Published in final edited form as:

Ultrasound Med Biol. 2015 January ; 41(1): 208–221. doi:10.1016/j.ultrasmedbio.2014.08.021.

Loss of echogenicity and onset of cavitation from echogenic liposomes: pulse repetition frequency independence

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Abstract

Echogenic liposomes (ELIP) are being developed for the early detection and treatment of atherosclerotic lesions. An 80% loss of echogenicity of ELIP (Radhakrishnan et al. 2013) has been shown to be concomitant with the onset of stable and inertial cavitation. The ultrasound pressure amplitude at which this occurs is weakly dependent on pulse duration. Smith et al. (2007) have reported that the rapid fragmentation threshold of ELIP (based on changes in echogenicity) is dependent on the insonation pulse repetition frequency (PRF). The current study evaluates the relationship between loss of echogenicity and cavitation emissions from ELIP insonified by duplex Doppler pulses at four PRFs (1.25 kHz, 2.5 kHz, 5 kHz, and 8.33 kHz). Loss of echogenicity was evaluated on B-mode images of ELIP. Cavitation emissions from ELIP were recorded passively on a focused single-element transducer and a linear array. Emissions recorded by the linear array were beamformed and the spatial widths of stable and inertial cavitation emissions were compared to the calibrated azimuthal beamwidth of the Doppler pulse exceeding the stable and inertial cavitation thresholds. The inertial cavitation thresholds had a very weak dependence on PRF and stable cavitation thresholds were independent of PRF. The spatial widths of the cavitation emissions recorded by the passive cavitation imaging system agreed with the calibrated Doppler beamwidths. The results also show that 64%–79% loss of echogenicity can be used to classify the presence or absence of cavitation emissions with greater than 80% accuracy.

Keywords

stable cavitation; inertial cavitation; passive cavitation imaging; Doppler ultrasound; echogenic liposomes

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Introduction

Echogenic liposomes (ELIP) are being developed as novel theragnostic ultrasound contrast agents (UCAs) for early detection and treatment of atherosclerotic lesions (Huang and MacDonald 2004; Sutton et al. 2013a). ELIP are lipid bilayer vesicles that encapsulate air and aqueous cores (Huang et al. 2001). ELIP conjugated with antibodies have also been shown to target and highlight atherosclerotic biomarkers (Demos et al. 1999; Hamilton et al. 2002; Hamilton et al. 2004) on intravascular ultrasound images. ELIP have been loaded with drugs (Tiukinhoy et al. 2004; Tiukinhoy-Laing et al. 2007; Shaw et al. 2009), genes (Buchanan et al. 2010), stem cells (Herbst et al. 2010), and vasoactive gases (Huang et al. 2009; Britton et al. 2010) for localized ultrasound-mediated delivery of therapeutics into thrombi and atherosclerotic lesions.

Microbubble-based UCAs serve as cavitation nuclei and can initiate both beneficial and deleterious bioeffects (Miller et al. 2008). Stable cavitation of microbubbles facilitates the lysis of clots (Datta et al. 2008; Sutton et al. 2013b) and enhances the permeability of tissue for drug delivery (Sutton et al. 2013a; Hitchcock et al. 2010). Stable cavitation is characterized by subharmonic, ultraharmonic, and harmonic acoustic emissions from microbubbles (Eller and Flynn 1969; Neppiras 1969). Inertially cavitating microbubbles emit broadband frequency components (Neppiras 1980; Roy et al. 1990; Holland and Apfel 1990). Inertial cavitation has been shown to facilitate the ablation of cancerous lesions (Coussios et al. 2007) and enhance gene delivery (Bazan-Peregrino et al. 2012). However, inertial collapse of UCAs has also been shown to cause cell damage, apoptosis, hemolysis and petechial hemorrhage (Miller et al. 2008; Miller and Dou 2004; Samuel et al. 2009; Skyba et al. 1998; Chen et al. 2003).

It is therefore, important to determine the type of cavitation emissions that occur from ELIP during imaging and therapeutic applications. Single-element transducers have been used in active and passive cavitation detection systems (Roy et al. 1990; Atchley et al. 1988). Such detection schemes are limited by the spatial sensitivity of the transducer and do not resolve the spatial distribution of cavitation activity (Haworth et al. 2012; Choi and Coussios 2012). Passive cavitation imaging is being developed to spatially resolve cavitation emissions (Haworth et al. 2012; Salgaonkar et al. 2009; Farny et al. 2009; Gyöngy et al. 2008).

Acoustically-induced UCA destruction has been categorized either as acoustically driven diffusion or rapid fragmentation based on temporal changes in UCA diameter (Chomas et al. 2001) and UCA echogenicity (Smith et al. 2007). Chen et al. (2002) observed that the rapid fragmentation of certain UCAs was followed by inertial cavitation and subsequent dissolution of the liberated microbubble. Ammi et al. (2006) also found that the post-excitation signals from fragmented UCAs matched the inertial cavitation response of free microbubbles. These studies (Chen et al. 2002; Ammi et al. 2006) suggest that rapid changes in echogenicity due to UCA fragmentation are concomitant with inertial cavitation. In a recent study of ELIP and Definity®, neither stable nor inertial cavitation emissions were detected until an approximately 80% loss of echogenicity or greater was observed (Radhakrishnan et al. 2013). Numerical calculations suggested that UCA shell rupture may initiate the loss of echogenicity at insonation pressures below the stable and inertial

cavitation thresholds (Radhakrishnan et al. 2013). The stable and inertial cavitation thresholds were weakly dependent on the pulse duration.

