I, Kirthi Radhakrishnan, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering.

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Abstract

Cardiovascular disease is the leading cause of death and disability in the United States and worldwide. Echogenic liposomes (ELIP) are theragonistic ultrasound contrast agents (UCAs) being developed for the early detection and treatment of cardiovascular disease. Stability of the echogenicity of ELIP in physiologic conditions is crucial to their successful translation to clinical use. The stability of ELIP echogenicity was determined in vitro under physiologic conditions of total dissolved gas concentration, temperature, and hydrodynamic pressure in porcine plasma and whole blood.

Ultrasound contrast agents (UCAs) have the potential to nucleate cavitation and promote both beneficial and deleterious bioeffects in vivo. Previous studies have elucidated the pressure amplitude threshold for rapid loss of echogenicity due to UCA fragmentation as a function of pulse duration and pulse repetition frequency (PRF). Previous studies have also demonstrated that UCA fragmentation was concomitant with inertial cavitation. The purpose of this study was to evaluate the relationship between stable and inertial cavitation thresholds and loss of echogenicity of ELIP as a function of pulse duration and pulse repetition frequency. Determining the relationship between cavitation thresholds and loss of echogenicity of ELIP would enable monitoring of cavitation based upon the on-screen echogenicity in clinical applications.

ELIP were insonified by a clinical ultrasound scanner in duplex spectral Doppler mode at four pulse durations and four PRFs in a static fluid and in a flow system. Cavitation emissions from the UCAs insonified by Doppler pulses were recorded using a single-element passive cavitation detection (PCD) system and a passive cavitation
imaging (PCI) system. Stable and inertial cavitation thresholds were ascertained. Loss of echogenicity from ELIP was assessed within regions of interest on B-mode images.

Stable cavitation thresholds were found to be lower than inertial cavitation thresholds. Stable and inertial cavitation thresholds of ELIP were found to have a weak dependence on pulse duration. However, the stable cavitation threshold of ELIP had no dependence on PRF. The inertial cavitation threshold of ELIP had a weak dependence on PRF. Cavitation thresholds ascertained using a PCI agreed with the thresholds ascertained using a single-element PCD. The azimuthal beamwidth of the cavitation emissions detected by the PCI system agreed with the calibrated beamwidth of the insonation Doppler pressure exceeding the cavitation threshold. The power of cavitation emissions was an exponential function of the loss of echogenicity over the investigated range of acoustic pressures. ELIP lost more than 80% echogenicity before the onset of stable or inertial cavitation. Once this level of echogenicity loss occurred, both stable and inertial cavitation emissions were detected in the physiologic flow phantom. These results indicate that 80% loss of echogenicity may be used as a qualitative metric to gauge the onset of stable and inertial cavitation from ELIP.
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CHAPTER I – Introduction
I.1 Background and Significance

I.1.1 Early detection of atherosclerotic lesions

Cardiovascular disease (CVD) is the leading cause of death accounting for 1 in every 3 deaths in the United States (Go et al. 2013) and worldwide (World Health Organization 2011). Atherosclerosis, a chronic inflammatory disease of the arteries, has been found to be the predominant precursor for CVD. Atherogenesis is complex and asymptomatic (Naghavi et al. 2003). Several factors, such as high levels of low-density-lipid (LDL) in the blood, free radicals caused by smoking, diabetes, and hypertension, genetic predisposition, hemodynamics at bifurcations in the vasculature, and infectious microorganisms, have been found to trigger endothelial dysfunction, inflammation and subsequent lesion formation (Epstein and Ross 1999). Some lesions develop lipid-rich, necrotic cores with thin unstable fibrous caps which are vulnerable to rupture (Finn et al. 2010). 70% of fatal acute myocardial infarctions (Naghavi et al. 2003) and 50% of transient ischemic attacks (Kwee et al. 2008) are caused by the rupture of vulnerable atherosclerotic plaques. Early detection and treatment of atherosclerotic lesions is therefore necessary for preventing acute cardiovascular events.

Current clinical methods to identify atherosclerotic plaques include invasive imaging techniques such as X-ray angiography, intravascular ultrasound, optical coherence tomography, and near-infrared spectroscopy, as well as non-invasive imaging techniques such as transesophageal ultrasound, ultrafast computed tomography and magnetic resonance imaging (Fayad and Fuster 2001). Invasive techniques employing catheterization have limited clinical utility in symptomatic patients (Davies et al. 2004) and can result in inadvertent vascular dissection (Vesely 2003). Clinical imaging
protocols currently employ macroscopic metrics based on plaque morphology such as luminal diameter, intima-media thickness, wall thickness and plaque volume to assess the stage of atherosclerosis and the vulnerability of the plaques (Fayad and Fuster 2001; Davies et al. 2004; Choudhury et al. 2004). Several clinical studies have demonstrated that plaque morphology and carotid intima-media thickness can be used to assess the risk of acute events (Sharma et al. 2009). However, some studies show that the morphological features of atherosclerotic lesions do not correlate with the risk of plaque rupture and therefore have limited diagnostic value (Finn et al. 2010; Davies et al. 2004).

Because the progression of atherosclerotic lesions into vulnerable plaques tends to be clinically asymptomatic and difficult to detect using current clinical approaches, molecular imaging methods are being developed to highlight the inflammatory stages of atherosclerotic lesions (Davies et al. 2004). In particular, ultrasound contrast agents (UCAs) designed to target specific molecular receptors are being developed for both intravascular and transvascular ultrasound imaging of atherosclerotic lesions (Kaufmann and Lindner 2007). Endothelial dysfunction triggers the endothelial cells to express various selectins, such as P-selectin and E-selectin, as well as adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Huo and Ley 2001). Both ICAM-1 and VCAM-1 play a role in recruiting monocytes during early stages of atheroma. As the lesion develops into thrombogenic plaque, the luminal surface of the lesion expresses fibrin I, fibrin II, fibrinogen and tissue factor (Bini et al. 1989; Falk and Fernández-Ortiz 1995; Marmur et al. 1996). The formation of thrombi involves platelet activation which is indicated by the expression of membrane glycoprotein (GPIIb/IIIa) on the platelet surface (Gawaz et al. 2005).
Angiogenesis in the vasa vasorum leading to neovascularization of the adventitia has also been implicated in the formation of vulnerable plaques and in restenosis (O'Brien et al. 1994). The upregulation of $\alpha_v\beta_3$ integrin and vascular endothelial growth factor (VEGF) promotes intraplaque angiogenesis (Hoshiga et al. 1995; Winter et al. 2003). Thus various molecular biomarkers have the potential to serve as indicators of the degree of progression of atherosclerosis.

I.1.2 Ultrasound contrast agents in detection of atherosclerosis

Gramiak and Shah (1968) first demonstrated the use of microbubbles in agitated saline to enhance contrast on echocardiographic images. Microbubble-based ultrasound contrast agents (UCAs) have been developed over the past 40 years for diagnostic and therapeutic applications. First generation UCAs consisted of air microbubbles encapsulated in thin shells composed of lipids, polysaccharides, polymers or albumin (Klibanov 1999). Second generation UCAs incorporated gases such as perfluorocarbons and sulfur hexafluoride having high molecular weight, low solubility and low diffusivity in blood to extend the persistence of the contrast in vivo (Mayer and Grayburn 2001). The United States Food and Drug Administration has approved two second generation UCAs, Definity® and Optison®, for left ventricular opacification (Mulvagh et al. 2008). European and Asian governments have approved UCAs for a broader range of applications, including detection of focal lesions in the liver, kidney and pancreas, assessment of abdominal trauma and evaluation of brain perfusion (Claudon et al. 2008). In addition to enhancing the contrast of blood pool and tissue perfusion, third generation
UCAs are being designed to target molecular specific biomarkers to highlight pathologic conditions (Klibanov 1999).

Several strategies are being investigated to target UCAs to molecular biomarkers (Kornmann et al. 2010). Active targeting can be achieved by direct conjugation of monoclonal antibodies to the ultrasound contrast agent or through indirect avidin-biotin mediated attachment of biotinylated ultrasound contrast agents (Klibanov 1999; Lanza et al. 1996). Indirect targeting through avidin-biotin interactions provides the advantage of multiple biotin binding sites per avidin molecule thus amplifying the contrast-to-noise-ratio at the target sites. However, indirect targeting requires sequential infusions of the biotinylated antibody, followed by streptavidin and finally the biotinylated UCAs, which may not translate readily to clinical applications. Direct conjugation of antibodies to the UCA shell may allow easy and clinically feasible methods to administer the UCAs.

Perfluorobutane microbubbles with lipid shells have been conjugated to various antibodies targeting VCAM-1 (Kaufmann et al. 2007), P-selectins (Lindner et al. 2001); (Christiansen et al. 2002), leukocytes (Christiansen et al. 2002) and ICAM-1 (Weller et al. 2003; Weller et al. 2002; Villanueva et al. 1998) using avidin-biotin links. These novel agents have been shown to highlight inflammation and early atherosclerotic lesions on diagnostic transvascular ultrasound images. Other groups (Schumann et al. 2002; Wang et al. 2006; Hagisawa et al. 2011) have employed GPIIb/IIIa-targeted UCAs to highlight the surface of thrombi on ultrasound images. Angiogenic biomarkers such as αvβ3 integrins (Dayton et al. 2004; Leong-Poi et al. 2003) and VEGF receptors (Willmann et al. 2008; Anderson Christopher et al. 2010; Korpanty et al. 2007) have been highlighted on ultrasound images using peptide-conjugated and antibody-conjugated UCAs.
Perfluorocarbon emulsions have also been engineered to target fibrin (Lanza et al. 1996) and tissue factor (Lanza et al. 2000) to delineate the surface of thrombi on diagnostic ultrasound images.

### I.1.3 Echogenic liposomes for detection of cardiovascular diseases

In addition to providing contrast for molecular imaging and detection of lesions, novel UCAs, such as echogenic liposomes (ELIP), are also being designed to deliver therapeutics to lesions. Echogenic liposomes are theragnostic UCAs that are being developed for the early detection and treatment of cardiovascular disease. ELIP are lipid bilayer vesicles that encapsulate gas and aqueous cores. ELIP have been loaded with various therapeutics, including thrombolytics (Tiukinhoy-Laing et al. 2007b; Shaw et al. 2009), vasodilators (Kee et al. 2008), an anti-angiogenic agent (Naji 2013), an antibiotic (Tiukinhoy et al. 2004), stem cells (Herbst et al. 2010), genes (Buchanan et al. 2010), and bioactive gases (Huang et al. 2009; Britton et al. 2010) to treat cardiovascular disease. Therapeutic agents such as genes (Buchanan et al. 2010; Akhtar et al. 1991) and vasoactive gases (Huang et al. 2009; Tsao et al. 1994) cannot be administered systemically because they are easily scavenged within the vasculature before they reach the target tissue. Other therapeutic agents such as thrombolytics (Shaw et al. 2009; Tanne et al. 1999) and antibiotics (Tiukinhoy et al. 2004; Eccleston et al. 1996) have systemic dose limits due to their toxic side-effects. Liposomal formulations, such as ELIP, provide a "stealth" vehicle for therapeutic agents to be loaded and administered systemically so that the therapeutic agents can be transported to the target tissue without being scavenged
or causing harmful side-effects (Allen and Moase 1996; Al-Jamal and Kostarelos 2011; Allison 2007).

Alkan-Onyuksel et al. (1996) first demonstrated ELIP echogenicity on intravascular ultrasound images and proposed a lipid formulation containing phosphatidylcholine (PC) to mimic the phospholipid composition of cell membranes, cholesterol (Chol) to provide rigidity to the lipid bilayer, phosphatidylethanolamine (PE) to enable antibody conjugation, and phosphatidylglycerol (PG) to prevent aggregation of the liposomes by rendering a slightly negative charge to the liposomes. The lipid composition and preparation protocols of ELIP have been optimized to maximize echogenicity and encapsulation efficiency (Huang et al. 2001; Huang et al. 2002; Huang and MacDonald 2004). Huang et al. (2001; 2004) found that increasing the concentration of the cryoprotectant, mannitol, improved the echogenicity of ELIP and hypothesized that mannitol introduces defects in the lipid bilayer, thereby allowing the encapsulation of air upon ELIP reconstitution. The ELIP formulation has been further modified by Buchanan et al. (2008) to incorporate dipalmitoyl phosphocholine (DPPC), a rigid saturated phospholipid, to enhance the stability of ELIP echogenicity at 37 °C. However, a critical gap in knowledge was the assessment of the stability of ELIP as a blood pool contrast agent.

Methods to conjugate antibodies directly to the ELIP shell have been developed and tested using immunoblot assays (Klegerman et al. 2002). Anti-fibrinogen-conjugated ELIP have been shown to adhere to in vitro fibrin clots under physiological shear rates (Demos et al. 1997; Demos et al. 1999). Further, in vivo studies have been carried to demonstrate the highlighting of thrombi on intravascular and transvascular images using
anti-fibrinogen-conjugated ELIP (Demos et al. 1999; Hamilton et al. 2002). Other antibodies such as anti-ICAM-1, anti-VCAM-1, anti-fibrin and anti-tissue factor have been directly conjugated to the lipid shell of ELIP and atheromatous components on porcine arteries have been highlighted on intravascular images using these antibody-conjugated ELIP (Hamilton et al. 2004). Antibody-conjugated ELIP also serve as targeted drug delivery vectors. Anti-ICAM-1-conjugated ELIP labeled with rhodamine (Hitchcock et al. 2010) have been delivered to murine aortic tissue upon exposure to 1-MHz continuous wave ultrasound. Bi-functional ELIP conjugated with anti-ICAM-1 and stem cells have also been delivered to porcine aortic tissue upon exposure to continuous wave ultrasound (Herbst et al. 2010). The fibrin binding sites on the thrombolytic agent tissue plasminogen activator (t-PA) remain exposed after loading onto ELIP and provide the necessary targeting to thrombi for contrast enhancement and image-guided drug delivery (Tiukinhoy-Laing et al. 2007a; Klegerman et al. 2008). These studies demonstrate that antibody-conjugated ELIP can be used as targeted UCAs for the early identification of atheromatous components and for targeted drug delivery.

I.1.4 Acoustic cavitation from ultrasound contrast agents and ELIP

The encapsulated microbubbles in ELIP also serve as nuclei for acoustic cavitation, which plays an important role in ultrasound-induced bioeffects. Acoustic cavitation is the non-linear volumetric oscillation of microbubbles in an acoustic field. Sustained oscillation of microbubbles about their equilibrium radius is called stable cavitation (Flynn 1964). Stable cavitation is detected by the presence of subharmonic, ultraharmonic and harmonic frequency components (Eller and Flynn 1969; Neppiras
The subharmonic response of UCAs has been employed in subharmonic imaging to enhance contrast to tissue ratio (Sridharan et al. 2013) and to estimate blood pressure non-invasively (Dave et al. 2013). Stable cavitation of microbubbles also causes microstreaming in the surrounding fluid (Elder 1959; Collis et al. 2010). Microstreaming is hypothesized to permeabilize cell membranes and enhance the removal of fibrin degradation products (Sutton et al. 2013). Sustained stable cavitation has been shown to facilitate the delivery of ELIP into arteries (Hitchcock et al. 2010) and enhance the lysis of clots (Datta et al. 2008).

Inertial cavitation is transient and involves large amplitude expansion of microbubbles followed by a violent collapse (Holland and Apfel 1989; Holland and Apfel 1990). The inertial collapse of microbubbles results in broadband frequency components in the acoustic emissions (Neppiras 1980). Inertial cavitation has been shown to facilitate the ablation of cancerous tissue (Coussios et al. 2007) and the delivery of genes into tissue through extravasation (Bazan-Peregrino et al. 2012). Ultrasound-mediated destruction of UCA contrast and the resultant acoustic emissions have been used in stimulated acoustic emissions imaging (SAE) (Tiemann et al. 2000), flash echo imaging (Kamiyama et al. 1999), and destruction-reperfusion imaging (Wei et al. 1998). However, ultrasound-induced UCA destruction at high mechanical indices (MI > 0.4) can trigger inertial cavitation near cellular membranes resulting in microvascular damage, cellular apoptosis and petechial hemorrhage (Miller et al. 2008; Skyba et al. 1998; Miller and Dou 2004; Chen et al. 2003a; Samuel et al. 2009).

