the transducer used for transmission had limited sensitivity at the subharmonic frequency.

Figure 4(a) shows the “baseline” spectral response, which was acquired before adding contrast agents to the plexiglass chamber. Figure 4(b) shows the spectral response of the native agent, which had a peak over 20 dB higher than the baseline at the subharmonic frequency.

Figures 5(a) and 5(b) show the number and volume-weighted diameters of the contrast agent populations. Their median/mean diameters, and full-width-half maximum range, computed from three independent measurements, are listed in Table I. As the median size of the agent was increased from 1.35 to $2.15 \mu\text{m}$, the agent became more polydisperse—the full-width-half-maximum breadth (number-weighted) of the distribution increased from 0.44 to $2.13 \mu\text{m}$, and the decreased to $1.67 \mu\text{m}$ when the median size increased to $2.99 \mu\text{m}$.

Figure 6 shows the spectral response of UCA populations with median diameters (a) 1.35 , (b) 1.44 , (c) 1.63 , (d) 1.89 , (e) 2.15 , and (f) $2.99 \mu\text{m}$, to 20 MHz excitation. The subharmonic

signal is clearly observable in each case over the noise floor, which is denoted by the dashed curve. Note that the subharmonic response peaked when the median diameter of the agent was $2.15 \mu\text{m}$.

FIG. 4. The (a) “baseline” spectral response obtained without contrast agent in the plexiglass chamber, and (b) the spectral response of native Targestar-P® computed for a pressure amplitude of 398 kPa. The subharmonic response of native agent was over 20 dB higher than the baseline spectral power at 10 MHz.

FIG. 5. The estimated (a) number-weighted, and (b) volume-weighted size distribution of size-manipulated agent samples. The size distributions were normalized to the maximum in each case for comparison.

Figure 7 shows the of the subharmonic response of the agent relative to the baseline spectrum, for the range of pressures which resulted in observable subharmonic response (190–398 kPa). The error bars shown in Fig. 7 represent ± 1 standard deviation, computed over five statistically independent measurements. All the error bars were very small, except for a single data point, which may have been affected by outliers.

Figure 8 shows the peak subharmonic response plotted against the median diameter for populations with median diameters ranging from 1.35 to 2.15 μm —the range of sizes for which subharmonic response increased monotonically. The peak subharmonic response ratio increased almost linearly ($R^2 = 0.987$) as the median diameter of the number-weighted agent size was increased. However, this was not the case when the median diameter of the volume-weighted agent size was increased ($R^2 = 0.532$).

5. DISCUSSION

High-frequency SHI is hampered by the lack of ultrasound contrast agents that demonstrate high subharmonic activity for frequencies > 10 MHz.^{9,32} The goal of this work was to investigate size-manipulated populations of UCA to determine the range of microbubble sizes that produce the strongest sub-

harmonic response at 20 MHz. A secondary goal was to assess if the number or the volume-weighted size distribution better represents the efficacy of the agent for high-frequency subharmonic imaging.

5.A. Size-dependent subharmonic response

Figures 6 and 7 demonstrate that the agent with 2.15 μm mean diameter produced the strongest subharmonic signal. The microbubble populations with median diameter 1.35 and 2.99 μm demonstrated weak subharmonic behavior. The 1.35 μm UCA had a large number of microbubbles in the range of 0.7–1.3 μm (nearly 60% of its number fraction). Also, the agent population which showed the strongest subharmonic response (2.15 μm) had $> 78.7\%$ of its number fraction larger than 1.3 μm . Therefore, the differences in SH response between this agent and the 1.35 μm agent likely corresponded to microbubbles with diameters larger than approximately 1.3 μm .

Also note that the subharmonic response of the agent decreased drastically by nearly 20 dB when the median size was increased to from 2.15 to 2.99 μm . Since this size-manipulated population peaks at 3 μm , it appears that microbubbles larger than 3 μm may not be contributing appreciably to the subharmonic response. Unfortunately, the UCA

TABLE I. Size distribution estimates of agents size distributions created from Targestar-P®.