Using echogenicity measurements, Smith et al. (2007) ascertained that the acoustically driven diffusion threshold was weakly dependent on the pulse repetition frequency (PRF) and the rapid fragmentation threshold was strongly dependent on the PRF. Chang et al. (2001) found that the pressure threshold initiating the disappearance of contrast from Albunex decreased with increasing PRF. However, the inertial cavitation threshold of Albunex did not change as a function of PRF (Chang et al. 2001). Shi et al. (Shi et al. 2000) hypothesized that the ultrasound-mediated fragmentation of UCAs at high pressure amplitudes (> 2 MPa) and high PRFs (>1 kHz) may initiate inertial cavitation. The PRF dependence of cavitation emissions and bioeffects from UCAs has been investigated in several studies (Chen et al. 2003; Karshafian et al. 2009; Miller and Gies 2000; Ter Haar 2002). Other studies have reported conflicting results regarding the lack of PRF dependence for inertial cavitation and cavitation-induced bioeffects (Miller and Quddus 2000; McDannold et al. 2008; Child et al. 1990).

Recently, Choi and Coussios (2012) evaluated the passive cavitation images of flowing SonoVue as a function of PRF. They observed that with increasing PRF and insonation pressure the spatial distribution of the cavitation activity became more asymmetric. Therefore, the PRF of the insonation pulses may impact the loss of echogenicity due to rapid destruction of contrast, the cavitation thresholds, and the spatial distribution of cavitation emissions from UCAs.

The goal of this study was to evaluate the PRF dependence of the stable and inertial cavitation thresholds, to determine the spatial distribution of cavitation emissions from ELIP insonified by duplex Doppler pulses, and to compare the onset of cavitation activity with the loss of echogenicity. Cavitation emissions from ELIP were acquired with a single element passive cavitation detector and with a linear array. The spatial distribution of the stable and inertial cavitation activity was compared with the calibrated beamwidth of the Doppler pressure profile exceeding the stable and inertial cavitation thresholds.

Materials and Methods

Preparation of echogenic liposomes (ELIP)

ELIP were prepared and shipped overnight on ice packs from the University of Texas Health Science Center, Houston to the University of Cincinnati. The lipid formulation as described by Buchanan et al. (2008) consisted of L- α -phosphatidylcholine (chicken egg; EPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-[phosphorac-1-glycerol] (DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) (Avanti Polar Lipids, Alabaster, AL, USA), and cholesterol (CH) (Sigma Aldrich St. Louis, MO, USA) in the molar ratio 27:42:8:8:15. Each 6 mg ELIP vial was reconstituted with 0.6 ml of 0.2 μ m-filtered deionized water (NANOPure, Barnstead International, Dubuque, IA, USA). ELIP were diluted in porcine plasma (Lampire Biologicals, Pipersville, PA, USA) at 37 °C and 93 $\pm$ 2 % dissolved oxygen (DO) to yield a final lipid concentration of 0.05

mg/ml (6.4×10^8 liposomes/ml) (Hamilton et al. 2004; Smith et al. 2007; Kopechek et al. 2011; Raymond et al. 2013).

Experimental Setup

The in vitro flow system shown in Figure 1 was used to determine the cavitation thresholds and loss of echogenicity from ELIP. A peristaltic pump (Mettler Toledo, Columbus, OH, USA) was used to convect the ELIP through latex tubing (McMaster-Carr, Aurora, OH, USA) coupled to ethyl vinyl acetate (EVA) tubing (1 mm inner diameter, 0.38 mm wall thickness) (McMaster-Carr). Variation in volumetric flow was less than 2% over a single pump cycle and thus significantly less pulsatile than our prior work (Radhakrishnan et al. 2013). The latex and EVA tubes were immersed in a tank of degassed water at 37 °C. The EVA tubing had a thinner wall (0.38 mm) and higher acoustic transmission coefficient (94%) compared to the latex tubing (0.8 mm wall thickness and 86% acoustic transmission coefficient) used in a prior setup (Radhakrishnan et al. 2013). The EVA tubing was used at the location of insonation to because it had a lower acoustic attenuation. Further, the EVA tubing had an inner diameter of 1 mm, which was comparable to the -3 dB elevational beamwidth of the Doppler pulses (0.8 mm). The narrower inner diameter tube ensured that no ELIP were outside the -3 dB elevational beamwidth, providing a relatively uniform insonation. The dimensions of the EVA tubing were also consistent with the inner diameter and thickness of small arteries (Dodge et al. 1992; Villablanca et al. 2007).

An after-load with an adjustable height was used to maintain a hydrostatic pressure corresponding to a mean arterial pressure of 93 mmHg. The flow rate was 1.5 ml/min so that the average flow velocity matched that used in a previous study (Radhakrishnan et al. 2013). ELIP were also infused into the insonation location and flow was turned off prior to insonation to measure cavitation emissions and loss of echogenicity in the absence of flow. The temperature and pressure in the flow system were monitored throughout the experiments using inline pressure and temperature sensors (PendoTECH, Princeton, NJ, USA).