Such bioeffects indicate that there is a need to monitor the onset and type of cavitation in order to optimize the nonlinear response of UCAs for improved imaging.
contrast and therapeutic effects, while ensuring bio-safety in the presence of ultrasound-induced UCA destruction. Previous studies have classified the regimes of UCA destruction based on the rate of change of microbubble diameter on high-speed optical images (Chomas et al. 2001) and the temporal changes in acoustic backscatter and echogenicity from UCAs (Porter et al. 2006; Smith et al. 2007). The regimes of UCA destruction have been classified as passive diffusion, acoustically driven diffusion, and rapid fragmentation (Chomas et al. 2001; Smith et al. 2007). Smith et al. (2007) ascertained the acoustically driven diffusion threshold and rapid fragmentation threshold of ELIP insonified by duplex Doppler pulses from a clinical scanner. The acoustically driven diffusion threshold was defined as the insonation pressure at which acoustically-induced diffusion of gas out of the ELIP causes an exponential decay of echogenicity. The rapid fragmentation threshold was determined as the lowest insonation pressure at which the ELIP echogenicity reduces to the background level within 5 s. The acoustically driven diffusion threshold of ELIP was weakly dependent on the insonation pulse duration and pulse repetition frequency (PRF), whereas the rapid fragmentation threshold of ELIP was strongly dependent on the insonation pulse duration and PRF. Chen et al. (Chen et al. 2002; Chen et al. 2003b) acoustically interrogated UCAs undergoing rapid fragmentation and found that the gas microbubbles liberated from rapid fragmentation nucleate inertial cavitation. These studies have suggested that rapid changes in echogenicity due to UCA fragmentation are concomitant with inertial cavitation and could be used to monitor the likelihood of inertial cavitation nucleated by UCAs.

Previous studies have investigated cavitation emissions from ELIP in the context of drug release and drug delivery. Cavitation emissions from ELIP insonified by color
Doppler and spectral Doppler pulses have been detected, even though a significant amount of drug release was not measured (Kopechek et al. 2013). Kopechek (2011) also noted a loss of echogenicity of calein-loaded ELIP and rosiglitazone-loaded ELIP on B-mode images concomitant with the detection of inertial cavitation. However, there have been no previous studies that evaluated the relationship between onscreen loss of echogenicity and cavitation thresholds of ELIP.

**I.1.5 Cavitation detection techniques**

Single-element transducers have been employed to detect cavitation using active and passive techniques (Roy et al. 1990; Atchley et al. 1988). In active cavitation detection (ACD) systems, the transducer is operated in the pulse echo mode to transmit a high-frequency narrowband pulse and receive the acoustic backscatter from microbubbles. In passive cavitation detection (PCD) systems, the transducer is used to passively record cavitation emissions from microbubbles. As noted by Roy et al. (1990), ACD provides better spatial and temporal resolution compared to PCD. However, the interrogation acoustic pulses used in ACD should be selected to be low enough not to initiate cavitation or microbubble destruction. These ACD pulses may also initiate acoustic streaming and primary and secondary radiation pressures (Roy et al. 1990).

In order to provide spatiotemporal resolution of cavitation activity within a region, imaging array transducers are being used to passively record the cavitation activity. The emissions recorded on each channel of the imaging array can be beamformed to create passive acoustic maps (also referred to as passive cavitation images). Several studies have demonstrated the use of passive acoustic maps to spatially
localize cavitation activity during continuous-wave high intensity focused ultrasound insonation (Gyongy et al. 2008; Gyongy and Coussios 2010; Salgaonkar et al. 2009; Jensen et al. 2012; Farny et al. 2009). Recent studies have also demonstrated the use of passive cavitation imaging in recording cavitation emissions from UCAs insonified by pulsed ultrasound (Haworth et al. 2012; Choi and Coussios 2012). Haworth et al. (2012) showed that the passive cavitation image resolution was independent of the insonation ultrasound pulse shape. Choi and Coussios (2012) observed that the spatial distribution of the cavitation energy from UCAs was dependent on the flow rate and pulse repetition frequency of insonation. At higher PRFs and higher insonation pressures, the spatial distribution of the cavitation energy from flowing UCAs was asymmetric (Choi and Coussios 2012). These results have motivated the use of passive cavitation imaging as a second detection system along with the single-element PCD to record cavitation emissions from flowing ELIP.

1.2 Hypothesis and specific aims

Previous studies have investigated the regimes of loss of echogenicity from ELIP insonified by diagnostic ultrasound transducers (Porter et al. 2006; Smith et al. 2007). The interaction of ultrasound with UCAs has been shown to initiate both therapeutic (Datta et al. 2008) and adverse bioeffects (Miller et al. 2008) through cavitation. The relationship between loss of echogenicity and cavitation emissions from ELIP could potentially provide an onscreen measure to gauge the likelihood of cavitation-induced bioeffects during therapy. The central hypothesis of this dissertation is that loss of echogenicity is concomitant with acoustic cavitation emissions from echogenic liposomes.
insonified by clinical Doppler. This hypothesis has been tested for ELIP and Definity® in an in vitro physiologic flow phantom. Cavitation thresholds and loss of echogenicity from ELIP have been quantified as a function of pulse duration and pulse repetition frequency. The following specific aims were designed to investigate the relationship between loss of echogenicity and cavitation from ELIP:

Specific Aim #1 (Chapter II): Evaluate the effect of dissolved gas content, temperature, hydrodynamic pressure and whole blood on the stability of ELIP echogenicity in a physiological flow phantom.

Specific Aim #2(a) (Chapter III): Quantify the loss of echogenicity and stable and inertial cavitation thresholds of ELIP insonified by 6-MHz clinical Doppler pulses as a function of pulse duration.

Specific Aim #2(b) (Chapter IV): Quantify the loss of echogenicity and stable and inertial cavitation thresholds and spatial distribution of cavitation emissions from ELIP insonified by 6-MHz clinical Doppler pulses as a function of pulse repetition frequency.

The overarching goal of the research described in this dissertation is the identification of ultrasound parameters necessary for initiating cavitation during ultrasound image-guided therapy whilst ensuring the safe use of ELIP. The experiments were carried out in a flow setup with thermodynamic parameters such as temperature, hydrodynamic pressure and dissolved oxygen controlled to be in the physiological range for humans.
I.3 Thesis overview

The focus of this dissertation is to elucidate the relationship between cavitation and loss of echogenicity from ELIP. A physiological flow system was developed to evaluate the stability of ELIP echogenicity. Cavitation thresholds and loss of echogenicity of ELIP were ascertained as a function of pulse duration and PRF. The spatial distribution of cavitation emissions was also determined using passive cavitation imaging. Please note that the vertical line in the margin denotes text and figures verbatim from published papers (Radhakrishnan et al. 2012) and (Radhakrishnan et al. 2013).

In Chapter II, the stability of echogenic liposomes in physiological flow has been evaluated. Prior to this study, ELIP had not been evaluated as a blood pool contrast agent. Previous in vivo studies primarily investigated the targeting of ELIP to atherosclerotic components after intra-arterial infusions to visualize contrast enhancement on intravascular ultrasound images. The study described in Chapter II has elucidated the effect of dissolved gas content, temperature transitions, hydrodynamic pressure and presence of formed elements in the diluent on the echogenicity of ELIP. This work has been published in Ultrasound in Medicine and Biology (Radhakrishnan et al. 2012).

In Chapter III, the relationship between cavitation and loss of echogenicity in ELIP and Definity® has been investigated. Previous studies have highlighted the dependence of cavitation thresholds and loss of echogenicity on pulse duration. In this study, ELIP and Definity® were insonified by duplex Doppler pulses at four pulse durations. Cavitation thresholds and loss of echogenicity were evaluated in the presence and absence of
physiologic flow. ELIP and Definity® lost 80% echogenicity at insonation pressures corresponding to the stable and inertial cavitation threshold. The loss of echogenicity threshold of ELIP in the static system was found to match the rapid fragmentation threshold (Smith et al. 2007). This work has been published in Physics in Medicine and Biology (Radhakrishnan et al. 2013).

In Chapter IV, the relationship between loss of echogenicity and cavitation emissions from echogenic liposomes as a function of pulse repetition frequency (PRF) has been described. Smith et al. (2007) determined that the rapid fragmentation threshold of ELIP in static fluid decreased with increasing PRF. In this study, cavitation thresholds were determined using a single-element passive detector and a passive imaging array. The loss of echogenicity threshold of ELIP determined in the static system was lower than the rapid fragmentation threshold determined by Smith et al. (2007). In the flow system approximately 80% loss of echogenicity was observed at the stable and inertial cavitation thresholds. The azimuthal width of cavitation activity above the cavitation threshold on the passive cavitation images agreed with the azimuthal beamwidth of the Doppler pressures exceeding the cavitation threshold after applying a correction for the point spread function of the passive cavitation imaging array. This work will be submitted to Ultrasound in Medicine and Biology.

Chapter V summarizes the findings of this dissertation and provides a direction for future studies. Studies to evaluate the cavitation emissions and loss of echogenicity from echogenic liposomes targeted to a substrate will be explored.
CHAPTER II – Stability of echogenic liposomes as a blood pool contrast agent in a physiologic flow phantom
II.1 Introduction

Ideal ultrasound contrast agents (UCAs) should be biocompatible, intravenously injectable, and stable in the pulmonary, cardiac and capillary circulation (Goldberg et al. 1994). Echogenic liposomes (ELIP) have been formulated to have a non-toxic and biocompatible phospholipid shell (Smith et al. 2010) which encapsulates air (Huang et al. 2001) or other bioactive gases (Huang et al. 2009; Britton et al. 2010). Previous studies have demonstrated the targeting of ELIP to thrombi (Demos et al. 1997; Demos et al. 1999; Hamilton et al. 2002) and atheromatous arteries (Hamilton et al. 2004) in vivo. However, the stability of ELIP as a blood pool contrast agent has not been evaluated.

The stability of UCA echogenicity depends on the persistence of backscatter intensity from the encapsulated microbubbles. The backscatter intensity depends on the sixth power of the radius of the encapsulated microbubbles and is enhanced by the presence of resonant-sized microbubbles at the incident acoustic frequency (Goldberg et al. 1994). The microbubble size is dictated by equilibrium of the vapor pressure and gas pressure inside the microbubble, the diffusion of gas between the microbubble and the surrounding medium, the Laplace pressure of the shell, and the hydrostatic pressure in the surrounding medium (Leighton 1994; Epstein and Plesset 1950). Because the thermodynamic and hydrodynamic parameters are tightly regulated through homeostasis in vivo, the type of gas and shell properties of UCAs need to be carefully selected to ensure stable echogenicity of UCAs.

In order to improve the persistence of contrast in vivo, second-generation UCAs, such as Definity® and Optison®, encapsulate perfluorocarbon gases having low solubility, higher molecular weight and low diffusivity (Mayer and Grayburn 2001). However,
recently there have been concerns about the safety of perfluorocarbon-based UCAs resulting in black-box warnings and contraindications on the use of these UCAs in patients suffering from various cardiovascular and hypertensive conditions (FDA 2008). In order to preclude these safety concerns and ensure biocompatibility, ELIP have been designed to primarily encapsulate air. However, air-filled UCAs have been more susceptible to dissolution due to air’s faster diffusion and higher solubility into blood, especially in the presence of a hydrostatic pressure above atmospheric pressure (Vuille et al. 1994; Padial et al. 1995; Mor-Avi et al. 1994). The high solubility results in a shorter half-life in vivo, potentially limiting the ability to perform contrast enhanced ultrasound imaging or therapy. Therefore, the effects of physiologically relevant thermodynamic and hydrodynamic parameters on the stability of air-filled UCAs need to be assessed during their development.

II.1.1 Effect of dissolved gas content on UCA stability

The diffusion of gas out of microbubble-based UCAs will depend on the concentration gradient of gas within versus outside the microbubble. In vivo studies have demonstrated that the concentration and type of anesthetic gas circulating in the blood stream impacts the longevity of UCAs (Mullin et al. 2011; Itani and Mattrey 2011). Both studies showed that compared to medical air, the use of oxygen as the anesthetic carrier gas significantly reduced the in vivo persistence of Definity®. Several groups have mathematically modeled the effect of gas diffusion on microbubble size. Duncan and Needham (2004) evaluated the effect of the diluent’s dissolved air content on the rate of dissolution of air-filled microbubbles and found that the Epstein-Plesset model slightly
overestimated the dissolution rates. Both Van Liew and Burkard (1995) and Kabalnov et al. (1998) used diffusion models to predict the effect of dissolved gas levels in blood on the size of perfluorocarbon gas-filled microbubbles in cardiopulmonary circulation.

However, these models did not consider the effect of the UCA shell in gas permeability and diffusion. Sboros et al. (2000a; 2000b) observed that the longevity of UCA backscatter depended on the partial pressure of oxygen in the surrounding fluid and the UCA shell properties. Borden and Longo (2002) incorporated the gas permeation resistance of lipid monolayer shells in a modified Epstein-Plesset model to describe the dissolution of shelled microbubbles. Recently, Kwan and Borden (2010) showed that the rigidity, solubility, and dynamic surface tension of the surfactant coating the microbubbles affected the rate of growth and dissolution of SF₆-filled microbubbles in air-saturated media. Smith (2008) developed a protocol to use 100% air-saturated 0.2 μm filtered deionized water for reconstitution of ELIP. However, there were no previous studies investigating the effect of dissolved gas levels in the diluent on the echogenicity of ELIP.

II.1.2 Effect of diluent temperature on microbubble stability

Thermodynamic parameters also play an important role on the size of the microbubbles and the stability of the UCA shell. Webb et al. (2010) evaluated the dynamics of unshelled air microbubbles undergoing heating and found three size-dependent threshold behaviors. Small microbubbles (10 μm radius) underwent an instantaneous decrease in bubble size and eventual dissolution. Larger microbubbles (40 μm radius) expanded upon heating and grew by diffusion. Intermediate sized
microbubbles (20 μm radius) underwent shrinkage due to outward diffusion of gas, followed by expansion due to diffusion of gas into the microbubble. It should be noted that the effect of temperature on UCA shells was not considered in the study. However, the study demonstrates the complex relationship between microbubble size and temperature-dependent gas diffusion. Mulvana et al. (2010) illustrated that lipid-shelled SF₆-filled microbubbles expanded in size at body temperature. Some microbubbles became unstable and underwent rapid shrinkage caused by lipid shedding at 40 °C. Other investigators (Mulvana et al. 2011; Vos et al. 2008) have examined the temperature dependence of microbubble dynamics and nonlinear acoustic emissions.

Pu et al. (2006) demonstrated that the acyl-chain length and transition temperatures of lipids used in the shell of UCAs play an important role on the dissolution of air-filled UCAs upon heating. Buchanan et al. (2008) also found that the incorporation of lipids with longer acyl chains and higher transition temperatures improved the stability of ELIP at physiologic temperature. Typical in vivo administration of ELIP as an UCA would involve reconstitution of ELIP at room temperature and intravenous injection. This process induces a temperature transition of ELIP from room temperature to body temperature. Some clinical procedures may also involve dilution of the UCA in PBS warmed to 37 °C prior to administration. If the PBS is not maintained at 37 °C, this process may induce a temperature transition of ELIP from body temperature to room temperature. Previous in vitro studies have not evaluated the stability of ELIP echogenicity under temperature transitions in order to ensure proper handling and administration of ELIP for in vivo applications.
II.1.3 Effect of hydrodynamic pressure on microbubble stability

Hydrodynamic overpressure in conjunction with the Laplace pressure dictates the gas diffusion and therefore the size distribution of the microbubbles. Air-filled UCAs are particularly susceptible to changes in hydrostatic pressure and dissolved gas content in the diluent. Vuille et al. (1994) observed a rapid decay of contrast within seconds from several first generation air-filled UCAs including Albunex, Angiovist and Levovist at 200 mmHg hydrostatic pressure. Mor-Avi et al. (1994) and Padial et al. (1995) observed cyclic variations in the UCA diameter during systole and diastole and an overall temporal decay of contrast from air-filled UCAs under pressures found in the left ventricle. Other groups elucidated changes in the subharmonic response of UCAs as a function of hydrodynamic pressures in the surrounding fluid (Forsberg et al. 2005; Adam et al. 2005; Andersen and Jensen 2010a; Andersen and Jensen 2010b). The UCA shell elasticity and the encapsulated gas influence the response of UCAs to changes in hydrostatic pressures in the surrounding fluid (Shi et al. 2004).

Huang et al. (2002) observed that hydrostatic overpressures exceeding 380 mmHg caused 50% loss of echogenicity from ELIP within 60 s on intravascular ultrasound images. However, the effect of normotensive and hypertensive hydrodynamic pressures on the echogenicity of ELIP has yet to be investigated quantitatively. Assessment of physiologically relevant arterial hydrodynamic pressures on the stability of ELIP is an important step in the development of ELIP as UCAs for highlighting and treating cardiovascular diseases.

In summary, the host fluid's total dissolved gas, temperature and hydrodynamic pressure will likely influence the size, stability and acoustic response of UCAs. Prior
studies have demonstrated that ELIP can be imaged with both transvascular and intravascular ultrasound systems and are more stable than Optison® in static fluid (Smith et al. 2007). Although these studies investigated various parameters that affected the stability of ELIP, the effects of dissolved gas content, temperature transitions, and hydrodynamic pressure in the host fluid on the echogenicity of ELIP had not been studied.