Serial number	Median/mean (number) (μm)	Median/mean (volume) (μm)	Full-width-half-maximum breadth (μm)
1	1.35/1.54	2.54/2.79	0.44/2.53
2	1.44/1.65	2.71/ 3.09	0.57/2.76
3	1.63/1.90	3.56/4.02	0.77/2.72
4	1.89/2.31	5.06/3.90	1.06/7.31
5	2.15/2.34	4.04/4.54	2.13/3.76
6	2.99/3.08	3.06/1.49	1.67/2.48

FIG. 6. The spectral response of the size-manipulated agent populations with median diameters (a) 1.35, (b) 1.44, (c) 1.63, (d) 1.89, (e) 2.15, and (f) 2.99 μm , computed at 398 kPa. The noise floor in each case is denoted with the dotted curve.

populations investigated in this study had a range of sizes, therefore it is not possible to determine the optimum microbubble size that would produce the strongest subharmonic response. Nonetheless, these results demonstrate that the sub-

FIG. 7. The subharmonic response of the agent relative to the baseline signal at 10 MHz, computed for different agent sizes when the peak negative pressure was increased progressively from 190 to 398 kPa.

harmonic response was strongest for microbubbles with diameters between 1.3 and 3 μm . Consequently, the subharmonic signal from the 2.15 μm diameter UCA was the strongest—it had the highest number fraction of its microbubbles in the 1.3–3 μm size range (60.2%).

Our results are specific to Targstar-P®—the differences in shell properties in UCA from other manufacturers may change the range of microbubble sizes that are responsible for the overall subharmonic response. However, the size range of “active” microbubbles can be expected to be similar for other phospholipid-encapsulated UCA if the shell properties are comparable. Our observations are consistent with the results of experimental studies conducted with individual phospholipid-encapsulated microbubbles by Helfield and

FIG. 8. The peak subharmonic response relative to baseline plotted against the median diameter of (a) number-weighted and (b) volume-weighted agent population. The linear regression was computed for the range of sizes over which the subharmonic response demonstrated a monotonic increase (1.35–2.15 μm).

colleagues, who found that microbubbles with diameters between 1.7 and 3 μm produced the strongest subharmonic response at 25 MHz.¹⁸ Our results also compare favorably with those reported by Cheung and co-workers, who concluded that microbubbles with the size range 1.2–5 μm were active in subharmonic mode when excited at 20 MHz.¹⁶

It can also be observed from Fig. 6 that the maximum subharmonic signal relative to the fundamental was nearly –35 dB, while Cheung and colleagues¹⁶ reported subharmonic-to-fundamental ratios approaching –10 dB with Definity®—another phospholipid-encapsulated contrast agent. This could be because of the through-transmission experiments that were employed in our work. The spectrum of the received RF echoes had a substantial contribution at the fundamental frequency from the transmitted signal that reached the received transducer directly, while the subharmonic signal was produced primarily by scattering phenomenon. This effect decreased measured subharmonic-to-fundamental ratios. Therefore, we did not use the subharmonic-to-fundamental ratio as a metric to compare the performance of the agents. Instead, the subharmonic response of different agent size distributions was computed relative to the baseline signal at 10 MHz.

Figure 7 demonstrates three interesting observations. First, the measured subharmonic response increased as the pressure amplitudes were increased, which is consistent with previous reports. Second, the subharmonic response peaked when the median diameter of the agent was 2.15 μm . Specifically, the agent with a 2.15 μm median diameter demonstrated up to 11 dB higher subharmonic response than the 1.35 μm agent, and up to 20 dB higher than the 2.99 μm agent. Third, the threshold pressure for the appearance of subharmonic signal was 190 kPa for the agent populations with median diameter 1.44–2.15 μm . However, a threshold pressure of 245 and 290 kPa was observed for the 3 and 1.35 μm agent, respectively. The subharmonic signal from these agent populations was very weak, presumably due to a lesser number fraction of active microbubbles. Therefore, the subharmonic response was apparent above the baseline only at higher pressures.