A linear array transducer (L12-5) connected to an HDI 5000 clinical diagnostic scanner (Philips, Bothell, WA, USA) (Fig. 1) was operated in the duplex spectral Doppler mode to insonify the ELIP. The Philips HDI 5000 is an FDA-approved clinical diagnostic scanner. It has been used in previous studies (Porter et al. 2006; Smith et al. 2007; Kopechek et al. 2013; Radhakrishnan et al. 2013) to demonstrate the use of diagnostic pulses to nucleate cavitation from ELIP and other contrast agents. Degassed water ($DO=25 \pm 2\%$) was pumped through the flow system to determine the acoustic noise and the minimum amount of cavitation that the system could detect. Cavitation emissions and images were acquired for a range of insonation pressures at four pulse repetition frequencies (PRFs) with a fixed pulse duration of 3.33 μ s, as indicated in Table 1. The number of Doppler pulses incident on ELIP flowing through the Doppler focus was calculated based on the average flow velocity and PRF as shown in Table 1. The range and step size of insonation pressures was limited by the Philips HDI 5000 scanner.

Calibration of diagnostic transducer

The *in situ* peak rarefactional pressure of the Doppler pulse at each mechanical index (MI) setting of the scanner was measured. In situ calibrations were performed in a tank of degassed water at 37 °C by placing a section of the EVA tubing (McMaster-Carr) at the Doppler sample volume of the L12-5 and aligning a 0.2 mm needle hydrophone (Precision Acoustics, Dorchester, UK) to the focus of the Doppler sample volume. A three-dimensional positioning system (Velmex NF90 Series, Velmex Inc., 291 Bloomfield, NY) was used to translate the hydrophone within the Doppler beam to measure the azimuthal beamwidth at each onscreen MI setting. The -3dB focal beam of the Doppler pulse was 0.8 mm×1.8 mm×13.2 mm (azimuth×elevation×range) at a focal distance of 1 cm from the transducer face.

Single-element passive cavitation detection

A single-element focused 10-MHz transducer (Valpey Fisher, Hopkinton, MA, USA) was used as a passive cavitation detector (PCD). The PCD was aligned confocally and orthogonally to the L12-5 Doppler sample volume (Figure 1b). The -3 dB beamwidth of the PCD was 0.8 mm×0.8 mm×2.4 mm (azimuth×elevation×range). The line trigger from the clinical scanner was used to range gate the acoustic emissions from the Doppler sample volume received by the passive detector. The acoustic emissions were filtered through a 35-MHz low pass filter (TTE, Los Angeles, CA, USA), amplified by 13 dB (Comlinear, Loveland, CO, USA), and recorded using a digital oscilloscope (LeCroy, Chestnut Ridge, NY, USA), which operated in the sequence mode. At each insonation pressure, two sequences were acquired, with each sequence containing 50 traces and each trace comprising 2000 samples at a sampling frequency of 100 MHz. The average power spectra of 100 traces were processed using a previously described method (Radhakrishnan et al. 2013) to ascertain the cavitation thresholds.

Passive cavitation imaging

A linear array transducer (L7-4) (Philips ATL, Bothell, WA, USA) was used to record acoustic emissions passively from ELIP and degassed water on 128 channels simultaneously (0.283 mm element pitch). The L7-4 transducer was aligned confocally and orthogonally to the L12-5 Doppler focus at the center of the lumen of the EVA tubing as shown in Figure 1(b). The emissions were recorded at 36 MHz sampling frequency and range gated to a depth of 40 mm from the face of the transducer, which corresponded to the distance between the EVA tubing and the L7-4. The recording channels were controlled by a Verasonics V-1-128 system (Verasonics, Inc., Redmond, WA, USA). The line trigger from the HDI 5000 scanner was used as an input trigger to synchronize the acquisition of a passive cavitation images (PCIs) with the single-element PCD. Because the B-mode pulses are interleaved with the Doppler pulses in duplex spectral Doppler mode on the HDI 5000 scanner, appropriate time delays were applied between each acquisition to ensure that emissions from at least 50 Doppler pulse insonations were recorded.

The raw radio frequency (RF) signals in the PCI frames containing Doppler pulses were beamformed using the delay-and-sum algorithm described in Haworth et al. (2012). Briefly, the frequency-dependent energy at each pixel location was computed by applying phase-

shifts to the frequency-domain RF signals received at each element and summing across all elements. From Haworth et al. (2012), the beamformed power spectrum $B(\omega, \vec{x})$ at each pixel location $\vec{x}$ and frequency ω was given by equation (1a)

$$B(\omega, \vec{x}) = \left| \sum_{n=1}^N S_n(\omega) A_{\cos}(\vec{x}_n, \vec{x}) e^{(i\omega \frac{|\vec{x}_n - \vec{x}|}{c})} \right|^2 \quad (1a)$$

where, $S_n(\omega)$ is the frequency domain representation of the signal received on the n^{th} element of the L7-4 transducer, $\vec{x}_n$ is the position of the n^{th} element, $A_{\cos}(\vec{x}_n, \vec{x})$ is a cosine apodization, and c is the sound speed. The cosine apodization is given by equation 1(b).

$$A_{\cos}(\vec{x}_n, \vec{x}) = \frac{\vec{x}_n \cdot \vec{x}}{|\vec{x}_n| |\vec{x}|} \quad (1b)$$

Cosine apodization was applied to reduce the grating lobes in the passive cavitation images. $B(\omega, \vec{x})$ yielded a power spectrum analogous to the processing used for the emissions recorded by the PCD.