II.2 Methods

II.2.1 Preparation of echogenic liposomes

Echogenic liposomes (ELIP) were prepared at the University of Texas Health Science Center at Houston in 6 mg vials and shipped with refrigerant packs overnight. Briefly, the lipids, L-\(\alpha\)-phosphatidylcholine (chicken egg; EPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-[phosphor-rac-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 chloroform were mixed in the molar ratio 27:42:8:8:15. After evaporation of the chloroform at 50 °C, the lipid film was formed by placing the lipid mixture under vacuum (<100 mTorr pressure) for 4h. The dry lipid film was rehydrated with deionized water and incubated at 55 °C for 30 minutes. The lipid mixture was sonicated in a water bath for 5 minutes. Mannitol solution (0.32 M) was added to the lipid mixture in vials. The vials were frozen on dry ice and lyophilized for 24 h. This lipid formulation as described by Buchanan et al. (Buchanan et al. 2008) has been found to enhance the stability of echogenic liposomes at body temperature. Each vial of lyophilized ELIP
containing 6 mg lipid was reconstituted with 0.6 ml of 0.2 μm filtered deionized water (NANOPure, Barnstead International, Dubuque, IA, USA) to give a lipid concentration of 10 mg/ml. Reconstituted ELIP were diluted in porcine plasma or whole blood to give a final lipid concentration of 0.05 mg/ml (Hamilton et al. 2004; Smith et al. 2007).

II.2.2 Physiologic flow phantom

The stability of ELIP was evaluated in a physiologic flow system shown in Figure 2.1. It consisted of a 250 mL water-jacketed reservoir and 2 mm inner diameter water-jacketed tubing (Radnoti, Monrovia, CA, USA). The water-jacketed system was connected to a thermostatic circulator (LKB, Bromma, Sweden) to control the temperature of the intra-luminal fluid. A pulsatile pump (Model 1407, Harvard Apparatus, Holliston, MA, USA) was set to a stroke rate of 60 beats per minute. The pulse pressure and the mean hydrodynamic pressure in the flow system were controlled to mimic normotensive or stage 1 hypertensive conditions (Chobanian et al. 2003) by adjusting the stroke volume of the pump and the height of an after-load as shown in Figure 2.1.
Figure 2.1: Schematic of the experimental setup. ELIP diluted in porcine plasma or whole blood were added to the water jacketed reservoir and pumped to the through the flow system. The linear array transducer (L12-5) of the Philips HDI 5000 clinical scanner was used to acquire fundamental B-mode images of the ELIP flowing in the lumen of a 0.4 mm thick latex tubing. The tubing was placed 1 cm away from the face of the transducer. Inline temperature and pressure sensors were used to monitor the in situ temperature and hydrodynamic pressure in the system.

The hydrodynamic pressure in the flow system (Figure 2.2) was measured using an inline pressure transducer (PendoTECH, Princeton, NJ, USA). The signal from the pressure transducer was conditioned (Omega Electronics, Stamford, CT, USA), digitized, and recorded at 500 Hz (Signal Express, National Instruments, Austin, TX, USA). The outlet of the pulsatile pump was connected to water-jacketed tubing. Once the water-jacketed tubing entered the tank containing degassed water (22 °C or 37 °C), compliant latex tubing (3.18 mm inner diameter (ID) 0.8 mm wall thickness) (Mcmaster Carr Aurora, OH, USA) was used. The temperature in the flow system was monitored during the experiment using an inline temperature probe (PendoTECH). To minimize acoustic
attenuation through the tubing wall at the imaging location, a 3.18 mm ID latex tube with
a 0.4 mm wall thickness (Piercan, San Marcos, CA, USA) was used.

![Pressure Graph]

**Figure 2.2:** Hydrodynamic pressures in the flow system measured by inline pressure
transducer. Note that the pressure transducer was calibrated using a KAL 84 pressure
calibrator (Halstrup Walcher GmbH, Kirchzarten, Germany). The signals were
recorded using Signal Express (National Instruments) at 500 Hz sampling frequency
and digitally filtered using a second order IIR filter having a 100 Hz cutoff frequency.
The RMS values were recorded continuously during the experiment into a comma-
separated-values log file and exported into Microsoft Excel® (Redmond, WA, USA).
Examples of hypertensive pressure 145/90 mmHg (––), normotensive pressure
120/80 mmHg (—), 60/20 mmHg (– – –) and atmospheric pressure 1 mmHg (···)
are shown.

**II.2.3 Image acquisition and processing**

Fundamental B-mode images of ELIP in the flow system were acquired with the
L12-5 linear array transducer (center frequency 6.9 MHz) of the Philips HDI 5000
diagnostic ultrasound scanner (Philips, Bothell, WA, USA). During image acquisition,
the acoustic output (mechanical index or MI) was set sufficiently low (MI=0.04) to avoid
loss of echogenicity due to acoustically driven diffusion (Smith et al. 2007). The frame
rate, persistence, grayscale map, time gain compensation (TGC) and 2-D gain were kept constant for all experiments. In each experiment, an initial B-mode image of ELIP in flow at atmospheric pressure (denoted as 1 mmHg throughout this document) was acquired, and subsequently three images were acquired at 5 s intervals at a 60/20 mmHg hydrodynamic pressure. Finally, to capture the temporal decay of ELIP echogenicity at arterial hydrodynamic pressures (120/80 mmHg), images were acquired every 5 s over 75 s. Images were stored on a magneto-optical disk and post-processed using MATLAB® (Mathworks, Natick, MA, USA).

Previous studies by Smith et al. (Smith et al. 2007) and Porter et al. (Porter et al. 2006) determined that mean digital intensity (MDI), unlike mean gray scale value (MGSV), was directly proportional to the backscattered acoustic power and independent of the grayscale map used by the scanner. A technique developed by Porter et al. (2006) was modified to establish the relationship between the MGSV and the MDI for the scanner settings used in these experiments. Both cine loops and freeze-frame images of a tissue mimicking phantom with wire targets (Model 539, ATS Laboratories, Bridgeport, CT, USA) were stored. A large dynamic range of brightness values was obtained by using several different TGC settings. Porter et al. (2006) defined ROIs within the tissue mimicking speckle (shown as ROI$_1$ in Figure 2.3(a)) thus computing MGSV over regions of varying intensity. In the current study, ROIs were defined within the hyperechoic regions of uniform intensity created by the wire targets (shown as ROI$_2$ in Figure 2.3(a)).

The MGSV measured on freeze-frame images using MATLAB and the MDI measured on corresponding cine loops using QLab (Philips, Bothell, WA, USA) within ROIs were fit with a polynomial fit (equation 2.1) as shown in figure 2.3(b).
CHAPTER II

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\text{MDI(dB)} = -0.0011(MGSV^2) + 0.48(MGSV),
\]  

(2.1)

All images were first converted to linear digital intensity prior to quantifying MDI for an ROI defined within the lumen of the tubing as shown in figure 2.3(c). The average MDI was computed over 15 images that exhibited steady-state echogenicity at 120/80 mmHg.

Figure 2.3: (a) Image of wire targets in tissue mimicking phantom used to calibrate mean digital intensity (MDI) based on mean gray scale value (MGSV). Region of
interest, (ROI\textsubscript{1}) defined within the tissue mimicking speckle was used by Porter et al. (Porter et al. 2006). ROI\textsubscript{2} defined in the hyperechoic region of uniform intensity is used in the current study. (b) Relationship between MDI and MGSV for a fixed grayscale map. (c) B-mode image of echogenic liposomes (ELIP) in plasma with the ROI defined within the tubing.

II.2.4 Effect of dissolved gas concentration in plasma on ELIP echogenicity

The main dissolved gases \textit{in vivo} include nitrogen, oxygen and carbon dioxide. Nitrogen and oxygen contribute to 95% of the total dissolved gases. Therefore, the relative partial pressures of nitrogen and oxygen, both in the diluent and in the ELIP affect in-gassing and out-gassing of the air encapsulated in ELIP and the echogenicity. Nitrogen in the blood is dissolved in the plasma and is slightly super-saturated (101-102\%) relative to alveolar air (Aksnes and Rahn 1957; Canfield and Rahn 1957). The super-saturation is due to incomplete transfer of gases due to ventilation perfusion inequalities in the lungs (Canfield and Rahn 1957). Oxygen in the blood is both dissolved in the plasma and bound to hemoglobin. The amount of oxygen bound to hemoglobin is 64 times the amount of oxygen dissolved in plasma, however, this bound oxygen is unavailable to contribute to in-gassing or out-gassing of microbubbles due to the oxygen dissociation curve (Allan H Mines 1986). The oxygen dissolved in plasma is undersaturated relative to alveolar levels (Altman 1959) due to ventilation perfusion inequalities in the lungs and oxygen consumption in the tissue (Allan H Mines 1986).

A dissolved oxygen (DO) meter (DO 110, Oakton Instruments, Vernon Hills, IL, USA) was used to measure the oxygen saturation in plasma. Because the dissolved gas content of each gas (nitrogen and oxygen) could not be measured individually, the oxygen saturation value was used as a measure of total dissolved air. The use of dissolved
oxygen as a surrogate measure for total dissolved air has been used in previous studies (Duncan and Needham 2004). Using the partial pressures and solubilities of dissolved gases in arterial and venous plasma (which can contribute to in-gassing and out-gassing) the total dissolved gas saturations in arterial and venous plasma (with respect to alveolar levels) are 94-98% and 87-91%, respectively (Aksnes and Rahn 1957; Altman 1959).

The citrated pooled porcine plasma used in these experiments (8304906, Lampire Biological Labs, Pipersville, PA, USA) had 63% ± 5.5% saturated dissolved oxygen when thawed from -80 °C to room temperature. To increase the dissolved gas content to one of the physiologic values listed above, the plasma was put in a pressure vessel (Millipore, Billerica, MA, USA) at 200 kPa of air for 2-3 hours. Then 30 ml plasma samples were placed in sealed syringes (309680, Becton Dickson, Franklin Lakes, NJ, USA) and held in a water bath at 37 °C for 60 min to warm the samples to body temperature, while maintaining the same dissolved gas concentration.

The measured dissolved oxygen content at 37 °C is shown in Table 2.1. The DO meter was calibrated in air-saturated water at 37 °C prior to measurement of DO of the plasma at 37 °C. Reconstituted ELIP were added to each 30 ml plasma aliquot in the water-jacketed reservoir, pumped through the flow system. B-mode images of the ELIP in plasma were acquired at hydrodynamic pressures of 1 mmHg, 60/20 mmHg and 120/80 mmHg above atmospheric pressure. Two-tailed paired Student’s t-tests were performed using Microsoft Excel® (Redmond, WA, USA) to determine if there were statistically significant (p<0.05) changes in ELIP echogenicity at pressures above 1 mmHg at each dissolved gas concentration.
Table 2.1: Dissolved oxygen content in plasma measured at 37 °C. Numbers represent the mean and standard deviation of the dissolved oxygen measurements (N=5).

<table>
<thead>
<tr>
<th></th>
<th>Degassed plasma</th>
<th>Plasma simulating arterial dissolved gas content</th>
<th>Plasma simulating venous dissolved gas content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured dissolved oxygen saturation at 37 °C</td>
<td>67 ± 5.6%</td>
<td>97.4 ± 1.5%</td>
<td>90.1 ± 3.1%</td>
</tr>
</tbody>
</table>

II.2.5 Effect of temperature transitions in plasma on ELIP echogenicity

Porcine plasma was warmed to room temperature, and the gas saturation increased to 91% ± 3.8%. Thirty-milliliter plasma samples in sealed syringes were maintained at room temperature or warmed to 37 °C prior to being added to the reservoir of the flow system. Reconstituted ELIP were added to the plasma in the reservoir. The initial temperature of the plasma ($T_p$) and the temperature of the flow system ($T_s$) are shown in Table 2.2. The difference between the initial temperature of plasma ($T_p$) and the temperature of the flow system ($T_s$) is denoted as ΔT in this table. Table 2.2 also shows the inline temperature of the ELIP in plasma as they flowed from the reservoir to the imaged section of the tubing (within 20 s). ELIP echogenicity at each temperature combination was tested at hydrodynamic pressures of 1 mmHg, 60/20 mmHg, and 120/80 mmHg above atmospheric pressure. B-mode images were acquired every 5 s over 75 s for all trials. A two-way ANOVA was performed using Microsoft Excel® to determine if there were significant differences (p<0.05) between ELIP in plasma at 22 °C and ELIP in plasma at 37 °C (ΔT = 0 °C) at the three pressure settings. Also, two-tailed paired Student’s t-tests were performed to ascertain if there were statistically significant (p<0.05) changes in ELIP echogenicities at pressures above 1 mmHg at each temperature transition.
Table 2.2: Temperature of plasma before being added to the flow system, temperature of the flow system and inline temperature of plasma downstream of the imaging location (N=5).

<table>
<thead>
<tr>
<th>Temperature of plasma ($T_p$) °C</th>
<th>Temperature of flow system ($T_s$) °C</th>
<th>$\Delta T = (T_s - T_p)$ °C</th>
<th>Measured inline temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>22</td>
<td>0</td>
<td>22 + 1.2</td>
</tr>
<tr>
<td>37</td>
<td>37</td>
<td>0</td>
<td>36 + 0.2</td>
</tr>
<tr>
<td>22</td>
<td>37</td>
<td>15</td>
<td>34.7 + 1.1</td>
</tr>
<tr>
<td>37</td>
<td>22</td>
<td>-15</td>
<td>24.7 + 0.5</td>
</tr>
</tbody>
</table>

II.2.6 Effect of normotensive and hypertensive pressures on ELIP echogenicity

Porcine plasma at room temperature with a dissolved air content of 91.5% + 2.9% was aliquoted into sealed syringes and maintained at 37 °C. Plasma aliquots were added to the 37 °C water-jacketed reservoir along with reconstituted ELIP and pumped through the flow system. After acquiring initial B-mode images at 1 mmHg pressure, the hydrodynamic pressure in the flow system was adjusted to normotensive (120/80 mmHg) or stage 1 hypertensive (145/90 mmHg) (Chobanian et al. 2003) and B-mode images were acquired every 5 s. A two-tailed unpaired Student’s t-test was performed using Microsoft Excel® to evaluate significant changes (p<0.05) in echogenicity of ELIP in plasma at normotensive and hypertensive hydrodynamic pressures.

II.2.7 Echogenicity of ELIP in whole blood

Porcine whole blood samples with sodium citrate (citrated) or with citrate-phosphate-dextrose (CPD) were shipped overnight from Lampire Biological Labs at 2-4 °C to the University of Cincinnati. Aliquots of 30 ml of either citrated or CPD whole blood were drawn into 60 ml syringes and were held at 37 °C for 60 min. The samples
were mixed with reconstituted ELIP and pumped through the flow system and B-mode images were acquired every 5 s. The overpressure in the flow system was maintained at 1 mmHg, 60/20 mmHg or 120/80 mmHg. The background echogenicity of whole blood without ELIP was measured as an echogenicity baseline at each overpressure setting. Blood samples were also sent to Antech Diagnostics (Oakbrook, IL, USA) for complete blood count (CBC) and fibrinogen assays.

II.3 Results

II.3.1 Effect of dissolved gas concentrations in plasma on ELIP echogenicity

ELIP were echogenic at arterial and venous dissolved gas concentrations and at 1 mmHg, 60/20 mmHg and 120/80 mmHg hydrodynamic pressures (Figure 2.4 (a)). When the dissolved gas concentrations in plasma were well below physiologic levels (DO ~ 67%), ELIP were echogenic at 1 mmHg and 60/20 mmHg hydrodynamic pressures but rapidly lost echogenicity (within 25 s) at physiologic (120/80 mmHg) pressure (Figure 2.4 (b)). This result provided the rationale to perform subsequent experiments with plasma at DO>87% (physiologic levels). These results suggest that low dissolved air concentration in the surrounding fluid accelerates the leakage of gas out of the ELIP.
Figure 2.4: The echogenicity of ELIP was constant at pressures up to 120/80 mmHg for both arterial and venous dissolved gas levels. Only at physiologic pressures in degassed plasma did the ELIP lose echogenicity. (a) Echogenicity of ELIP in plasma at different dissolved gas concentrations and hydrodynamic overpressures. (b) Change in echogenicity of ELIP over 75 s in porcine plasma with arterial dissolved gas level (◊), venous dissolved gas level (∗), or degassed (▲) at 37 °C and 120/80 mmHg (N=5).
II.3.2 Effect of temperature transitions on ELIP echogenicity

Figures 2.5 (a) and (b) show the effect of temperature transitions on the echogenicity of ELIP in plasma. In plasma at 37 °C, without a temperature transition (ΔT = 0 °C), ELIP echogenicity remained consistent at 1 mmHg, 60/20 mmHg and 120/80 mmHg hydrodynamic pressures. In plasma at 22 °C (both ΔT = 0 °C and ΔT= 15 °C) the echogenicity of ELIP at 120/80 mmHg was lower than the echogenicity at 1 mmHg (p=0.04 and p=0.02 respectively). ELIP in plasma warmed from room temperature to body temperature (ΔT= 15 °C) appeared to develop a hyperechoic line of scatterers on the upper wall of the tubing at 120/80 mmHg (Figure 2.5 a). Despite the reduced echogenicity at 120/80 mmHg, ELIP maintained good intra-luminal contrast (19.8 ± 0.6 dB) (Shi et al. 2004; Okada et al. 2005).