These results may have implications for the design of microbubble contrast agents. For instance, Definity®, an FDA approved agent commonly used in the context of preclinical studies is quoted to have mean diameters in the range of 1.1–3.3 μm . Such a wide variability could be due, in part, to the mechanical agitation process based method with which these particular agents are manufactured. The results of our study demonstrated a strong dependence of SHI efficacy on microbubble size distribution, suggesting that vial-to-vial variability in the agent size distribution may lead to considerable differences in SHI performance. Additionally, the fact that microbubbles larger than sub-micrometer size are well suited for HF-SHI, may be fortuitous because sub-micrometer sized bubbles have poor persistence *in vivo*.^{25,26}

5.B. Agent preparation and size distribution assessment

Figure 5 and Table I reveal that the volume-weighted size distribution may show different trends compared to the num-

ber weighted size distribution. The largest agent based on the number-weighted median diameter may not be the largest based on the volume-weighted diameter, because of the effect of the “tail” of the distribution. It has been previously argued that the volume-weighted size distribution should be used as an indicator of the acoustic performance, rather than the number-weighted size distribution.²⁴ However, our studies demonstrate that the volume-weighted diameter may not always be a reliable indicator of the imaging performance of the agent, at least in the context of high-frequency SHI (see Fig. 8). Specifically, even though the shape of the distributions was different, the number-weighted median size could predict the subharmonic response of the agent size distribution. This observation was expected, because the largest microbubbles (>3 μm diameter) that are weighted highly in the volume distribution may not contribute to the subharmonic response at high frequencies. However, if the size distribution of the agent is modified specifically for high frequency subharmonic imaging by eliminating the large microbubbles in the “tail” of the distribution, we would expect similar trends from the number and volume weighted size distributions.

5.C. Excitation pulse parameters

Although SH imaging is done in pulse-echo mode, however, our motivation was to assess the relative difference in the high-frequency subharmonic response of different agent sizes. Therefore, we employed the through-transmission experimental configuration because of the (1) excellent signal to noise ratio achievable, and (2) low variability in the measured subharmonic response.^{39,40} Our processing methods ensured that the measured subharmonic response was not affected by nonlinear propagation or overlap with the fundamental signal. Therefore, we believe that the trends observed in transmission mode will be similar to those observed in pulse-echo experiments.

Although broadband coded-excitation pulses have demonstrated potential for high-frequency SHI, such pulses typically excite subharmonic response from a broader size-range of microbubbles than sine-bursts.¹⁷ We conducted this study with narrowband sine-burst pulses in order to identify the size range of microbubbles that are most active at high frequencies—20 MHz in our case, and to reduce spectral overlap between the subharmonic and the fundamental signal. These studies employed pressures lower than 400 kPa because microcirculation imaging is typically conducted at low pressures to increase UCA persistence. The choice of excitation frequency was governed by possible applications in coronary and carotid *vasa vasorum* imaging. Intravascular ultrasound scanners commonly employ frequencies in the 20–45 MHz frequency range for coronary imaging. Carotid imaging is typically performed in linear mode at 7–15 MHz. However, we envisage that subharmonic imaging at 20 MHz transmission frequencies is feasible, as subharmonic imaging employs long duration pulses capable of achieving higher penetration depth.