Cavitation thresholds

To obtain the stable and inertial cavitation thresholds, spectral analysis was performed on the cavitation emissions acquired by the single element PCD and the passive cavitation imaging array using previously published methods (Radhakrishnan et al. 2013). Briefly, the subharmonic and odd multiples of the subharmonic (ultraharmonic) frequency components and broadband frequency components excluding the fundamental, harmonic, subharmonic and ultraharmonic bands (Haworth et al. 2012) were extracted. The root-mean-squared (RMS) values of the subharmonic (and ultraharmonic) emissions and broadband emissions are given by

$$\text{Cavitation Signal}_{\text{RMS}} = \sqrt{\sum_{f_{\text{Band}}} \text{Power}_{\text{ELIP}}} - \sqrt{\sum_{f_{\text{Band}}} \text{Power}_{\text{DW}}}, \quad (2)$$

where $\text{Power}_{\text{ELIP}}$ and Power_{DW} are the detected acoustic emission powers from ELIP in the tubing and degassed water in the tubing, respectively. f_{Band} includes the subharmonic and ultraharmonic frequency bands to calculate the stable cavitation threshold or the broadband frequency bands to calculate the inertial cavitation threshold. As described by Radhakrishnan et al. (2013), piecewise linear fits of the RMS subharmonic emissions and RMS broadband emissions was performed using the 'slmengine' function in MATLAB. The peak rarefactional pressure at the inflection between the first two segments was defined as the stable or inertial cavitation threshold. Stable and inertial cavitation thresholds were ascertained at each PRF.

Comparison of spatial distribution of cavitation emissions and insonation beamwidths

The goal of this analysis was to compare the measured spatial distribution of cavitation activity on the PCI with the calibrated beamwidth of the *in situ* Doppler pressure profile of the insonation region above the cavitation thresholds. Cavitation emissions were expected to be detected within the spatial beamwidth of the Doppler pressure profile exceeding the cavitation thresholds. The spatial width of cavitation emissions on the PCI was defined as the width over which the PCI pixels had an amplitude greater than the PCI pixel amplitude detected at the cavitation threshold. The comparison between calibrated beamwidths and PCI spatial widths was carried out in the azimuthal direction along the direction of flow. The widths were computed at each insonation pressure for both stable and inertial cavitation emissions.

To compare the measured spatial width of cavitation activity on the PCI with the calibrated L12-5 Doppler beamwidth, the point spread function (PSF) of the passive cavitation imaging array (L7-4) was taken into account. This comparison was achieved by convolving the PSF with the calibrated spatial profile of the Doppler beam exceeding the stable and inertial cavitation thresholds. The PSF of the PCI was computed using the 'transientrectarray' function in the Fast Object-oriented C++ Ultrasound Simulator software (McGough et al. 2009). Prior to convolution, the amplitude of the PSF was normalized such that the PSF had unitary energy. The percent error between the spatial widths of the cavitation activity on the PCI and the calibrated beamwidth of the Doppler pressure exceeding the stable and inertial cavitation thresholds was computed at all four PRFs using equation 3.

$$\text{Percent error} = \frac{BW_{\text{PCI}} - BW_{\text{Calibration}}}{BW_{\text{Calibration}}} \times 100, \quad (3)$$

where BW_{PCI} is the spatial width of cavitation emissions measured on the PCI and $BW_{\text{Calibration}}$ is the calibrated beamwidth of the Doppler pressure exceeding the cavitation threshold.

Image processing

At each insonation pressure, a B-mode image of ELIP was saved on the HDI-5000 ultrasound scanner. The frame rate, persistence, grayscale map, time gain compensation (TGC) and 2-D gain were kept constant throughout the experiments. In order to measure the echogenicity of ELIP flowing in a tubing of 1mm inner diameter, the high definition zoom function on the scanner was employed. Using previously established techniques (Radhakrishnan et al. 2012), the grayscale values on the image were converted into the linear digital intensities. The loss of echogenicity of ELIP in the static system was assessed by computing the mean digital intensity (MDI) within regions of interest (ROIs) defined at locations within the Doppler focus and upstream to the Doppler pulse as shown in Figure 2(a). The Bmode pulses interleaved with the Doppler pulses in the duplex Doppler mode were not found to cause a significant loss of echogenicity over the regions of interest. The percent loss of echogenicity (LOE) was computed using equation (4a).

$$\text{Percent LOE}_{\text{No flow}} = \frac{\text{MDI}_{\text{No Doppler}} - \text{MDI}_{\text{With Doppler}}}{\text{MDI}_{\text{No Doppler}}} \times 100, \quad (4a)$$

where $\text{MDI}_{\text{No Doppler}}$ and $\text{MDI}_{\text{With Doppler}}$ are the mean digital intensities in the ROIs corresponding to locations unexposed and exposed to Doppler insonation respectively. Smith et al. (2007) defined the rapid fragmentation threshold as the lowest pressure at which the MDI of ELIP reduced to the background value, which corresponded to 90% LOE, within 5 s from the start of Doppler insonation. Thus the lowest peak rarefactional pressure causing a LOE of 90% was used to define the loss of echogenicity threshold for ELIP in the static system.