There was no difference in ELIP echogenicities (p=0.056) when a comparison was made at all three hydrodynamic pressures between echogenicities at room temperature and at body temperature (ΔT = 0 °C). A rapid loss of echogenicity was observed in ELIP cooled from 37 °C to 22 °C (ΔT= -15 °C) at 120/80 mmHg (p=0.002). Within 10 s of increasing the overpressure from 60/20 mmHg to 120/80 mmHg, the ELIP appeared to float to the upper wall of the tubing with a few sparse scatterers within the lumen. Therefore, a decrease in temperature tends to cause gas diffusion out of the ELIP.
Figure 2.5: (a) B-mode images of ELIP in plasma at temperature transitions (ΔT) of -15 °C (37 °C to 22 °C), 0 °C (at 22 °C and at 37 °C), and 15 °C (22 °C to 37 °C) at 1 mmHg, 60/20 mmHg, and 120/80 mmHg pressures. (b) ELIP in plasma cooling from 37 °C to 22 °C showed a loss of echogenicity (p=0.002 (†)). Significant
decreases in ELIP echogenicity at 120/80 mmHg were observed at room temperature (p<0.05 (*)) (N=5).

II.3.3 Effect of normotensive and hypertensive pressures on the echogenicity of ELIP

In hypertensive conditions (145/90 mmHg) there was a marginal loss in echogenicity compared to the echogenicity of ELIP at 1 mmHg pressure (p=0.05) (Figure 2.6). Despite the lowered echogenicity (3 dB), ELIP provided good intra-luminal contrast relative to plasma alone (18.6 + 1 dB). Further, ELIP echogenicity at 145/90 mmHg was not different from the ELIP echogenicity at 120/80 mmHg. Thus the ELIP echogenicity was not affected by physiologic blood pressure variations.

**Figure 2.6:** Effect of increased pulse pressure on echogenicity of ELIP in plasma at 37 °C. A marginal loss of ELIP echogenicity was observed (p=0.05 (*)) at 145/90 mmHg compared to 1 mmHg (N=6).
II.3.4 Echogenicity of ELIP in whole blood

ELIP were found to be echogenic in whole blood. ELIP exhibited a tendency to float in whole blood at 120/80 mmHg when compared to plasma, as evidenced by a hyperechoic line that developed along the top of the flow tube (Figure 2.7). The complete blood count (CBC) assays of the whole blood indicated poikilytosis and spherocytosis (abnormal red blood cell (RBC) morphology) in some samples of citrated whole blood used for ELIP dilution. These samples were analyzed separately. ELIP in citrated blood containing normal RBCs was not different than CPD blood. ELIP in citrated blood containing abnormal RBCs had lower echogenicities after 45 s at 120/80 mmHg overpressure as compared to the echogenicity of ELIP in CPD blood or citrated blood with normal RBCs (Figure 2.7). These findings indicate that ELIP are echogenic in whole blood at physiologic pressure and body temperature.
Figure 2.7: An insignificant decrease in echogenicity over time was observed at 120/80 mmHg in citrated whole blood and citrate-phosphate-dextrose (CPD) whole blood, but a significant decrease was observed in citrated whole blood with abnormal RBCs. Temporal changes in echogenicity of ELIP in CPD blood (♦) citrated blood (▲) and citrated blood with abnormal red blood cells (RBCs) (■) at 120/80 mmHg over 75 s is shown. Background echogenicity of CPD blood (◊) citrated blood (∆) and citrated blood with abnormal RBCs (□) at 120/80 mmHg are also shown. (N=4).

II.4 Discussion

In the current study, the stability of ELIP echogenicity was examined in a physiologic flow system mimicking hydrodynamic pressures in small arteries in one pass without recirculation. ELIP maintained echogenicity at physiologic total dissolved gas levels (>87%), body temperature (37 °C) and physiologic arterial hydrodynamic pressures (120/80 and 145/90 mmHg) in plasma and whole blood. The following discussion will focus on the impact of total dissolved gas content, temperature transitions
and hydrodynamic overpressures on the echogenicity of ELIP in plasma and the interaction of erythrocytes on the echogenicity of ELIP in whole blood.

**II.4.1 Effect of dissolved gas concentration in plasma on ELIP echogenicity**

In this study, dissolved oxygen was used as a surrogate measure of total dissolved gas in plasma. Since nitrogen is the dominant gas component in both blood and in ELIP and does not change from arterial blood to venous blood, ELIP echogenicity in plasma at arterial and venous dissolved gas levels were not different. However, in under-saturated plasma (DO<75%), an overpressure of 120/80 mmHg caused rapid loss of echogenicity of ELIP in the air-depleted surrounding plasma. The effect of fluid under-saturation on the rate of dissolution of microbubbles has been documented in other studies (Duncan and Needham 2004; Sboros et al. 2000a). Sboros et al. (2000a) and Kwan and Borden (Kwan and Borden 2010) showed that the solubility and rigidity of the shell plays an important role in determining the rate of dissolution of UCAs in under-saturated fluids. Duncan et al. found that distearoyl-phosphocholine (DSPC) lipid-coated air microbubbles dissolved within 20 s in fluids with a DO of 84%. It is possible that, like the stiff DSPC-shelled air microbubbles, the rigidity provided by DPPC in the lipid shell inhibits the rapid dissolution of gas from ELIP.

Subsequently, other studies using ELIP (Radhakrishnan et al. 2013; Raymond et al. 2013; Gruber et al. 2013) have also confirmed and emphasized that the dissolved air in the host fluid should be maintained at physiological levels to ensure stable ELIP echogenicity and acoustic attenuation. A limitation of this study was the use of air as a
composite gas to saturate the plasma prior to addition of ELIP. Studies that include the physiologic exchange of gases, specifically carbon dioxide, also need to be carried out in the future to determine whether the stability of ELIP echogenicity is affected. Further, ELIP encapsulating other bio-active gases such as nitric oxide, xenon, hydrogen sulfide, and argon also need to be carefully evaluated in host fluids with physiological levels of dissolved gases to ensure robust echogenicity.

**II.4.2 Effect of the temperature transitions on ELIP echogenicity**

At body temperature (37 °C), ELIP maintained good intra-luminal contrast at 1 mmHg, 60/20 mmHg, and 120/80 mmHg. Temperature transitions may occur during clinical applications. For instance, ELIP reconstituted and diluted at room temperature (22 °C) may subsequently be injected into the vascular system at 37 °C. Despite a drop in echogenicity at 120/80 mmHg compared to the echogenicity at 1 mmHg pressure good intra-luminal contrast was maintained (19.8 ± 0.6 dB) (Shi et al. 2004; Okada et al. 2005) when the ELIP underwent a temperature transition from room temperature to body temperature. One likely explanation is that nitrogen and oxygen, the main constituent gases in air have lower solubilities in the plasma at 37 °C compared to 22 °C. As a result, these gases will preferentially diffuse into the ELIP, resulting in larger, more buoyant liposomes that rise in a fluid more rapidly. This is consistent with the observation of a hyperechoic line along the upper wall of the tubing. The lower echogenicity measured in the lumen could have originated from a shadowing artifact from the hyperechoic line along the upper wall of the tubing.
Most *in vitro* studies of microbubble stability and dynamics are conducted at room temperature and assumed to be applicable at body temperature. Recently, Vos et al. (2008) observed lower thresholds of acoustic response and higher radial expansions of phospholipid-coated UCAs such as SonoVue® and Definity® at body temperature (37 °C) compared to room temperature (22 °C). Also, Mulvana et al. (2010) observed an increase in the mean initial microbubble diameter and a corresponding higher acoustic signal from SonoVue® at 40 °C compared to 22 °C. They also noted dramatic reductions in microbubble diameter at discrete intervals over ten minutes at 40 °C compared to 22 °C. They suggested that temperature dependent changes in the surface tension of the shell affected the rate of dissolution of gas from the UCA.

ELIP echogenicity was not statistically different at 22 °C and at 37 °C (ΔT = 0 °C). In our study, echogenicity was recorded over 75 s, which may be too short to observe changes in echogenicity caused by dramatic reduction in microbubble diameter as evidenced by Mulvana et al. (2010). Recently, Raymond et al. (2013) showed that the broadband acoustic attenuation of ELIP was higher at 37 °C compared to 25 °C. These authors hypothesized that the number of ELIP microbubbles contributing to the attenuation as well as the shell parameters played a role in the increased attenuation of ELIP at 37 °C. Unlike the broadband attenuation measurement, the clinical imaging system measures the backscatter from ELIP at 6.9 MHz and may not have been sensitive enough to measure changes in the microbubble size and shell parameters as observed by Raymond et al. (2013).

Figures 2.5 (a) and (b) show that ELIP diluted in plasma at 37 °C and subsequently cooled to room temperature, 22 °C (ΔT = -15 °C) lost echogenicity rapidly at physiologic
overpressures (120/80 mmHg). Plasma at 22 °C has higher oxygen and nitrogen solubility coefficients and lower vapor pressure than plasma at 37 °C. Thus, as the plasma cooled from 37 °C to 22 °C, the diffusion gradient between the ELIP and the surrounding plasma increased. An overpressure of 120/80 mmHg may have further accelerated the diffusion of gas out of the ELIP by increasing the partial pressures within the ELIP. This result reiterates the need for temperature-controlled water-jacketing of the tubing and reservoir to maintain the echogenicity of ELIP. Further, this finding suggests that ELIP premixed in fluid at 37 °C should not be allowed to cool to 25 °C prior to intravenous injection.

II.4.3 Effect of normotensive and hypertensive pressures on the echogenicity of ELIP

ELIP had similar echogenicity under stage 1 hypertensive arterial pressures (145/90 mmHg) and under normotensive (120/80 mmHg) pressure. Other studies (Andersen and Jensen 2010b; Frinking et al. 2010) showed that at low acoustic pressures (< 500kPa), the fundamental acoustic response of UCAs was independent of the overpressure in the ambient fluid. Frinking et al. (2010) found that there was no change in the linear acoustic response of phospholipid coated microbubbles at overpressures up to 120 mmHg. Similarly, Andersen and Jensen (2010a; 2010b) noted that at insonifying pressures below 500 kPa, the energy of the fundamental component of the acoustic emissions from SonoVue® reduced by only 0.6 dB when the hydrostatic pressure was changed from 0 mmHg to 180 mmHg. The observations in these investigations are consistent with our observation that there is no difference in echogenicity of ELIP at
120/80 mmHg and 145/90 mmHg as measured using fundamental B-mode imaging. Further, the ELIP were instantaneously exposed to an overpressure of 120/80 mmHg as they flowed out of the pulsatile pump thus mimicking the instantaneous hydrodynamic overpressure in the left ventricle.

**II.4.4 Echogenicity of ELIP in whole blood**

Previous studies have documented the interaction of liposomal vesicles with whole blood and plasma proteins. Plasma proteins have been shown to adsorb onto and intercalate into the bilayer membrane (Bonté and Juliano 1986; Semple et al. 1998). Several proteins including albumin, apolipoproteins, fibronectin, C-reactive protein, α-, β- and immuno-globulins, as well as clotting factor-VIII interact with and destabilize liposomes (Bonté and Juliano 1986). Gregoriadis and Davis (1979) observed that liposomes were more stable in whole blood rather than in blood serum. They hypothesized that the erythrocytes in whole blood donated cholesterol to the liposomes and also interacted strongly with the lipoproteins, thus inhibiting adsorption of plasma proteins on the liposomes. The ELIP formulation used in the current study also contains cholesterol, which based on studies by Gregoriadis and Davis (1979), has been hypothesized to hinder plasma protein adsorption. The results of the current study show that ELIP were echogenic in whole blood at physiologic hydrodynamic pressures for 75 s. Recent acoustic attenuation studies by Raymond et al. (2013) also confirmed that ELIP were stable and had comparable attenuation over the frequency range of 3-25 MHz in whole blood and in phosphate-buffered saline.
Whole blood containing citrate-phosphate-dextrose (CPD), unlike citrated whole blood, maintains the pH, osmolality and ATP supply to the RBCs and mimics \textit{in vivo} conditions. In both citrated and CPD whole blood, it was found that some ELIP aggregates migrated to the upper wall of the tubing. This behavior may be attributed to aggregation of ELIP due to interaction with leukocytes and erythrocytes. Studies by Schwartz et al. (1983) demonstrated that phosphatidylserine-phosphatidylcholine liposomes adhered to the surface of normal RBCs. Liposomes in mesenteric arteries were observed to be pushed away from the center of the lumen towards the vessel wall due to interactions with RBCs on intravital images and micro-particle image velocimetry (Jeong et al. 2007). Addition of polyethylene glycol to the lipid shell of ELIP could inhibit their interaction with RBCs and possible aggregation. On the other hand, such migration towards the vessel wall may allow for better interaction of antibody-conjugated and drug-loaded ELIP with atherosclerotic components for better targeting and drug delivery (Hamilton et al. 2004).

In clinical applications, a contrast enhancement of 8-16 dB above the background is required for unambiguous delineation of myocardial perfusion (Shi et al. 2004). An enhancement of least 3 dB above normal liver parenchyma is required for visualization of lesions on contrast enhanced ultrasound images (Okada et al. 2005). The addition of ELIP provided 14-17 dB enhancement above background in CPD whole blood and 10-13 dB enhancement above background in citrated whole blood with normal RBCs. However, ELIP showed only 5-10 dB enhancement above background in citrated blood containing abnormal RBCs (Figure 2.7). The overall echogenicity of ELIP in citrated blood containing abnormal RBCs decayed to 50% of its initial value within 75 s. Poikilocytosis
is a non-specific indicator of abnormal RBCs, of which spherocytosis is the most common morphology caused by hemolytic anemia (Lynch 1990). Schwartz et al. (1983) also observed that abnormal surface morphology of hypoxia-induced sickled RBCs promoted increase in liposome-RBC adhesion and fusion. *In vivo* and *in vitro* studies are required to determine if the interaction between ELIP and RBCs can be used to detect poikilocytosis and other abnormal RBC morphologies and whole blood pathologies.

There were some limitations noted in this study. The studies focused on immediate changes (75 s) in echogenicity and did not investigate the longevity of ELIP in physiologic conditions over the typical half life of an ultrasound contrast agent (10-15 minutes) (Mullin et al. 2011; Landmark et al. 2008). The effect of other *in vivo* processes such as filtration during passage through the lungs (Hogg 1987), dynamic gas exchange in the lung, and the effect of opsonization and phagocytosis on ELIP stability also need to be evaluated. These studies are necessary for all contrast agents as characteristics in one physiological setting may not translate to other settings.

**II.5 Conclusion**

In conclusion, this in vitro study evaluated the persistence of ELIP as a blood pool contrast agent. ELIP were found to be stable in physiologic flow conditions when proper care was taken to adjust the pressure, dissolved gas content and temperature of the fluids containing the ELIP before and during use. A rapid temperature drop from 37 °C to 22 °C and low dissolved gas content in the surrounding medium caused ELIP to lose echogenicity. Abnormal RBCs in whole blood caused more aggregation of ELIP and
decay of echogenicity. Future *in vivo* studies need to be carried out to evaluate the robustness and longevity of ELIP as blood pool agents.
CHAPTER III – Relationship between cavitation thresholds and loss of echogenicity of ultrasound contrast agents as a function of pulse duration
III.1 Introduction

In Chapter II the stability of ELIP under physiological conditions was assessed by evaluating ELIP echogenicity on B-mode images acquired at low insonation pressures (MI = 0.04). Previous studies have shown that diagnostic pulses at higher insonation pressures and longer pulse duration can cause acoustically-mediated destruction of ultrasound contrast agents (UCAs) (Porter et al. 2006; Smith et al. 2007). Ultrasound-mediated destruction of UCAs has been categorized as passive diffusion, acoustically driven diffusion, and rapid fragmentation (Chomas et al. 2001). Smith et al. (2007) determined the acoustically driven diffusion and rapid fragmentation thresholds for ELIP based on the temporal decay of echogenicity. The acoustically driven diffusion threshold was weakly dependent on the pulse duration whereas the rapid fragmentation threshold was strongly dependent on pulse duration (Smith et al. 2007). The interaction of UCAs with pulsed ultrasound can also be investigated by assessing the cavitation emissions from the UCAs (Chen et al. 2002; Chen et al. 2003b). Chen et al. (2003b) and Chomas et al. (2001) have described rapid fragmentation as the acoustically-induced inertial collapse of microbubbles resulting in microbubble fragments. These studies have suggested that rapid changes in echogenicity due to UCA fragmentation are concomitant with inertial cavitation and could be used to monitor the likelihood of inertial cavitation nucleated by UCAs. Previous studies have also elucidated the pulse duration dependence of stable cavitation thresholds (Vykhodtseva et al. 1995) and inertial cavitation thresholds (Atchley et al. 1988; Fowlkes and Crum 1988; Lo et al. 2007; Ammi et al. 2006). This Chapter describes experiments performed to determine the relationship between loss of echogenicity and cavitation emission from UCAs as a function of pulse duration.
The hypothesis of this Chapter is that the onset of rapid loss of UCA echogenicity is concomitant with the threshold of inertial cavitation. Two different lipid-shelled UCAs, ELIP and Definity®, were insonified by 6-MHz duplex spectral Doppler ultrasound from a clinical ultrasound imaging system (HDI 5000, Philips, Bothell, WA, USA). Acoustic cavitation emissions and B-mode ultrasound images of ELIP and Definity® were acquired. Stable and inertial cavitation thresholds and loss of echogenicity (LOE) from UCAs were quantified. Cavitation thresholds and LOE thresholds in a static fluid system were compared to the rapid fragmentation thresholds as defined by Smith et al. (2007). Because these UCAs are being investigated for use in small and medium arteries (Lindner 2004; Laing and McPherson 2009) the cavitation emissions and LOE were also measured in a physiologic flow phantom (Radhakrishnan et al. 2012).