Goertz and colleagues reported subharmonic *vasa vasorum* imaging studies conducted at 30 MHz transmit

frequency, which is higher than that used in our study (20 MHz).⁸ While their study was conducted with a custom-made system, commercially available IVUS systems for coronary imaging—Volcano Corporation and Boston Scientific operate in the range of 20–45 MHz and 20–40 MHz, respectively. The advantage of imaging at low frequencies is that we expect stronger subharmonic response from agent microbubbles—because of the lower encapsulation damping and larger scattering cross sections involved. We are currently investigating these tradeoffs for subharmonic imaging of the coronary artery, using a modified commercial IVUS system. The findings of the present study could help create agent formulations that could improve the performance of subharmonic imaging *in vivo*, facilitating the rapid clinical translation of *vasa vasorum* imaging.

5.D. Study limitations

We demonstrated that using agents enriched in 1.3–3 μm diameter microbubbles can considerably improve the overall subharmonic response of the agent. However, the actual improvement in SHI sensitivity can only be assessed under physiological conditions; therefore, we plan to conduct *in vivo* imaging studies with a clinical ultrasound system to assess the improvement in imaging performance. Further, we assumed that changes in the subharmonic response of different agent populations were primarily due to the difference in the agent size. However, methods to create smaller agent populations could potentially induce changes in the encapsulating shell. Such changes usually induce asymmetry in the shell.⁴¹ Helfield and colleagues investigated the impact of shell heterogeneity on the subharmonic response, but no clear relationship between the extent of heterogeneity and the subharmonic response was observed.¹⁸ Further, they demonstrated that shell heterogeneity did not have a noticeable effect on subharmonic pressure threshold. Therefore, we believe that the differences in the subharmonic response of the agent populations were predominantly due to the differences in their size distribution.

We assumed the same number density of microbubbles while comparing the subharmonic response from agents with different median sizes. The conclusions of this study may not hold if the same gas volume is compared. Given that clinical dosage is likely to be limited by the gas volume, it would be of interest to develop techniques to eliminate bubbles larger than 3 μm diameter, which were not found to contribute appreciably to the subharmonic response in this study. Such large bubbles still form a considerable volume fraction of many commercially available agents.

The subharmonic signal strength reported in this study should be interpreted carefully. Since this study was conducted in transmission mode, the reported subharmonic response may not correspond to the raw imaging signal obtained in pulse-echo studies. However, we expect the trends to be consistent across agent variants, because scattering was the dominant source of the measured subharmonic signal.

Another limitation of the study is that attenuation and nonlinear propagation may have altered the spectrum of the re-

ceived signal. Although we did not correct for the attenuation at the subharmonic frequency, the agent with the highest scattering to attenuation ratio is expected to produce the highest subharmonic relative to the baseline signal—a characteristic that is desirable for UCA.⁴²

We believe that nonlinear propagation is not a major issue in SHI, because subharmonic signal originates primarily from the agent. Also, the pressures used in this study were lower than previously reported studies,^{16,32} which should reduce nonlinear propagation. Nonlinear propagation could produce some artifacts at the subharmonic frequency, because of the self-demodulation phenomenon.²⁰ This phenomenon generates a low-frequency broadband signal, which is proportional to the second derivative of the excitation signal envelope. For rectangular gated signals such as those used in this study, the self-demodulation signal is primarily contained in the first and last few cycles, where the slope of the envelope changes sharply. Therefore, we ensured that the self-demodulation signal was eliminated from our analysis—we rejected the first and last two cycles from the received signal, and employed a Chebyshev window to estimate its spectrum. Therefore, we do not expect nonlinear propagation to impact the conclusions of this study.

6. CONCLUSIONS

The results of this study demonstrate that the subharmonic response can be considerably improved by modifying the size distribution of the contrast agent for high frequency applications. The agent microbubbles with diameters between 1.3 and 3 μm appear to be most active in subharmonic mode at 20 MHz frequencies. Further, the number-weighted size distribution of the agent is a better indicator of the efficacy of the agent for subharmonic imaging at high frequencies, instead of the volume-weighted size distribution. We envisage that these results may help design contrast agents for high-frequency nonlinear imaging applications such as *vasa vasorum* imaging.

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