For flowing ELIP, the ROI was not defined within the Doppler focus because of continuous replenishment of the destroyed contrast within the Doppler focus. Therefore, regions of interest (ROIs) were defined upstream (proximal) and downstream (distal) of the Doppler focus as shown in Figure 2(b). LOE was computed for each insonation pressure using equation (4b).

$$\text{Percent LOE}_{\text{Flow}} = \frac{\text{MDI}_{\text{Up}} - \text{MDI}_{\text{Down}}}{\text{MDI}_{\text{Up}}} \times 100, \quad (4b)$$

where MDI_{Up} and MDI_{Down} are the mean digital intensities in the ROIs upstream (proximal) and downstream (distal) of the Doppler focus respectively.

Comparison of Loss of Echogenicity and Cavitation emissions

A receiver operating characteristic (ROC) analysis was performed to determine the efficacy of using loss of echogenicity to classify whether stable and inertial cavitation emissions occurred. Cavitation emissions lower than the cavitation threshold were the gold standard negative condition, whereas cavitation emissions greater than the cavitation threshold were the gold standard positive condition. The area under the ROC curve, accuracy, sensitivity, and specificity were computed.

Statistical Analysis

Statistical analysis was performed in MATLAB (The MathWorks Inc). An unequal variance t-test was performed to compare the stable and inertial cavitation thresholds. A three-way Analysis of variance (ANOVA) test was performed to compare the cavitation thresholds of ELIP with and without flow exposed to spectral Doppler at four pulse repetition frequencies. Bonferroni multiple comparison post-hoc tests were conducted to elucidate the factors that contributed to significant changes in cavitation thresholds. A two-way ANOVA with Bonferroni multiple comparison was performed to compare the LOE thresholds in the static system with the rapid fragmentation thresholds determined by Smith et al. (2007). Kolmogorov–Smirnov (K-S) tests were performed to determine the agreement between the spatial widths of cavitation emissions on the PCI and calibrated Doppler beamwidths exceeding the cavitation thresholds. The use of LOE to classify the presence or absence of

cavitation emissions was tested using the null hypothesis that the area under the ROC curve was 0.5 (Hanley and McNeil 1982).

Results

Cavitation thresholds

Figures 3(a) and 3(b) show the stable and inertial cavitation thresholds of ELIP as a function of PRF in static and flow conditions. The stable and inertial cavitation thresholds ascertained using the single-element PCD system (Figure 3(a)) agreed with the thresholds determined using the passive cavitation imaging system (Figure 3(b)) ($p=0.13$). The stable cavitation thresholds did not depend on the PRF ($p=0.23$). As seen in figures 3(a) and (b), the inertial cavitation thresholds have a weak dependence on the PRF. The inertial cavitation threshold of ELIP at the highest PRF (8.33 kHz) was significantly different from the inertial cavitation threshold at lower PRFs ($p=0.002$). The inertial cavitation thresholds were moderately higher than the stable cavitation thresholds ($p<0.05$). The cavitation thresholds measured in flow were the same as those measured in static fluid ($p=0.4$).

Spatial distribution of cavitation emissions

The spatial distribution of cavitation activity detected on the PCI was compared to the calibrated beamwidth of the Doppler pressure profile. Figures 4 and 5 show a comparison of the beamwidths of the calibrated Doppler pressure profile and the measured beamwidth of the cavitation activity on the PCI exceeding the stable and inertial cavitation thresholds, respectively. Table 2 shows the percent error between the spatial widths of the cavitation activity on the PCI and the calibrated beamwidth of the Doppler pressure exceeding the stable and inertial cavitation thresholds at all four PRFs. The spatial widths of stable and inertial cavitation activity measured on the PCI were not significantly different than the expected beamwidth based on the pressure calibration data as ascertained by the K-S test (Table 3). The spatial widths of cavitation emissions detected on the PCI were equal to or higher than the calibrated beamwidths at pressures below 1 MPa. However, at pressures higher than 1 MPa, the spatial widths of cavitation emissions on the PCI appeared to saturate and did not increase linearly with pressure. This saturation is likely due to the ELIP destruction.

Loss of echogenicity

Figure 6 shows a comparison of the cavitation thresholds, the loss of echogenicity thresholds and the rapid fragmentation thresholds (Smith et al. 2007) for ELIP in a static fluid. The loss of echogenicity threshold was determined as the lowest peak rarefactional pressure at which 90% loss of echogenicity occurred (Smith et al. 2007). The rapid fragmentation threshold determined by Smith et al. (2007) were higher than the stable and inertial cavitation thresholds and the loss of echogenicity thresholds ascertained in the present study ($p<0.05$). The loss of echogenicity threshold was found to be the same as the stable cavitation threshold.