**III.2 Materials and Methods**

**III.2.1 Ultrasound contrast agents**

The UCAs used in this study, ELIP and Definity®, were diluted in porcine plasma (Lampire Biologicals, Pipersville, PA, USA) at 37 °C and at 93 ± 2 % dissolved oxygen. ELIP were prepared at the University of Texas Health Science Center, Houston as described by Buchanan et al. (2008) and were shipped on dry ice to the University of Cincinnati. This particular lipid formulation has been found to be stable under physiologic temperature (Buchanan et al. 2008) and hydrodynamic pressure (Radhakrishnan et al. 2012). Each vial containing 6 mg of lyophilized lipids were reconstituted with 0.6 ml of 0.2 μm filtered deionized water (NANOPure, Barnstead International, Dubuque, IA, USA) and diluted in porcine plasma to yield a final lipid
concentration of 0.05 mg/ml ($6.4 \times 10^8$ liposomes/ml) (Hamilton et al. 2004; Smith et al. 2010; Kopechek et al. 2011). Definity® (Lantheus Medical Imaging, North Billerica, MA, USA) was activated at room temperature by agitating the vials for 45 s using a VialMix® (Lantheus Medical Imaging) according to the manufacturer’s instructions. Based on the manufacturer's recommended maximum bolus dose of 20 μl/kg and assuming a blood volume of 65 ml/kg (CIBA-Geigy and Lentner 1984), Definity® was diluted in porcine plasma to a final concentration of 0.31 μl/ml ($3.7 \times 10^6$ microbubbles/ml). Degassed water (dissolved oxygen=25 ± 2%) was used as a control to assess acoustic emissions and background echogenicity from the tubing alone. All UCAs were used in flow experiments within 10 minutes after reconstitution or withdrawal from an activated vial.

### III.2.2 Experimental setup

Cavitation thresholds and loss of echogenicity from ELIP and Definity® were measured in the absence of flow in a static fluid shown in Figure 3.1. UCAs diluted in porcine plasma were pumped by a peristaltic pump (Mettler Toledo, Columbus, OH, USA) into a compliant flow system consisting of latex tubing with dimensions based on physiologically relevant values for small and medium arteries (Holubkov et al. 2002; Korosoglou et al. 2008; Masuo et al. 2002; Weidinger et al. 2002). An after-load with an adjustable height was used to maintain a hydrostatic pressure corresponding to a mean arterial pressure of 93 ± 4 mmHg. ELIP or Definity® were infused into the sample chamber and flow was turned off prior to insonation. The temperature and pressure in the flow system were monitored throughout the experiments using inline pressure and temperature sensors (PendoTECH, Princeton, NJ, USA).
The stable and inertial cavitation thresholds and loss of echogenicity from ELIP and Definity® were also measured in the physiological flow system previously described in Chapter II (Figure 3.1). ELIP or Definity® were diluted in porcine plasma and added to a temperature-controlled reservoir (Radnoti, Monrovia, CA, USA) and pumped (Model 1407, Harvard Apparatus, Holliston, MA, USA) through temperature-controlled tubing (Radnoti) at 37 °C. The stroke rate of the pulsatile pump was maintained at 60 strokes per minute and the stroke volume was adjusted in conjunction with the height of the afterload to maintain a hydrodynamic pressure of 120/80 mmHg in the flow system.

**Figure 3.1:** Schematic of the experimental setup. Measurements of cavitation and echogenicity were carried out in a static fluid by infusing UCAs or degassed water into the sample chamber using a peristaltic pump and turning the flow off prior to insonation. For measurements in physiologic flow, the peristaltic pump was replaced with a pulsatile pump to infuse fluid (UCAs or degassed water) continuously into the sample chamber. The linear array transducer (L12-5) was driven by the Philips HDI.
5000 scanner operated in the duplex spectral Doppler mode and insonified the fluid in the sample chamber. The 10-MHz passive cavitation detector was confocally aligned with the Doppler beam and acquired cavitation emissions.

**III.2.3 Ultrasound parameters**

A linear array transducer (L12-5) driven by a clinical diagnostic ultrasound scanner (HDI 5000, Philips, Bothell, WA, USA) (Figure 3.1) in duplex spectral Doppler mode (6-MHz center-frequency) insonified ELIP or Definity® or degassed water in a tubing placed 1 cm from the transducer face. Measurements in degassed water determined the baseline noise level of the cavitation detection system. The Doppler pulse duration and peak rarefactive pressures were varied as shown in Table 3.1 and cavitation emissions and images were acquired. The range and step size of insonation pressures was limited by the Philips HDI 5000 scanner output. The acoustic peak rarefactive pressure of the Doppler pulse was measured inside the tubing at each transducer output setting.

Calibration of the L12-5 transducer output were performed in a tank of degassed water at 37 °C by placing a section of the thin-walled latex tubing (Piercan, San Marcos, CA, USA) at the Doppler focus 1 cm from the transducer face. A 0.2 mm needle hydrophone (Precision Acoustics, Dorchester, UK) was aligned to the focus of the Doppler sample volume inside the latex tubing through a slit cut into the tubing. A three-dimensional positioning system (Velmex NF90 Series, Velmex Inc., 291 Bloomfield, NY) was used to translate the hydrophone within the latex tubing. The -3dB focal beam of the Doppler pulse was 0.8 mm x 1.8 mm x 13.2 mm (azimuth x elevation x range) at a focal distance of 1 cm from the transducer face. The acoustic pressure waveforms of the Doppler pulse and the B-mode pulse were also recorded at their respective focii for each
transducer output setting and pulse duration. Figure 3.2 (a) shows the transverse beam profile of the transducer. The axial beam profile of the transducer shown in Figure 3.2(b) also shows an overlay of the cross-section of the latex tubing. The Doppler insonation pressure profile was therefore not uniform across the cross-section of the tubing.

**Figure 3.2:** (a) Transverse beam profile and (b) Axial beam profile of the Doppler beam from the L12-5 array.

<table>
<thead>
<tr>
<th>Pulse duration (μs)</th>
<th># Cycles</th>
<th>Range of mechanical indices (MI)</th>
<th>Range of peak rarefactionsal pressures (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.67</td>
<td>10</td>
<td>0.04-0.9</td>
<td>0.06-1.84</td>
</tr>
<tr>
<td>3.33</td>
<td>20</td>
<td>0.04-0.7</td>
<td>0.06-1.48</td>
</tr>
<tr>
<td>5.83</td>
<td>35</td>
<td>0.04-0.56</td>
<td>0.06-1.17</td>
</tr>
<tr>
<td>8.33</td>
<td>50</td>
<td>0.02-0.34</td>
<td>0.03-0.7</td>
</tr>
</tbody>
</table>

**III.2.4 Cavitation thresholds**

Previous studies have established spectral methods to identify different types of bubble activity as stable cavitation (Flynn 1964; Eller and Flynn 1969; Datta et al. 2008; Hitchcock et al. 2011) and inertial cavitation (Coussios et al. 2007; Chen et al. 2003b;
Leighton 1994; Cramer and Lauterborn 1982). In the present study, a focused single element 10-MHz transducer (Valpey Fisher, Hopkinton, MA, USA) was used as a passive cavitation detector (PCD) (Roy et al. 1990). The frequency response of the passive cavitation detector is shown in Figure 3.3 (a). The 10 MHz transducer was driven by a pulser receiver (Panametrics, Waltham, MA, USA) using a broadband pulse. The broadband acoustic reflections off a metal plate reflector were received by the 10 MHz and used to calculate the frequency response of the transducer. Figure 3.3 (b) and (c) show the transverse and axial beam profiles of the 10 MHz detector. The spatial beam profile of the 10 MHz transducer was obtained by aligning a 0.2 mm needle hydrophone (Precision Acoustics) to the transducer focus. A three-dimensional positioning system (Velmex NF90 Series, Velmex Inc., 291 Bloomfield, NY) was used to translate the hydrophone within the 10 MHz transducer. The -3dB focal beam of the Doppler pulse was 0.8 mm x 1.8 mm x 2.4 mm (azimuth x elevation x range) at a focal distance of 2 cm from the transducer face.
Figure 3.3: (a) Frequency response (b) Transverse beam profile and (c) Axial beam profile of the 10-MHz single-element, focused passive cavitation detector. Note that in Figure 3.3(c) the axes are not equal.

The PCD was aligned confocally and orthogonally to the L12-5 pulsed Doppler focus (Figure 3.1). A custom trigger from the clinical scanner was used to synchronize data acquisition and to range gate the acoustic emissions received by the PCD. 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) operated in sequence mode. At each insonation pressure, two sequences were recorded over 3 s. Each sequence contained 50 traces and each trace comprised 2000 samples at a sampling frequency of 100 MHz. Spectral analysis was performed using MATLAB (Mathworks, Natick, MA, USA) to obtain the stable and inertial cavitation thresholds.
Examples of the average power spectra from ELIP and degassed water are shown in Figure 3.3(a). The subharmonic and odd multiples of the subharmonic (ultraharmonic) frequency components (blue bands) and inharmonic or broadband frequency components (red bands) were extracted (Figure 3.4(a)). Inharmonic bands are broadband frequency components excluding the fundamental, harmonic, subharmonic and ultraharmonic bands (Haworth et al. 2012). The root-mean-squared (RMS) values of the subharmonic (and ultraharmonic) emissions and broadband emissions are given by equation (3.1).

\[
\text{Cavitation Signal}_{\text{RMS}} = \sqrt{\sum_{\text{Band}} \text{Power}_{\text{UCA}}} - \sqrt{\sum_{\text{Band}} \text{Power}_{\text{DW}}},
\]

(3.1)

where Power\text{\text{UCA}} and Power\text{\text{DW}} are the detected acoustic emission powers from the UCAs (ELIP or Definity®) in the tubing and degassed water in the tubing, respectively. \(f_{\text{Band}}\) includes the subharmonic and ultraharmonic frequency bands to calculate the stable cavitation threshold or the inharmonic (broadband) frequency bands to calculate the inertial cavitation threshold indicated in (Figure 3.4(a)).

Figure 3.4(b) shows the RMS subharmonic emissions as a function of the Doppler pulse peak rarefractional pressure. The cavitation threshold was defined as the lowest insonation pressure that elicited emissions which increased linearly with insonation pressure. This definition was used to ascertain the lowest insonation pressure at which cavitation emissions were detected and therefore the threshold of a particular type of cavitation (Fabiilli et al. 2009). A three segment piecewise linear fit of the RMS subharmonic emissions was performed using the 'slmengine' function in MATLAB. The slopes of the first and the third segments were forced to be zero. The peak rarefractional pressure at the inflection between the first two segments was defined as the stable
cavitation threshold as shown in Figure 3.4(b). A two-segment piecewise linear fit was performed on the RMS broadband emissions (Figure 3.4(c)) because the broadband signals, unlike the subharmonic signals, did not saturate at the higher peak rarefractional pressures. The peak rarefractional pressure at the inflection between the first two segments was defined as the inertial cavitation threshold as shown in Figure 3.4(c). Stable and inertial cavitation thresholds were ascertained for the range of pulse durations shown in Table 3.1.
Figure 3.4: (a) Example of cavitation spectrum from ELIP (solid line) and degassed water (DW) (dotted line). The blue bands indicate the sub- and ultraharmonics (centered at 3, 9, 15, 21 MHz). The red bands indicate the inharmonic bands or broadband at frequencies outside the fundamental (6 MHz), harmonics (12, 18 and 24 MHz).
MHz) and sub- and ultraharmonics. (b) Example of subharmonic emissions from ELIP as a function of insonation peak rarefational pressures. A three-segment piecewise linear fit was performed and the inflection between the first two segments was defined as the stable cavitation threshold. (c) Example of broadband emissions from ELIP as a function of insonation peak rarefational pressures. A two-segment piecewise linear fit was performed and the inflection between the two segments was defined as the inertial cavitation threshold.

III.2.5 Assessment of Loss of echogenicity

At each insonation pressure a B-mode image of ELIP or Definity® was saved on the ultrasound scanner and later post-processed in MATLAB. The B-mode imaging center frequency was 6.9 MHz in the duplex spectral Doppler mode. The frame rate, persistence, grayscale map, time gain compensation (TGC) and 2-D gain were kept constant throughout the experiments. Previous studies demonstrated that unlike the mean gray scale value (MGSV) computed from B-mode images, the mean digital intensity (MDI) computed on cine-loops, is directly proportional to the backscattered acoustic power and independent of the grayscale color map (Porter et al. 2006; Smith et al. 2007). Using a previously established technique (Radhakrishnan et al. 2012) the linear MDIs were computed.

The ROIs were defined at locations unexposed to Doppler insonation and within the Doppler pulse to evaluate the loss of echogenicity of ELIP and Definity® in the static system as shown in Figure 3.5(a). The percent loss of echogenicity (LOE) was computed using equation (3.2a).

\[
\text{Percent LOE}_{\text{No flow}} = \frac{\text{MDI}_{\text{No Doppler}} - \text{MDI}_{\text{With Doppler}}}{\text{MDI}_{\text{No Doppler}}} \times 100, \quad (3.2a)
\]

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 a 90% LOE within 5 s from the start of Doppler insonation. Thus the lowest peak rarefractional pressure causing a LOE of 90% was used to define the threshold for rapid loss of echogenicity of ELIP and Definity® in the static system.

For flowing ELIP or Definity®, the same definition of LOE threshold could not be used due to continuous replenishment of the destroyed contrast at the Doppler focus. Therefore, regions of interest (ROIs) were defined upstream and downstream of the Doppler focus as shown in Figure 3.5(b). The mean transit time between the upstream and downstream ROIs was 600 ms and the time to traverse the -6 dB beamwidth of the spectral Doppler pulse was 50 ms, which corresponds to approximately 62 Doppler pulses at the 1250 Hz pulse repetition frequency. LOE was computed for each insonation pressure using equation (3.2b).

\[
\text{Percent LOE}_{\text{Flow}} = \frac{\text{MDI}_{\text{Up}} - \text{MDI}_{\text{Down}}}{\text{MDI}_{\text{Up}}} \times 100, \tag{3.2b}
\]

where MDI_{Up} and MDI_{Down} are the mean digital intensities in the ROIs upstream and downstream of the Doppler focus respectively. A numerical model was also developed to predict the cavitation phenomena causing LOE in ELIP and Definity® in the flow system.
Figure 3.5: (a) Example of ELIP in the 0.4 mm thick latex tubing with no flow. The gray 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 0.4 mm thick latex tubing. The gray 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”.