As the cavitation emission thresholds and the LOE both showed either a very weak dependence or no dependence on the PRF, the LOE and cavitation data across all four PRFs

were combined to compute the ROC curves. The specificity and sensitivity of the LOE as a classifier for the absence or presence of stable cavitation emissions and inertial cavitation emissions is plotted as ROC curves in Figures 7(a) and (b) respectively. The area under the ROC curve was found to be 0.93 and 0.92 for stable and inertial cavitation emissions, respectively. The null hypothesis that the area under the ROC curve is equal to or less than 0.5 was rejected ($p < 0.05$) for both types of cavitation emissions. LOE values between 57% and 79% could be used to classify the presence and absence of stable cavitation emissions with an accuracy of greater than 80%. In addition, LOE values between 64% and 89% could be used to classify the presence and absence of inertial cavitation emissions with an accuracy of greater than 80%. The LOE values that simultaneously maximizes the sensitivity and specificity of detecting stable and inertial cavitation emissions are 63% and 64%, respectively.

Discussion

The PRF dependence of the stable and inertial cavitation thresholds of ELIP exposed to duplex Doppler pulses was measured. The spatial distribution of the cavitation emissions from ELIP was compared to the calibrated beamwidth of the Doppler ultrasound pressure amplitude profile. The relationship between these bubble activity metrics and the loss of echogenicity was also determined. Each of these measurements will be subsequently discussed.

Cavitation thresholds

Previously, Radhakrishnan et al. (2013) ascertained the stable and inertial cavitation thresholds of ELIP as a function of pulse duration at 1.25 kHz PRF. The stable and inertial cavitation thresholds were found to be weakly dependent on the insonation pulse duration. In the current study, the stable and inertial cavitation thresholds have been evaluated as a function of the PRF at a fixed 3.33 μ s pulse duration. The stable and inertial cavitation thresholds of ELIP at a 3.33 μ s pulse duration and 1.25 kHz PRF in the current study are 0.42 ± 0.04 MPa and 0.55 ± 0.03 MPa respectively. These thresholds agree with the stable and inertial cavitation thresholds of ELIP (0.41 ± 0.04 MPa and 0.59 ± 0.08 MPa respectively) in a previous study (Radhakrishnan et al. 2013) despite differences in the flow setup such as inner diameter of the tubing and pulsatility of the pump.

There were qualitative differences observed in the power spectra of each passive cavitation measurement approach. The dynamic ranges, transducer frequency responses, and spatial sensitivities of the two systems differ. These differences likely contribute to the observed spectral differences. Additionally, beamforming for PCIs increased the signal-to-noise ratio (SNR) due to the summation across channels. Despite these differences, the cavitation thresholds ascertained using the piecewise linear fit were indistinguishable in both systems. In general, the sensitivity of the piecewise linear fit in determining the cavitation thresholds may be limited by the SNR of the detection system. However, because both the PCD and passive cavitation imaging systems measured the same thresholds, the SNR likely did not affect the threshold measurement. Farny et al. (2009) employed different methods to beamform passive cavitation data obtained from an array and observed the same trend of

cavitation activity at the insonation focus between the single-element and array-based cavitation data.

The stable cavitation thresholds did not change with increasing PRF. The inertial cavitation thresholds had a very weak dependence on the PRF. Previous studies investigating the PRF dependence of cavitation thresholds and bioeffects have had contradictory results. Cavitation-induced bioeffects such as hemolysis (Chen et al. 2003), cell permeability (Karshafian et al. 2009), and cell death (Miller and Gies 2000) are dependent on PRF. Chen et al. (2003) have suggested that long pulses and high PRFs can induce a “cascading effect” causing explosive bubble generation resulting in increased hemolysis at higher PRFs. Karshafian et al. (2009) observed increased cell permeability and decreased cell viability at higher PRFs. Miller and Gies (2000) observed that cavitation-induced cell membrane damage was weakly dependent on the PRF of spectral Doppler pulses. Fowlkes and Crum (1988) suggested that high-pressure pulses repeatedly impinging on the microbubbles could weaken the shell resulting in lower cavitation thresholds. Therefore, at higher PRFs, repeated pulses (Table 1) are hypothesized to weaken the ELIP shell resulting in a lower inertial cavitation threshold as observed in the current study. However, other studies have shown that the inertial cavitation threshold nucleated by a UCA (Chang et al. 2001), disruption of the blood brain barrier (McDannold et al. 2008), and petechial hemorrhage (Miller and Quddus 2000) are independent of PRF.

Spatial distribution cavitation emissions

The spatial distribution of the cavitation emissions detected on the PCI agreed with the calibrated beamwidth of the Doppler pulse. The agreement was observed for stable and inertial cavitation activity at all four PRFs (Figures 4 and 5). Previously, Gyöngy and Coussios (2010) used the spatial calibration of a HIFU transducer and the inertial cavitation threshold to co-localize the HIFU transducer focus with a passive cavitation image. Due to relatively poor resolution along the axis of the passive cavitation imaging array, the spatial distribution of the cavitation emissions was compared to the predicted cavitation region qualitatively. In this study, the spatial distribution of the cavitation emissions was assessed along the azimuth of the passive cavitation imaging array, which has submillimeter resolution for the L7-4 array. Figures 4 and 5 and Table 2 confirm that the passive cavitation image system provides good lateral resolution to map the spatial distribution of the cavitation activity. The resolution is diffraction limited by the point spread function of the array transducer (Haworth et al. 2012).

The large standard deviations in the spatial widths of cavitation emissions on the PCIs (Table 2) were predominantly due to measurements near the cavitation threshold. Near the cavitation threshold, the percent error values were very high due to the combination of the stochastic nature of cavitation yielding varying widths and the calibrated beamwidths being in the submillimeter range. Therefore, small variations in the signal strength and location of cavitation emissions on the PCIs were magnified in the percent error calculation based on equation (3). Vial-to-vial variability also may have contributed to higher percent error values. Another potential systematic error could be that the PSF of the passive cavitation imaging array was computed using a simulator and not measured experimentally.

Recently Choi and Coussios (2012) assessed the spatial distribution of cavitation emissions from SonoVue in the focal volume as a function of PRF and flow rate. Based on destruction-reperfusion imaging studies by Wei et al. (1998), Choi and Coussios (2012) defined a critical pulse repetition frequency (PRF) above which the UCAs flowing through the insonation volume were insonified by more than one acoustic pressure pulse. They observed that at PRFs higher than the critical PRF the distribution of the cavitation energy was spatially skewed upstream of the insonation volume. The critical PRF based on the flow rate and the lateral beamwidth of the Doppler pulse in the current study is 39.5 Hz. As shown in Table 1, the PRFs investigated in this study were limited by the settings on the HDI 5000 scanner and were above the critical PRF as described by Choi and Coussios (2012). However, the spatial asymmetry of the cavitation energy at PRFs higher than the critical PRF (Choi and Coussios 2012) was not evident in the current study. The presence of significant sidelobes in the pressure profile of the HIFU transducer may have contributed to the spatial asymmetry observed by Choi and Coussios (2012). However, in the current study the sidelobes of the Doppler pulse did not exceed the stable and inertial cavitation thresholds.

Loss of echogenicity

The loss of echogenicity threshold of ELIP in a static fluid was defined as the lowest peak rarefactional pressure at which ELIP lost 90% echogenicity (Radhakrishnan et al. 2013). As seen in Figure 6 the LOE threshold at 1250 Hz PRF was 0.49 ± 0.15 MPa based on 90% loss of echogenicity definition. By choosing 70% or 50% loss of echogenicity, this LOE threshold would have dropped to 0.39 ± 0.05 MPa and 0.17 ± 0.05 MPa respectively. The definition of 90% loss of echogenicity as the LOE threshold was used in the current study to allow a strict comparison with the rapid fragmentation threshold defined by Smith et al. (2007). In previously published results (Radhakrishnan et al. 2013), the loss of echogenicity thresholds of ELIP agreed with the rapid fragmentation threshold and the inertial cavitation threshold. In this study, as shown in figure 6 the loss of echogenicity thresholds of ELIP were lower than the rapid fragmentation thresholds of ELIP. The discrepancy between the loss of echogenicity threshold ascertained in the current study versus the previous study (Radhakrishnan et al. 2013) are likely due to the geometry of the experimental setup. In previous studies (Smith et al. 2007; Radhakrishnan et al. 2013) the -3 dB elevational beamwidth of the Doppler pressure profile was narrower than the sample of ELIP being insonified. In particular, in the previous study by Radhakrishnan et al. (2013) the inner diameter of the tubing was 3 mm but the 3 dB elevational beamwidth was 0.8 mm. Therefore the ELIP within the tube lumen were not uniformly insonified at the Doppler focus. The ELIP outside the -3 dB elevational beamwidth may have contributed to residual echogenicity thus leading to an underestimation of loss of echogenicity at each insonation pressure in previous studies. By using a tube with a narrower inner diameter in the current study, there were no ELIP outside the -3 dB elevational beamwidth.

In the current study, hyperechoic aggregates of ELIP exposed to Doppler pulses with a PRF of 8.33 kHz were observed on the lower wall of the tubing. Smith et al. (2007) also noted acoustic streaming or effects of radiation forces at PRFs greater than 2.5 kHz. Dayton et al. (1997; 1999) showed that insonation with PRFs in the kHz range can induce primary

radiation force on flowing UCAs. Optical studies and numerical calculations carried out by Dayton et al. (1997; 1999) showed that primary radiation force pushes the UCA away from the transducer and Bjerknes (secondary radiation) forces promote aggregation of UCA microbubbles. Thus, primary and secondary radiation forces may have played a role in the formation of hyperechoic aggregates of ELIP.

ROC analysis was used in this study to demonstrate that LOE can correctly classify the presence or absence of stable and inertial cavitation emissions with good specificity, sensitivity, and accuracy. The particular LOE value used to classify cavitation emissions depends on the relative weight of accuracy, sensitivity, and specificity chosen. In a previous study examining the pulse duration dependence of cavitation thresholds and loss of echogenicity (Radhakrishnan et al. 2013), a piece-wise linear fit approach was used to ascertain the correlation of LOE with stable and inertial cavitation. An 80% loss of echogenicity was correlated with the onset of stable and inertial cavitation, which agrees with the present study.