III.2.6 Statistical Analysis

Statistical analysis was performed in MATLAB (Mathworks). An unequal variance t-test was performed to compare the stable and inertial cavitation thresholds. A three-way ANOVA was performed to compare the cavitation thresholds ascertained for the two UCAs (ELIP and Definity®) with and without flow exposed to pulsed Doppler using four pulse duration settings. 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). This statistical test was also used to compare the LOE thresholds and the cavitation thresholds in the static system.
III.3 Results

III.3.1 Cavitation Thresholds

Figures 3.6 (a) and (b) show the stable and inertial cavitation thresholds of ELIP and Definity®, respectively, as a function of pulse duration in flow and static conditions. The cavitation thresholds of both UCAs had a weak dependence on pulse duration. For each pulse duration, flow setting, and type of UCA, the stable cavitation thresholds were found to be significantly lower than the inertial cavitation thresholds (p<0.01). There was no difference in the cavitation thresholds (stable and inertial) at the two shortest pulse durations 1.67 µs and 3.33 µs. However, the cavitation thresholds (stable and inertial) at the longer pulse durations (5.83 µs and 8.33 µs) were found to decrease with increasing pulse duration (p<0.01). This decrease indicated a weak dependence of cavitation thresholds on pulse duration. Flow conditions did not have a significant effect on the stable and inertial cavitation thresholds. The type of UCA (ELIP or Definity®) also did not have an effect on the cavitation thresholds.
Figure 3.6: Stable and inertial cavitation thresholds in (a) ELIP, and (b) Definity® in flow and static conditions.
III.3.2 Loss of echogenicity

The LOE thresholds in the static system were defined as the minimum insonation pressure at which the LOE was 90% based on criteria for rapid fragmentation ascertained by Smith et al. (2007). Figure 3.7(a) shows the LOE thresholds and cavitation thresholds of ELIP in a static system compared with the rapid fragmentation thresholds reported by Smith et al. (2007). There was no difference between the LOE thresholds of ELIP in the static system and the rapid fragmentation thresholds of ELIP measured by Smith et al. (2007)(p>0.05) (Figure 3.7(a)). Figure 3.7(b) shows the LOE thresholds and cavitation thresholds for Definity® in a static system. The thresholds of LOE from ELIP and Definity® were not different for the four pulse durations. For ELIP and Definity® in a static fluid exposed to short ultrasound pulse durations (1.67 µs and 3.33 µs) shown in Figures 3.7 (a) and (b), the threshold of LOE was higher than the threshold for stable and inertial cavitation. However, there was no difference between the LOE thresholds and the inertial cavitation thresholds at the longer pulse durations (5.83 µs and 8.33 µs) in the static system.
**Figure 3.7**: Comparison of loss of echogenicity, rapid fragmentation (Smith et al. 2007) and stable and inertial cavitation thresholds in (a) ELIP and (b) Definity® in the static fluid.
Because only a fraction of UCAs were exposed to the Doppler pulses in the flow system, the LOE did not have a well-defined threshold similar to that measured by Smith et al. (2007). The stable and inertial cavitation emissions had an exponential relationship with LOE from ELIP and Definity®. The relationship between subharmonic emissions and broadband emissions and LOE of ELIP for a 1.67 μs pulse duration are shown in Figures 3.8(a) and (b) respectively. The relationship between subharmonic emissions and broadband emissions and LOE of Definity® for a 1.67 μs pulse duration are shown in Figures 3.9(a) and 3.9(b) respectively. Also shown are exponential fits to the experimental data ($R^2 > 0.90$). The exponential fits ($S_{fit}$) were of the form given by equation (10)

$$S_{fit} = S_o e^{k(LOE)}$$

where $S_o$ is the cavitation signal corresponding to 0% LOE and $k$ is the exponential growth constant. Figures 3.8(b) and 3.9(b) also show insets of the onscreen echogenicity of ELIP and Definity®, respectively, as these agents flow through the Doppler focus. The exponential fit was used to allow comparison with a previous study which elucidated an exponential relationship between loss of echogenicity and cavitation emissions (Tung et al. 2010). Further, a piecewise linear fit (as described in section III.2.4) was also used to ascertain the threshold loss of echogenicity at which concomitant stable cavitation (Figure 3.10 a) and inertial cavitation (Figure 3.10 b) emissions were detected. As shown in Figure 3.10 (c) and (d), approximately 80% loss of echogenicity was concomitant with the detection of stable and inertial cavitation emissions from ELIP and Definity®.
Figure 3.8: Relationship between loss of echogenicity and (a) subharmonic emissions, and (b) broadband emissions from ELIP insonified by Doppler pulses of duration 1.67 μs. The open squares represent the experimental data. The x and y error bars represent the standard deviation of four measurements. The dotted line represents an exponential fit. The insets show the onscreen echogenicity of ELIP flowing through the Doppler focus and the corresponding percent loss of echogenicity.
Figure 3.9: Relationship between loss of echogenicity and (a) subharmonic emissions, and (b) broadband emissions from Definity® insonified by Doppler pulses of duration 1.67 μs. The solid squares represent the experimental data. The x and y error bars represent the standard deviation of four measurements. The dotted line represents an exponential fit. The insets show the onscreen echogenicity of ELIP flowing through the Doppler focus and the corresponding percent loss of echogenicity.
Figure 3.10: Relationship between loss of echogenicity and (a) subharmonic emissions, and (b) broadband emissions from ELIP insonified by Doppler pulses of duration 1.67 μs. The line represents the piecewise linear fit to determine the loss of echogenicity at which concomitant cavitation emissions were detected. Loss of echogenicity threshold at which concomitant stable cavitation (blue squares) and inertial cavitation emissions were detected from (c) ELIP and (d) Definity® are also shown.

III.4 Discussion

Cavitation thresholds and loss of echogenicity from ELIP and Definity® were assessed in a physiologic flow system. In the following discussion, the cavitation and LOE thresholds are compared with the cavitation and rapid fragmentation thresholds.
reported in the literature. The relationship between cavitation and loss of echogenicity and the limitations of the study are also discussed.

### III.4.1 Cavitation thresholds

The stable cavitation thresholds were found to be lower than the inertial cavitation thresholds at all four pulse duration settings, for both ELIP and Definity®, in the static and the flow systems. Theoretical computations by Bader and Holland (2013) have predicted that the stable cavitation threshold of resonant-sized bubbles exposed to 6-MHz pulsed ultrasound will be lower than their inertial cavitation threshold. Hitchcock et al. (2010) ascertained the stable and inertial cavitation thresholds of ELIP insonified by 1-MHz continuous wave (CW) ultrasound. These authors also found that the threshold for ultraharmonic emissions (characteristic of stable cavitation) was lower than the inertial cavitation threshold. The pressure amplitude of the cavitation threshold of ELIP exposed to 1-MHz CW ultrasound (Hitchcock et al. 2010) was found to be lower than the cavitation threshold determined in this study for ELIP exposed to 6-MHz pulsed ultrasound. This difference is likely due to the frequency dependence of the stable and inertial cavitation thresholds. A higher cavitation threshold is expected for higher frequency ultrasound exposure (Bader and Holland 2013). In addition, the cavitation threshold also exhibited a weak dependence on pulse duration, as seen in Figures 3.6(a) and (b), which may have contributed to the difference. The inertial cavitation pressure threshold of ELIP and Definity® at the lowest pulse duration (1.67 µs) was 0.63 ± 0.01 MPa and 0.56 ± 0.09 MPa respectively. These inertial cavitation thresholds agreed with
the inertial cavitation threshold (0.64 MPa) predicted for free microbubbles exposed to 6-MHz single cycle pulses (Apfel and Holland 1991).

The dependence of the cavitation threshold on pulse duration has also been investigated by other authors (Atchley et al. 1988; Fowlkes and Crum 1988; Lo et al. 2007). Ammi et al. (2006) found that the post-excitation inertial collapse thresholds in Optison® decreased with increasing number of cycles at three different insonation frequencies. The results in Figure 3.6 indicating a decrease in inertial cavitation threshold for ELIP and Definity® with increasing pulse duration were consistent with the observations by Ammi et al.(2006). A decrease in stable cavitation threshold with increasing pulse duration (Figure 3.6) is consistent with studies by Andersen and Jensen (2009) and Vykhodtseva et al. (1995) who observed an increase in the stable cavitation dose and a decrease in the stable cavitation threshold with increasing pulse duration.

The presence or absence of flow did not affect the cavitation thresholds. The type of UCA (ELIP or Definity®) also did not affect the cavitation thresholds. Both ELIP and Definity® are lipid shelled UCAs. However, ELIP and Definity® have different lipid compositions, microbubble concentrations and microbubble size distributions. Further, ELIP encapsulate air pockets and aqueous cores (Kopechek et al. 2011; Huang 2008) whereas Definity® microbubbles have lipid monolayers encapsulating octafluoropropane gas. However other studies (Bader and Holland 2013; Stride and Saffari 2003) have suggested that the UCA shells must first be disrupted in order to liberate the gas microbubbles to nucleate bubble activity once the pressure threshold is exceeded. Thus the shell properties of UCAs may not have affected the cavitation thresholds because gas was effectively liberated. Other studies (Kabalnov et al. 1998; Kwan and Borden 2010;
Sarkar et al. 2009) have shown that UCAs containing low-solubility gases, when diluted in air-saturated fluids, undergo gas equilibration and become saturated with air within a few seconds. For these reasons the type of gas in lipid-shelled UCAs also may not have influenced the cavitation thresholds in these experiments.

### III.4.2 Loss of echogenicity

Several studies have been carried out in static fluids to investigate the relationship between cavitation and ultrasound-induced UCA destruction, specifically rapid fragmentation (Stride and Saffari 2003; Sboros 2008). In some studies, the broadband emissions and post-excitation signals characteristic of inertial cavitation were used as indicators of rapid fragmentation of UCAs (Chen et al. 2003b; Ammi et al. 2006). However, other studies contend that inertial cavitation thresholds were higher than the rapid fragmentation thresholds of UCAs (Chen et al. 2000; Uhlendorf et al. 2000). The rapid fragmentation threshold of ELIP was defined in a previous study by Smith et al. (2007) as the minimum insonation pressure that induces a rapid destruction of ELIP echogenicity to background levels within 5s. The rapid destruction of ELIP was assessed by measuring the echogenicity of ELIP in a region of interest defined within the Doppler sample volume on B-mode images acquired every 5 s. It was not possible to acquire B-mode images using the method described by Smith et al. (2007) because the scanner suspends the duplex Doppler insonation while saving images to its hard disk. The suspension of Doppler insonation would have interfered with the acquisition of cavitation emissions because the passive cavitation detection system is synchronized with the line
Therefore, it was not possible to assess the temporal changes in loss of echogenicity as investigated by Smith et al. (2007).

However, in order to compare the loss of echogenicity assessed in the current study with the rapid fragmentation threshold defined by Smith et al. (2007), the LOE threshold was defined for ELIP in a static fluid insonified by duplex Doppler. The LOE threshold was the minimum insonation pressure at which 90% loss of echogenicity was observed which is consistent with the loss of echogenicity measured at the rapid fragmentation threshold (Smith et al 2007). The LOE threshold of ELIP in a static fluid agreed with the rapid fragmentation threshold (Figure 3.7(a)). At a pulse repetition frequency of 1.25 kHz, significant acoustic streaming and radiation force were not observed. These LOE thresholds were found to be statistically similar to the inertial cavitation thresholds at the longer pulse durations (5.83 µs and 8.33 µs). However, the LOE thresholds were found to be higher than the inertial cavitation thresholds at the shorter pulse durations (1.67 µs and 3.33 µs). Spectral broadening at the shorter pulse durations may have resulted in an underestimation of the inertial cavitation threshold.

In flow, the loss of echogenicity of ELIP and Definity® did not exhibit a threshold behavior. An exponential relationship between loss of echogenicity and insonation pressure was also observed by Yeh and Su (2008). As seen in Figures 3.8, 3.9 and 3.10, a significant loss of echogenicity was evident at insonation pressures below the stable and inertial cavitation thresholds. In order to predict the cavitation phenomenon causing loss of echogenicity of ELIP and Definity® numerical calculations have been carried out (Radhakrishnan et al. 2013). Briefly, the size-dependent pressure thresholds of four cavitation phenomena: UCA shell rupture, subharmonic emissions from UCAs,
subharmonic emissions from free microbubbles and inertial cavitation have been computed. Based on the axial beam profile of the Doppler pressures, the number of microbubbles surviving each cavitation phenomena was calculated. The backscatter intensities from the initial size distribution of microbubbles and the size distribution of the surviving microbubbles were used to predict the loss of echogenicity caused by each of the four cavitation phenomena. The numerical calculation of the loss of echogenicity from Definity® shell rupture agreed with the experimentally measured loss of echogenicity threshold for all four pulse durations. However, the numerical calculation of the loss of echogenicity of ELIP exposed to Doppler pulses based on shell rupture was found to be different from the measured loss of echogenicity using a Kolmogorov–Smirnov test. Even so, the numerical calculations of loss of echogenicity of ELIP based on shell rupture agreed with the functional form of the measured loss of echogenicity.

These numerical calculations suggest that the LOE depends on shell rupture. The threshold for UCA rupture can occur prior to subharmonic emissions or inertial cavitation (Bader and Holland 2013). Hence, the onset of LOE may occur without stable or inertial cavitation emissions, shown in Figures 3.8 and 3.9. The exponential relationship between the disappearance of contrast and inertial cavitation activity in Definity® was also observed by Tung et al. (2010) in a vessel phantom placed inside a murine skull. It is evident from Figures 3.10 (c) and (d) that ELIP and Definity® lost approximately 80% echogenicity even before the onset of inertial cavitation. As discussed in Radhakrishnan et al. (2013), the rupture of UCAs was predicted to be the main source of loss of echogenicity. Thus the loss of echogenicity may occur at low insonation pressures without concomitant cavitation activity. This result shows that an 80% loss of
echogenicity was concomitant with the increase in stable and inertial cavitation emissions and could serve as an indicator of the onset of stable and inertial cavitation.

### III.4.3 Limitations

The insonation pressures and pulse durations used in this study were limited by the settings on the Philips HDI 5000 scanner in the duplex Doppler mode. Also the elevational beamwidth of the clinical L12-5 transducer did not cover the entire diameter of the lumen. As a result there was residual echogenicity downstream of the Doppler even at high insonation pressures. However, the choice of transducer and tubing diameter in this study were both clinically and physiologically relevant (Holubkov et al. 2002; Masuo et al. 2002).

Cavitation emissions were acquired using a single element transducer aligned confocally with the Doppler sample volume. Note that, the Doppler beam profile had side-lobes that could have interacted with the UCAs as they flowed into the Doppler sample volume. Unlike other studies (Kopechek et al. 2013; Farny et al. 2009), no corrections were made to the power spectra based on the frequency response of the passive cavitation detector and frequency dependent attenuation of the tubing. Cavitation thresholds were defined as the lowest insonation pressure beyond which there was a steady increase in cavitation power with insonation pressure. Thus the thresholds are limited by the sensitivity of the detector, the detection electronics and the noise in the system. However, a fairly broadband detector with a -3dB bandwidth of 13 MHz was chosen to detect subharmonics, ultraharmonics and broadband emissions. Also, spectral
broadening at shorter pulse durations may have resulted in an underestimation of the inertial cavitation threshold.

**III.5 Conclusion**

This study ascertained the stable and inertial cavitation thresholds for two lipid-shelled UCAs, ELIP and Definity®, as a function of Doppler pulse duration. The cavitation threshold decreased with increasing pulse duration. For each pulse duration, the ELIP and Definity® cavitation thresholds did not differ substantially. The loss of echogenicity was quantified as a function of insonation pressure at the four pulse durations. Stable or inertial cavitation emissions were not detected until approximately 80% loss of echogenicity was observed. Once 80% loss of echogenicity occurred, both stable and inertial cavitation were evident.
CHAPTER IV – Relationship between cavitation thresholds and loss of echogenicity of ELIP as a function of pulse repetition frequency
IV.1 Introduction

Acoustic cavitation from UCAs can potentially initiate therapeutic (Datta et al. 2008; Coussios et al. 2007; Hitchcock et al. 2011; Song et al. 2011; Bazan-Peregrino et al. 2012) and deleterious bioeffects in the vasculature (Skyba et al. 1998; Chen et al. 2003a; Miller and Dou 2004; Miller et al. 2008; Samuel et al. 2009). Because echogenic liposomes (ELIP) are being developed as theragnostic UCAs for detection and treatment of atherosclerosis, it is 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). However they 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). Hence passive cavitation imaging arrays are being developed to monitor cavitation with better spatial resolution over a large area (Salgaonkar et al. 2009; Farny et al. 2009; Gyongy and Coussios 2010; Haworth et al. 2012).

As hypothesized in Chapter I, the relationship between cavitation emissions and loss of echogenicity of UCAs can provide an onscreen metric to monitor cavitation from UCAs. Smith et al. (2007) demonstrated that the rapid loss of echogenicity of UCAs on B-mode images within 5s was due to rapid fragmentation. 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 likened the post-excitation signals from fragmented UCAs with the inertial cavitation emissions from free microbubbles. Taken together, these studies (Smith et al 2007; Chen et al. 2002; Ammi et al 2006) suggest that the rapid loss of echogenicity of UCAs on B-mode images
is concomitant with inertial cavitation and can be potentially used to monitor cavitation from UCAs. In Chapter III, the relationship between loss of echogenicity and stable and inertial cavitation emissions from ELIP and Definity® was investigated as a function of pulse duration (Radhakrishnan et al. 2013). Approximately 80% loss of echogenicity of ELIP and Definity® was concomitant with the onset of stable and inertial cavitation emissions from these agents. 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. However, the effect of the pulse repetition frequency (PRF) on the cavitation emissions and loss of echogenicity of ELIP had not been evaluated.

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. (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 (Miller and Quddus 2000; Ter Haar 2002; Chen et al. 2003a; Karshafian et al. 2009). Fowlkes and Crum (1988) suggested that repeated insonation can weaken the cavitation nuclei thus lowering the inertial cavitation threshold at higher PRFs. However, other studies have reported
conflicting results regarding the lack of PRF dependence for inertial cavitation and
cavitation-induced bioeffects (McDannold et al. 2008; Miller and Gies 2000; 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 loss of echogenicity, the stable and inertial cavitation
thresholds, and the spatial distribution of cavitation emissions from ELIP insonified by
duplex Doppler pulses. Cavitation emissions from ELIP were acquired with a single
element passive cavitation detector and with a passive cavitation imaging 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.

**IV.2 Materials and Methods**

**IV.2.1 Experimental setup**

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 and method of reconstitution are the same as described by Radhakrishnan et
al. (Radhakrishnan et al. 2013) and in section II.2.1 of the dissertation. The in vitro flow system shown in Fig 4.1 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 compliant 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). The latex and EVA tubes were immersed in a tank of degassed water at 37 °C. The EVA tubing was used to minimize acoustic attenuation at the insonation location and to ensure that the ELIP were uniformly insonified by the Doppler beam. 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 Chapter III (Radhakrishnan et al. 2013). Cavitation emissions and loss of echogenicity from flowing ELIP were quantified. 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 a clinical diagnostic scanner (Philips, Bothell, WA, USA) (Fig. 4.1) was operated in the duplex spectral Doppler mode to insonify the ELIP. Degassed water (DO=25 ± 2%) was pumped through the flow system to determine the acoustic noise and the minimum amount of cavitation that the
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System could detect. Cavitation emissions and images were acquired for a range of
insonation pressures and for four pulse repetition frequencies (PRF) at a constant pulse
duration of 3.33 μs, as indicated in table 4.1. 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 4.1. The range and step size of insonation pressures
was limited by the Philips HDI 5000 scanner.

Table 4.1: Duplex spectral Doppler pulse parameters. Pulse duration was maintained
at 3.33 μs.

<table>
<thead>
<tr>
<th>Pulse repetition frequency (kHz)</th>
<th># Pulses insonifying ELIP in flow</th>
<th>Range of mechanical indices (MI)</th>
<th>Range of peak rarefactiveal pressures (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>31</td>
<td>0.04-0.7</td>
<td>0.07-1.7</td>
</tr>
<tr>
<td>2.5</td>
<td>63</td>
<td>0.04-0.53</td>
<td>0.07-1.28</td>
</tr>
<tr>
<td>5</td>
<td>126</td>
<td>0.04-0.39</td>
<td>0.07-0.8</td>
</tr>
<tr>
<td>8.33</td>
<td>211</td>
<td>0.04-0.33</td>
<td>0.07-0.64</td>
</tr>
</tbody>
</table>
Figure 4.1: (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 was programmed using the Verasonics system to acquire passive acoustic signals. (b) The cross-sectional view of the tubing and the alignment of the three transducers from the tubing are shown.

**IV.2.2 Calibration of diagnostic transducer**

The *in situ* peak rarefractional pressure of the Doppler pulse at each MI setting of the scanner was calibrated. 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 line-trigger from the clinical scanner was used to externally trigger the function generator (Agilent 33250A, Santa Clara, CA, USA) to generate a frame trigger corresponding to the frame rate of the scanner. At each spatial position and onscreen MI setting, the 0.2 mm needle hydrophone signals were preamplified (Precision Acoustics, Dorchester, UK) and averaged over 16 traces on the oscilloscope (LeCroy, Chestnut Ridge, NY, USA). The sensitivity of the hydrophone was used to compute the peak negative pressure at each location and onscreen MI. The -3dB focal beam of the Doppler pulse was 0.8 mm x 1.8 mm x 13.2 mm (azimuth x elevation x range) at a focal distance of 1 cm from the transducer face.

**IV.2.3 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. The -3 dB beamwidth of the PCD was 0.8 mm x 0.8 mm x 2.4 mm (azimuth x elevation x 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 (section III.2.4) in Chapter III (Radhakrishnan et al. 2013) to obtain the cavitation thresholds.

**IV.2.4 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. The emissions were recorded at 36 MHz sampling frequency and range gated to a depth of 40 mm from the face of the transducer. The recording channels were controlled by a Verasonics V-1-128 system (Verasonics, Inc., Redmond, WA, USA). 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 Fig 4.1(b). The L7-4 transducer has 128 elements and 0.283 mm element pitch. The line trigger from the HDI 5000 scanner was used as an input trigger to synchronize the acquisition of a passive cavitation images (PCI) 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 at least 50 PCI frames contained emissions from Doppler pulse insonations.

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 time delays to the RF signals received at each element and summing across all elements. From Haworth et al. (2012), the beamformed power spectrum $B(\omega, \bar{x})$ at each pixel location $\bar{x}$ and frequency $\omega$ was given by equation (4.1a)

$$B(\omega, \bar{x}) = \left| \sum_{n=1}^{N} S_n(\omega) A_{\cos}(\bar{x}_n, \bar{x}) e^{i \omega \left[ \frac{1}{c} (\bar{x}_n - \bar{x}) \right]} \right|^2$$  \hspace{1cm} (4.1a)

where, $S_n(\omega)$ is the frequency domain representation of the signal received on the $n^{th}$ element of the L7-4 transducer, $A_{\cos}(\bar{x}_n, \bar{x})$ is a cosine apodization, $\bar{x}_n$ is the position of the $n^{th}$ element, and $c$ is the sound speed. The cosine apodization is given by equation 4.1(b).

$$A_{\cos}(\bar{x}_n, \bar{x}) = \frac{\bar{x}_n \cdot \bar{x}}{|\bar{x}_n| |\bar{x}|}$$  \hspace{1cm} (4.1b)

Cosine apodization was applied to reduce the grating lobes in the passive cavitation images. In order to determine the cavitation thresholds of ELIP, the PCI power spectra were beamformed from the location of the Doppler focus and averaged over 50 PCI frames. The frequency bands indicated in section III.2.4 were used to compute the RMS subharmonic and broadband emissions detected by the PCI system. The cavitation emissions detected by the PCI were processed as described in section III.2.4 and IV.2.5 to ascertain the cavitation thresholds. An example of the broadband PCI from ELIP
insonified by Doppler at a peak rarefactional pressure of 1.3 MPa is shown in Figure 4.2 (a). PCI power spectra were beamformed along the azimuthal direction at a range location corresponding to the distance of the tubing from the L7-4 transducer. The azimuthal distributions of subharmonic and broadband emissions (example shown in Figure 4.2 (b)) averaged over 50 PCI frames were used to assess the spatial widths of cavitation activity from flowing ELIP as described in section IV2.6.

**Figure 4.2**: (a) PCI of broadband cavitation emissions from flowing ELIP insonified by Doppler pulse (PRF=1.25 kHz, PRP=1.3 MPa). The blue box indicated location of tubing. (b) Azimuthal distribution of broadband cavitation emissions from ELIP beamformed from the range location corresponding to the location of the tubing (indicated by the blue box in (a)).

### IV.2.5 Cavitation thresholds

Spectral analysis was performed on the cavitation emissions acquired by the single element PCD and the PCI array based on previously published methods (Radhakrishnan et al. 2013) to obtain the stable and inertial cavitation thresholds. 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 equation (4.2).

\[
\text{Cavitation Signal}_{\text{RMS}} = \sqrt{\sum_{f_{\text{Band}}} \text{Power}_{\text{UCA}}} - \sqrt{\sum_{f_{\text{Band}}} \text{Power}_{\text{DW}}}, \tag{4.2}
\]

where \(\text{Power}_{\text{UCA}}\) and \(\text{Power}_{\text{DW}}\) are the detected acoustic emission powers from ELIP in the tubing and degassed water in the tubing, respectively. \(f_{\text{Band}}\) 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 in Chapter III, piecewise linear fits of the RMS subharmonic emissions and RMS broadband emissions was performed using the 'slmengine' function in MATLAB. The peak rarefational 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 four PRFs.

**IV.2.6 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 spatial beamwidth of the insonation region. This comparison was done in the azimuthal direction along the direction of flow (Figure 4.2 b). The RMS subharmonic and broadband emissions detected at the stable and inertial cavitation thresholds were defined as the subharmonic and broadband noise.
floors of the PCI system. The spatial width of stable and inertial cavitation activity detected by the PCI was computed at each insonation pressure as the azimuthal width of the RMS subharmonic and RMS broadband emissions exceeding the subharmonic and broadband noise floors of the PCI system. The azimuthal beamwidth of the Doppler pressure profile greater than the stable and inertial cavitation thresholds was determined from the calibration data for the L12-5 (section IV2.2). To compare the measured spatial width of cavitation activity on the PCI with the calibrated beamwidth of the insonation region, the point spread function (PSF) of the PCI array (L7-4) was also taken into account. The PSF of the PCI array was computed using the ‘transientrectarray’ function in the Fast Object-oriented C++ Ultrasound Simulator software (McGough et al. 2009). The PSF was normalized by its own energy and was subsequently convolved with the azimuthal beamwidth of the Doppler beam exceeding the stable and inertial cavitation thresholds. Figures 4.3 (a) shows the calibrated azimuthal beamwidth of the Doppler (MI=0.56) with the shaded region indicating the Doppler pressures exceeding the inertial cavitation threshold. Figure 4.3 (b) show the PSF of the PCI array and Figure 4.3 (c) shows the convolution of the azimuthal beam profile of the Doppler pulse and the PSF of the PCI array exceeding the inertial cavitation threshold (shaded region).
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Figure 4.3: (a) Calibrated azimuthal beamwidth of Doppler pressure (MI=0.56) shaded region indicated Doppler pressure exceeding the inertial cavitation threshold. (b) Point spread function (PSF) of passive cavitation imaging array calculated using the FOCUS software. (c) Convolution of azimuthal Doppler pressure exceeding the inertial cavitation and the PSF. The shaded region indicates the beamwidth exceeding the inertial cavitation threshold.

IV.2.7 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 1 mm inner diameter, the high definition zoom function on the scanner was employed. Using previously established techniques, as discussed in Chapter II (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 4.4 (a). The percent loss of
echogenicity (LOE) was computed using equation (4.3a).

\[
\text{Percent LOE}_{\text{No flow}} = \frac{\text{MDI}_{\text{Upstream of Doppler}} - \text{MDI}_{\text{At Doppler focus}}}{\text{MDI}_{\text{Upstream of Doppler}}} \times 100, \quad (4.3a)
\]

where \( \text{MDI}_{\text{Upstream of Doppler}} \) and \( \text{MDI}_{\text{At Doppler focus}} \) 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
rarefactual 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 4.4 (b). LOE was computed for each insonation
pressure using equation (4.3b).

\[
\text{Percent LOE}_{\text{Flow}} = \frac{\text{MDI}_{\text{Up}} - \text{MDI}_{\text{Down}}}{\text{MDI}_{\text{Up}}} \times 100, \quad (4.3b)
\]

where \( \text{MDI}_{\text{Up}} \) and \( \text{MDI}_{\text{Down}} \) are the mean digital intensities in the ROIs upstream
(proximal) and downstream (distal) of the Doppler focus respectively.
Figure 4.4: (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 located within the Doppler focus and upstream of the Doppler focus. (b) Example of ELIP flowing through the 1 mm inner diameter ethyl vinyl acetate tubing. The gray bars indicate the location of the Doppler “sample volume” or spatial pulse length. The echogenicity was analyzed in regions of interest (ROIs) located upstream (proximal) and downstream (distal) of the Doppler “sample volume”.

IV.2.8 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 ANOVA 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.

**IV.3 Results**

**IV.3.1 Cavitation thresholds**

Figures 4.5 (a) and 4.5 (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 4.5(a)) agreed with the thresholds determined using the PCI system (Figure 4.5(b)) (p=0.13). The stable cavitation thresholds did not depend on the PRF (p=0.23). As seen in figures 4.5 (a) and (b), the inertial cavitation thresholds have a weak dependence on the PRF. The inertial cavitation threshold of ELIP at 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).
Figure 4.5: Stable and inertial cavitation thresholds of ELIP in flowing and static plasma measured using (a) a single-element PCD and (b) a PCI array.
IV.3.2 Spatial distribution of cavitation emissions

The spatial distribution of cavitation activity detected with the PCI was compared to the calibrated beamwidth of the Doppler pressure profile. Figure 4.6(a) and (c) show the measured subharmonic and broadband emissions indicating the spatial distribution of stable and inertial cavitation, respectively. Figure 4.6(b) and (d) show the calibrated beamwidth of the Doppler pressure profile exceeding the stable and inertial cavitation thresholds, respectively.

![Figure 4.6](image-url)
threshold. (c) Spatial distribution of the broadband emissions measured by the PCI exceeding the broadband emissions at the inertial cavitation threshold. (d) Calibrated azimuthal pressure profile of the Doppler pulse greater than the inertial cavitation threshold.

Figure 4.7(a) and (b) 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 4.2 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 percent error was computed using equation 4.4.

\[
\text{Percent error} = \left( \frac{BW_{PCI} - BW_{Calibration}}{BW_{Calibration}} \right) \times 100,
\]

where \(BW_{PCI}\) is the spatial width of cavitation emissions measured on the PCI and \(BW_{Calibration}\) is the calibrated beamwidth of the Doppler pressure exceeding the cavitation threshold. 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 4.3). However at pressures higher than 1 MPa, the spatial width of cavitation emissions detected by the PCI appeared to saturate.
Figure 4.7: (a) Comparison of the calibrated beamwidth of the Doppler pressure above the stable cavitation threshold and the measured widths of subharmonic emissions on the PCI (PRF=1.25 kHz). (b) Comparison of the calibrated beamwidth of the Doppler pressure above the inertial cavitation threshold and the measured widths of broadband emissions on the PCI (PRF=1.25 kHz).

Table 4.2: Percent error in the spatial width of stable and inertial cavitation activity detected using the passive cavitation imaging (PCI) system compared to the calibrated beamwidth of the Doppler pressure profile.

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

Table 4.3: K-S test between spatial widths of cavitation emissions measured on PCI and calibrated Doppler beamwidths exceeding the cavitation thresholds of ELIP.

<table>
<thead>
<tr>
<th>Pulse repetition frequency (kHz)</th>
<th>Stable cavitation emissions (p-value)</th>
<th>Inertial cavitation emissions (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>0.12</td>
<td>0.16</td>
</tr>
<tr>
<td>2.5</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>8.33</td>
<td>0.09</td>
<td>0.06</td>
</tr>
</tbody>
</table>
IV.3.3 Loss of echogenicity

Figure 4.8 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 rarefractional pressure at which 90% loss of echogenicity occurred, (Smith et al. 2007). The rapid fragmentation threshold determined by Smith et al. (2007) were found to be 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.

![Diagram showing comparison of Loss of Echogenicity (LOE), Rapid Fragmentation, Stable and Inertial Cavitation thresholds for ELIP in static fluid.](image)

**Figure 4.8:** Comparison of loss of echogenicity, rapid fragmentation (Smith et al. 2007) and stable and inertial cavitation thresholds of ELIP in static fluid.

In order to determine the relationship between loss of echogenicity and cavitation emissions from ELIP in the flow system, the RMS subharmonic emissions and RMS broadband emissions were plotted against the corresponding loss of echogenicity (Figure 4.9 (a) and (b)). Piecewise linear fits were applied as shown in Figure 4.9 (a) and (b) to
ascertain the minimum loss of echogenicity threshold at which concomitant stable and inertial cavitation emissions were detected. Figure 4.9 (c) shows that at approximately 80% loss of echogenicity, stable and inertial cavitation emissions also occurred. At the highest PRF (8.33 kHz) hyperechoic aggregates of ELIP were observed on the lower wall of the tubing indicative of radiation forces. Figure 4.10 shows an example of ELIP flowing through the EVA tubing insonified by the Doppler pulses at 8.33 kHz PRF and 0.64 MPa peak rarefactual pressure.

**Figure 4.9:** Relationship between loss of echogenicity and (a) subharmonic emissions and (b) broadband emissions from ELIP insonified by Doppler pulses of PRF 1.25 kHz. The open squares show the detected cavitation emissions concomitant with the measured loss of echogenicity. The abscissa and ordinate error bars represent the
standard deviation of four measurements. The dashed line represents a piecewise linear fit. (c) The loss of echogenicity threshold at which concomitant stable cavitation (blue squares) and inertial cavitation (red squares) emissions were detected at each PRF.

Figure 4.10: B-mode image of flowing ELIP in plasma insonified by Doppler at 0.64 MPa peak rarefractional pressure, 8.33 kHz PRF, 3.33 μs pulse duration. The flow direction and the direction of Doppler insonation are shown. Hyperechoic aggregates were observed along the distal wall of the tubing.

**IV.4 Discussion**

**IV.4.1 Cavitation thresholds**

In Chapter III, the stable and inertial cavitation thresholds of ELIP as a function of pulse duration at 1.25 kHz PRF were reported. 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 μs pulse duration. The stable and inertial cavitation thresholds of ELIP at a 3.33 μs 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 the previous study (Radhakrishnan et al. 2013).

The stable and inertial cavitation thresholds measured using a single-element PCD system agreed with the stable and inertial thresholds measured using passive cavitation imaging (Figure 4.5). Although the thresholds were not different, there were qualitative differences observed in the power spectra of each passive cavitation measurement approach. Figure 4.11 (a) and (b) show representative spectra from ELIP and degassed water detected by the single-element PCD system and the PCI, respectively, for a single set of experiments. 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 of the passive cavitation images increases the signal-to-noise ratio (SNR). Despite these differences, the cavitation thresholds ascertained using the piecewise linear fit were similar in both systems. The sensitivity of the piecewise linear fit in determining the cavitation thresholds may be limited by the SNR of the detection system. However, both the PCD and PCI have sufficiently high SNR to detect the onset of stable and inertial cavitation. Farny et al. (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. 2003a), cell permeability (Karshafian et al. 2009), and cell death (Miller and Quddus 2000) are dependent on PRF. Chen et al. (2003a) 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 Quddus (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 4.1) 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 Gies 2000) are independent of PRF.

### IV.4.2 Spatial distribution of cavitation emissions

The spatial distribution of the cavitation emissions detected by 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 (Figure 4.7 and Table 4.3). 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 PCI array, the spatial distribution of the cavitation emissions was compared to the predicted cavitation region qualitatively. In the current study, the spatial distribution of the cavitation emissions was assessed along the azimuth of the PCI array, which has submillimeter resolution for the L7-4 array. The calibrated beamwidth of the Doppler pressure profile exceeding the stable and inertial cavitation threshold was convolved with the PSF of the L7-4 to allow for a quantitative comparison. Figure 4.6 and Table 4.2 confirm that the passive cavitation image system provides good lateral resolution to map the spatial distribution of the cavitation activity. The resolution is limited by the point spread function of the array transducer.