Conclusions

In this study, the stable and inertial cavitation thresholds of ELIP insonified by duplex spectral Doppler at four PRFs have been ascertained using a single-element passive cavitation detector and a passive cavitation imaging system. The inertial cavitation thresholds had a very weak dependence on the PRF, whereas no dependence was observed for the stable cavitation thresholds. The spatial distribution of the cavitation emissions detected by the passive cavitation imaging system was compared with the calibrated beamwidth of the Doppler pulse and did not have a PRF dependence. The loss of echogenicity threshold of ELIP in a static fluid was found to be lower than the rapid fragmentation thresholds of ELIP (Smith et al. 2007). ROC analysis showed that LOE could be used to classify whether or not stable and inertial cavitation occurred. No dependence on PRF was observed for the loss of echogenicity. Thus if an 80% loss of echogenicity is observed from echogenic liposomes exposed to Doppler ultrasound, both stable and inertial cavitation are expected to occur. The importance of these data lies in the potential surrogate use of loss of echogenicity for cavitation onset.

Acknowledgments

This work was supported in part by the National Institutes of Health through grants R01HL059586 and R01HL074002.

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Figure 1.

(a) The in vitro flow system with controlled temperature and hydrodynamic pressure is shown. The flowing ELIP in plasma were insonified by the L12-5 transducer in duplex spectral Doppler mode. A single-element 10-MHz focused transducer and associated detection electronics was used for passive cavitation detection. The L7-4 imaging array

acquired passive acoustic signals. (b) The cross-sectional view of the tubing and the alignment of the three transducers from the tubing are shown.

Figure 2.

(a) Example of ELIP in the 1-mm inner diameter ethyl vinyl acetate tubing with no flow. The red bars indicate the location of the Doppler “sample volume” or spatial pulse length. The echogenicity was analyzed within regions of interest (ROIs) that were unexposed (No Doppler) and exposed (With Doppler) to Doppler pulses. (b) Example of ELIP flowing through the 1 mm inner diameter ethyl vinyl acetate tubing. The red bars indicate the location of the Doppler “sample volume” or spatial pulse length. The echogenicity was analyzed in regions of interest (ROIs) located upstream and downstream of the Doppler “sample volume”.

3 (a)

3 (b)

Figure 3. Stable and inertial cavitation thresholds of ELIP in flowing and static plasma measured using (a) a single-element passive cavitation detector (PCD) and (b) a passive cavitation imaging array.

Figure 4.

Comparison of the calibrated beamwidth of the Doppler pressure above the stable cavitation threshold and the measured widths of stable cavitation emissions on the passive cavitation images of ELIP insonified by Doppler pulses at PRFs (a) 1.25 kHz, (b) 2.5 kHz, (c) 5 kHz, and (d) 8.33 kHz.

Figure 5.

Comparison of the calibrated beamwidth of the Doppler pressure above the inertial cavitation threshold and the measured widths of inertial cavitation emissions on the passive cavitation images of ELIP insonified by Doppler pulses at PRFs (a) 1.25 kHz, (b) 2.5 kHz, (c) 5 kHz, and (d) 8.33 kHz.

Figure 6.

Comparison of loss of echogenicity, rapid fragmentation (Smith et al. 2007) and stable and inertial cavitation thresholds of ELIP in static fluid.

Figure 7.

ROC curves for (a) stable cavitation emissions and (b) inertial cavitation emissions as a predictor for loss of echogenicity from ELIP insonified by Doppler pulses. The cavitation emissions and loss of echogenicity datasets at all four PRFs were combined to compute the ROC curves.

Table 1

Duplex spectral Doppler pulse parameters. Pulse duration was maintained at 3.33 μ s. The number of pulses insonifying the ELIP was based on a plug flow assumption

Pulse repetition frequency (kHz)	# Pulses insonifying ELIP in flow	Range of mechanical indices (MI)	Range of peak rarefactional pressures (MPa)
1.25	31	0.04–0.7	0.07–1.7
2.5	63	0.04–0.53	0.07–1.28
5	126	0.04–0.39	0.07–0.8
8.33	211	0.04–0.33	0.07–0.64

Table 2

Percent error in the spatial width of stable and inertial cavitation activity detected using the passive cavitation imaging system compared to the calibrated beamwidth of the Doppler pressure profile

Pulse repetition frequency (kHz)	Percent error in PCI width of stable cavitation activity	Percent error in PCI width of inertial cavitation activity
1.25	-2.3 ± 15.3	-12.1 ± 22.5
2.5	7.5 ± 28.2	-6.05 ± 24.1
5	7.36 ± 16.1	9.37 ± 21.3
8.33	2.45 ± 15.35	5.46 ± 14.6

Table 3

K-S test between spatial widths of cavitation emissions measured on PCI and calibrated Doppler beamwidths exceeding the cavitation thresholds of ELIP

Pulse repetition frequency (kHz)	Stable cavitation emissions (p-value)	Inertial cavitation emissions (p-value)
1.25	0.12	0.16
2.5	0.11	0.12
5	0.15	0.09
8.33	0.09	0.06