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. The PRFs of the Doppler pulse used in the current study (1.25 kHz- 8.33 kHz) were much higher than the critical PRF. 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. Choi and Coussios (2012) defined the spatial asymmetry as the ratio of the spatial energy of the cavitation activity measured within a regions of interest located upstream and downstream of the insonation beam. In the current study, the average cavitation spectral power within the spatial width located upstream of the Doppler pulse was found to agree with the average cavitation spectral power within the spatial width located downstream of the Doppler pulse. Note that the range of insonation pressures used by Choi and Coussios (2012) (0.1 MPa-1.5 MPa and MI=0.14-2.1) was comparable to those used in the current study (0.06 MPa-1.6 MPa and MI=0.04-0.7). 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.
IV.4.3 Loss of echogenicity

The loss of echogenicity threshold of ELIP in a static fluid was defined in Chapter III as the lowest peak rarefractional pressure at which ELIP lost 90% echogenicity. This definition was developed to allow a comparison between the rapid fragmentation threshold defined by Smith et al. (2007) and the current work. In Chapter III (Radhakrishnan et al. 2013), the loss of echogenicity thresholds of ELIP agreed with the rapid fragmentation threshold and the inertial cavitation threshold. However, in this Chapter, as shown in figure 4.8 the loss of echogenicity thresholds of ELIP were lower than the rapid fragmentation thresholds of ELIP.

Figure 4.12 shows representative B-mode images of ELIP echogenicity in the 3 mm ID tubing and the 1 mm ID tubing insonified by Doppler at 0.56 MPa peak rarefractional pressure, 1.25 kHz PRF and 3.33 μs pulse duration. The discrepancy between the loss of echogenicity threshold ascertained in the current study versus the previous study (Chapter III) is 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 Chapter III 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 narrower inner diameter tube in the current study, there were no ELIP outside the -3 dB elevational beamwidth to contribute to the echogenicity.
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 (Figure 4.10). 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.

Figures 4.9 (a) and (b) plot the stable and inertial cavitation emissions as a function of the loss of echogenicity of ELIP in the flow system, respectively. ELIP in
flow did not exhibit a complete loss of echogenicity even at the highest Doppler pressures. ELIP lost 80% echogenicity at pressures just below the stable and inertial cavitation thresholds. These results were also evident in Chapter III examining the pulse duration dependence of cavitation thresholds and loss of echogenicity (Radhakrishnan et al. 2013). Numerical calculations based on the Marmonant model (Marmottant et al. 2005) published previously (Radhakrishnan et al. 2013), indicate that UCA shell rupture may cause loss of echogenicity at insonation pressures lower than the stable and inertial cavitation thresholds.

**IV.5 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. 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). ELIP lost more than 80% echogenicity at insonation pressures lower than the stable and inertial cavitation cavitation thresholds. Once 80% loss of echogenicity occurred, both stable and inertial cavitation emissions were detected in the physiologic flow phantom, thus confirming previous results reported in Chapter III (Radhakrishnan et al. 2013).
CHAPTER V – Conclusions and Future Work
V.1 Summary

The interaction of ultrasound contrast agents (UCAs) with pulsed ultrasound have been investigated by evaluating the acoustically-mediated destruction of UCAs (Porter et al. 2006; Smith et al. 2007) and by assessing the acoustic emissions from the UCAs (Chen et al. 2002; Chen et al. 2003a; Chen et al. 2003b). In previous studies, the destruction thresholds of ELIP have been determined by evaluating the rate of loss of echogenicity (Porter et al. 2006; Smith et al. 2007). Other investigators have also examined the role of acoustic cavitation from ELIP as a mechanism to induce drug release (Kopechek et al. 2013) and delivery to vascular tissue (Hitchcock et al. 2010). However, the relationship between cavitation thresholds and loss of echogenicity of ELIP had not been previously studied. **The central hypothesis of this dissertation was that loss of echogenicity is concomitant with acoustic cavitation emissions from echogenic liposomes insonified by clinical Doppler ultrasound.** This hypothesis was tested by measuring acoustic emissions, which indicate stable and inertial cavitation. Based on the studies outlined in Chapters II, III, and IV, this hypothesis was proven with the caveat that a minimum 80% loss of echogenicity was required for concomitant detection of stable and inertial cavitation emissions from ELIP. Up to an 80% loss of echogenicity was observed at insonation pressures lower than the stable and inertial cavitation thresholds. At these lower acoustic pressure amplitudes, loss of echogenicity was not accompanied by cavitation emissions. Unlike the cavitation emissions, the loss of echogenicity of flowing ELIP did not exhibit a threshold behavior. These results suggest that loss of echogenicity and nucleation of cavitation from ELIP are complex phenomena and require further investigation.
The stability of ELIP as a blood pool agent in an in vitro flow system exposed to physiological levels of total dissolved gas, temperature, and hydrodynamic pressures in porcine plasma and whole blood was described in Chapter II. Stable echogenicity and consistent intraluminal contrast were observed on B-mode images of ELIP for arterial and venous values of total dissolved gas in porcine plasma. However, ELIP in degassed plasma (DO < 70%) lost echogenicity rapidly at 120/80 mmHg hydrodynamic pressure. ELIP remained echogenic at room temperature and at body temperature in plasma. However, when cooled from body temperature (37 ºC) to room temperature (22 ºC) in porcine plasma, ELIP lost echogenicity within 10 s at 120/80 mmHg hydrodynamic pressure. The echogenicity of ELIP was also stable over 75 s when exposed to normotensive (120/80 mmHg) and hypertensive (145/90 mmHg) hydrodynamic pressures in flowing porcine plasma. ELIP were also stable in whole blood at normotensive pressures and body temperature. However, the echogenicity of ELIP appeared to decay in the presence of abnormal RBCs in whole blood. Apart from demonstrating the stability of ELIP in physiological conditions, these results emphasize the importance of careful thermodynamic handling of ELIP prior to in vivo administration.

In Chapter III the relationship between the stable and inertial cavitation thresholds and loss of echogenicity of ELIP and Definity® as a function of insonation pulse duration was discussed. Stable and inertial cavitation thresholds for ELIP were weakly dependent on the pulse duration were the same as the cavitation thresholds of Definity®. For both UCAs, the inertial cavitation threshold exceeded the stable cavitation threshold. The loss of echogenicity threshold of ELIP in a static fluid was found to agree with the rapid fragmentation threshold measured by Smith et al. (2007). The loss of echogenicity of
ELIP and Definity® in flow did not exhibit a clear pressure threshold (Radhakrishnan et al. 2013). A 80% loss of echogenicity was evident below the stable and inertial cavitation thresholds for both UCAs. Numerical calculations (Radhakrishnan et al. 2013) predicted that UCA shell rupture may initiate the loss of echogenicity of Definity® at insonation pressures below the stable and inertial cavitation thresholds.

In Chapter IV, the relationship between cavitation thresholds and loss of echogenicity of ELIP and Definity® has been discussed as a function of pulse repetition frequency was explored. The stable cavitation threshold was not dependent on PRF whereas the inertial cavitation threshold was weakly dependent on PRF. The loss of echogenicity threshold of ELIP in static fluid was lower than the rapid fragmentation threshold ascertained by Smith et al. (2007). As in Chapter III, a loss of echogenicity of 80% was observed at pressures below the stable and inertial cavitation thresholds in ELIP. In addition to the single-element passive cavitation detector, a passive cavitation imaging (PCI) system was also employed to record cavitation emissions from ELIP. Both independent detection methods yielded identical stable and inertial cavitation thresholds. The calibrated beamwidth of the Doppler pulse convolved with the point spread function of the PCI array was used to predict the spatial distribution of cavitation activity. The beamwidths of the Doppler pulse which exceeded the stable and inertial cavitation thresholds agreed with the spatial distribution of stable and inertial cavitation emissions detected by the PCI array at four PRFs.
V.2 Discussion and Future Directions

In Chapter II, the stability of ELIP under physiological conditions of total dissolved gas, temperature and hydrodynamic pressure in porcine plasma and whole blood was demonstrated as a blood pool agent. However, several limitations were noted in the study. The echogenicity of ELIP was measured over a limited duration of 75 s to mimic the duration of a single pass of ELIP through circulation in vivo. Recirculation of ELIP was not investigated. Further, various physiological conditions, such as the presence of carbon dioxide in venous blood, gas exchange and filtration in the lungs, and opsonization of liposomal vesicles, have not been investigated in this study. The effect of hydrodynamic pressures exceeding 145/90 mmHg on ELIP echogenicity also needs to be tested in future in vivo studies. These parameters would likely be better investigated in animal studies which include the full range of physiologic conditions. In vivo studies would also allow for the investigation of the removal of ELIP vesicles through the reticuloendothelial system and possible toxic effects from ELIP passage across the cardiopulmonary system. Previous in vivo studies have demonstrated that ELIP can be used to highlight atherosclerotic lesions and thrombi in vivo (Demos et al. 1999; Hamilton et al. 2002; Hamilton et al. 2004), but they have not investigated ELIP as a blood pool agent. These proposed blood pool agent studies could additionally be performed with an FDA-approved UCA such as Definity® for comparison.

The results discussed in Chapter II also provide guidelines to ensure proper handling of ELIP prior to injection. In summary, ELIP should not be diluted in degassed fluids (DO < 70 %). Also, ELIP diluted in a fluid at 37 °C should not be cooled to 25 °C. Additionally, the in vitro physiological flow system can be used to evaluate the stability
of future batches and formulations of ELIP. Recently, Raymond et al. (2013) demonstrated a broadband acoustic spectroscopy method to measure the frequency-dependent attenuation of three formulations of ELIP and to determine their shell parameters. Measurement of the attenuation provides the advantage of being able to monitor the stability of ELIP over a large frequency range (3-25 MHz). However, the Philips HDI 5000 scanner has the advantage of a wide dynamic range (170 dB) and provides qualitative echogenicity (MGSV) as well as quantitative backscatter intensity (MDI). However, the choice of the imaging transducer (L12-5) limits the B-mode frequency (6.9 MHz) used to monitor the ELIP. Future work to develop new formulations of ELIP for imaging and image-guided drug delivery may include evaluation of ELIP stability in the physiological flow system as well as the broadband acoustic spectroscopy system for quality control. Recently, Kandadai et al. (2013) used the physiological flow system and the broadband acoustic spectroscopy system to evaluate the stability and acoustic properties of novel plasmin-loaded ELIP.

As described in Chapters III and IV, up to 80% loss of echogenicity was evident at insonation pressures below the stable and inertial cavitation thresholds. At pressures sufficient to induce loss of echogenicity above 80%, both stable and inertial cavitation were evident in ELIP and Definity®. Thus, loss of 80% echogenicity may be used as a qualitative metric to gauge the onset of stable and inertial cavitation from ELIP and Definity®. In order to predict the mechanism underpinning the loss of echogenicity of ELIP and Definity®, numerical calculations were carried out (Radhakrishnan et al. 2013). Numerical calculations of the loss of echogenicity based on the Marmottant model (Marmottant et al. 2005) suggest that UCA shell rupture induces the loss of echogenicity
for Definity® at insonation pressures lower than the stable and inertial cavitation thresholds (Radhakrishnan et al. 2013). Based on these results, we postulated that the rupture of UCA shells liberates the encapsulated gas which undergoes dissolution and causes the loss of echogenicity on B-mode images.

Liberated gas microbubbles dissolve into the surrounding medium within several hundred milliseconds (Kabalnov et al. 1998; Kwan and Borden 2010; Sarkar et al. 2009; Bevan et al. 2008) and probably do not survive transport to the downstream ROI. Other groups have also posited that microbubbles liberated from a lipid shell may be stabilized by lipid fragments that re-organize after rupture (Chomas et al. 2001; Mehier-Humbert et al. 2007; Postema et al. 2004). Thus the post-rupture microbubbles may be smaller in size than the initial UCAs (Chomas et al. 2001) and therefore non-echogenic at the B-mode center frequency (6.9 MHz). As a larger fraction of the microbubble population undergoes rupture, an exponential increase in loss of echogenicity occurs as described in Chapter III and IV.

At a fixed insonation frequency, stable or inertial cavitation are nucleated at the lowest acoustic pressure thresholds, respectively, only when optimally-sized gas microbubbles are present (Bader and Holland 2013; Apfel and Holland 1991). One possible explanation for the lack of correlation between loss of echogenicity and stable and inertial cavitation emissions at lower pressures is that gas microbubbles liberated from ELIP and Definity® must coalesce, or grow to an optimal size before triggering stable or inertial cavitation. Microbubble coalescence, rectified diffusion, and Bjerknes forces may be necessary for the formation of optimally-sized gas microbubbles after liberation from UCAs. Another plausible explanation is that at lower acoustic pressures,
only a small amount of gas is liberated from the UCA with every acoustic cycle, representative of acoustically driven diffusion. These smaller than optimally-sized pockets of liberated gas would likely require much larger acoustic pressures to cavitate.

As noted by Radhakrishnan et al. (2013), the numerical calculations of loss of echogenicity in ELIP did not agree with the experimentally measured loss of echogenicity. The numerical calculations of UCA shell rupture are sensitive to the size distribution and shell parameters of ELIP. Further, unlike Definity®, ELIP encapsulate microbubbles as well as aqueous cores (Huang et al. 2001). The current understanding of the morphology, size, and location of gas microbubbles in ELIP vesicles is limited and further investigations are necessary to develop an appropriate theory. Moreover, the Marmottant model (Marmottant et al. 2005), which has been used to describe the dynamics of gas-filled UCAs like Definity® (Santin et al. 2010; King and O’Brien Jr 2011), may not be sufficient to predict the loss of echogenicity from vesicles encapsulating aqueous cores. A more suitable model may be required for numerical calculations in the future. Furthermore, Definity® and ELIP encapsulate octafluoropropane gas and air, respectively. The different rates of dissolution of octafluoropropane microbubbles and air microbubbles had not been taken into consideration in the numerical calculations of loss of echogenicity. Therefore, improved theoretical models need to be developed to understand the complex phenomena which trigger loss of echogenicity from UCAs.

Although loss of echogenicity can provide a qualitative metric to gauge the onset of cavitation from UCAs, passive cavitation imaging (PCI) can provide more quantitative spatiotemporal information of the cavitation activity. In Chapter IV, the beamwidth of the
Doppler pulse with pressures exceeding the cavitation thresholds was shown to be the same as the spatial distribution of cavitation emissions detected by the PCI. Ongoing and future studies investigating the role of cavitation in ultrasound-mediated drug delivery and sonothrombolysis may benefit from the spatiotemporal resolution provided by PCI arrays. Unlike the spectral Doppler pulses used in these studies, color Doppler pulses operate in a scanned mode, interleaved with B-mode pulses, and have a complex spatiotemporal insonation scheme. A focused single-element passive cavitation detector may provide high spatial specificity but it will be relatively insensitive to cavitation activity occurring outside the detector focus. On the other hand, an unfocused single-element passive cavitation detector may be sensitive to cavitation activity occurring over a large area, but will not provide spatial resolution on the location of cavitation activity within its beamwidth. PCI can be used in future studies to assess the spatial location and distribution of cavitation emissions from ELIP to coregister cavitation activity with bioeffects such as drug delivery and thrombolysis.

Because ELIP are being developed for molecular imaging of atherosclerosis and ultrasound-mediated targeted drug delivery, future studies should also investigate the relationship between cavitation emissions and loss of echogenicity from ELIP targeted to clots and endothelial cells. Recent studies have demonstrated that the acoustic response and backscatter intensity of unbound UCAs differ from targeted or bound UCAs (Zhao et al. 2005; Zhao et al. 2006; Couture et al. 2009; Helfield et al. 2010). Couture et al. (2009) demonstrated that multiple scattering effects an important role in modeling the reflection coefficient from UCAs bound to a surface. The destruction thresholds of targeted UCAs were found to be dependent on the pulse duration and number of
transmitted pulses (Sprague et al. 2010). The destruction thresholds of bound UCAs were found to be lower than the destruction thresholds of unbound UCAs (Helfield et al. 2010). Sprague et al. (2010) also noted that at insonation pressures above 1 MPa, the destruction of targeted UCAs was concomitant with inertial cavitation emissions. The size-dependent subharmonic response of bound UCAs was different from the unbound UCAs (Helfield et al. 2010). Furthermore, the stable cavitation threshold of targeted UCAs was found to be lower than the predicted stable cavitation threshold of unbound UCAs (Sprague et al. 2010).

These studies (Couture et al. 2009; Helfield et al. 2010; Sprague et al. 2010) suggest that the cavitation thresholds and loss of echogenicity from targeted ELIP may be different from the cavitation thresholds and loss of echogenicity of unbound ELIP. Thus, the stability, cavitation thresholds, and loss of echogenicity from targeted ELIP should be investigated. In these proposed future studies, the passive cavitation imaging system should also be employed to assess the spatial distribution of the cavitation activity.
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