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Stability of echogenic liposomes as a blood pool ultrasound contrast agent in a physiologic flow phantom

Kirthi Radhakrishnan^a, Kevin J. Haworth, Ph.D.^b, Shao-Ling Huang, M.D., Ph.D.^c, Melvin E. Klegerman, Ph.D.^c, David D. McPherson, M.D.^c, and Christy K. Holland, Ph.D.^b

^aProgram of Biomedical Engineering, University of Cincinnati, Cincinnati, OH, USA

^bDepartment of Internal Medicine, Division of Cardiovascular Diseases, University of Cincinnati, Cincinnati, OH, USA

^cDepartment of Internal Medicine, University of Texas Health Science Center, Houston, TX, USA

Abstract

Echogenic liposomes (ELIP) are multifunctional ultrasound contrast agents (UCAs) with a lipid shell encapsulating both air and an aqueous core. ELIP are being developed for molecular imaging and image-guided therapeutic delivery. Stability of the echogenicity of ELIP in physiologic conditions is crucial to their successful translation to clinical use. In this study we determined the effects of the surrounding media's dissolved air concentration, temperature transition and hydrodynamic pressure on the echogenicity of a chemically modified formulation of ELIP to promote stability and echogenicity.

ELIP samples were diluted in porcine plasma or whole blood and pumped through a pulsatile flow system with adjustable hydrodynamic pressures and temperature. B-mode images were acquired using a clinical diagnostic scanner every 5 s for a total duration of 75 s. Echogenicity in porcine plasma was assessed as a function of total dissolved gas saturation. ELIP were added to plasma at room temperature (22 °C) or body temperature (37 °C) and pumped through a system maintained at 22 °C or 37 °C to study the effect of temperature transitions on ELIP echogenicity. Echogenicity at normotensive (120/80 mmHg) and hypertensive pressures (145/90 mmHg) was measured. ELIP were echogenic in plasma and whole blood at body temperature under normotensive to hypertensive pressures. Warming of samples from room temperature to body temperature did not alter echogenicity. However, in plasma cooled rapidly from body temperature to room temperature or in degassed plasma, ELIP lost echogenicity within 20 s at 120/80 mmHg. The stability of echogenicity of a modified ELIP formulation was determined *in vitro* at body temperature, physiologic gas concentration and throughout the physiologic pressure range. However, proper care should be taken to ensure that ELIP are not cooled rapidly from body temperature to room

Correspondence: Name: Kirthi Radhakrishnan, Address: University of Cincinnati, Department of Internal Medicine, Division of Cardiovascular Diseases, 231 Albert Sabin Way, Cincinnati OH 45267-0586, USA, Fax: 513-558-6102, Telephone: 513-558-5505, radhakki@mail.uc.edu.

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temperature as they will lose their acoustic properties. Further *in vivo* investigations will be needed to evaluate the optimal usage of ELIP as blood pool contrast agents.

Keywords

ultrasound contrast agents; echogenic liposomes; stability; plasma; whole blood

Introduction

Since the first use of agitated saline to enhance contrast on echocardiographic images, ultrasound contrast agents (UCAs) have been developed over the past 40 years as diagnostic blood pool agents (Gramiak and Shah 1968). The United States Food and Drug Administration has approved UCAs for left ventricular opacification (Mulvagh et al. 2008). European and Asian governments have approved UCAs for a broader range of applications, including contrast-enhanced ultrasound imaging to detect focal lesions in the liver, kidney and pancreas, to assess abdominal trauma and to evaluate brain perfusion using transcranial color-coded sonography (Claudon et al. 2008). Current research focuses on the development of next generation UCAs as targeted molecular imaging agents to detect early atherosclerosis and lesion formation (Villanueva and Wagner 2008; Demos et al. 1999; Klibanov 1999; Kee and McPherson 2008; Hamilton et al. 2004). Recent findings on the role of acoustic cavitation in ultrasound-mediated delivery of therapeutics into thrombi (Datta et al. 2008), cell membranes (Deng et al. 2004) and endothelia (Alonso et al. 2010) have propelled the development of UCAs as theranostic vesicles (Dijkmans et al. 2004).

Echogenic liposomes (ELIP) are being developed as multifunctional theranostic agents for detection of atherosclerotic plaques and for image-guided ultrasound-mediated delivery of therapeutics to treat cardiovascular diseases (McCarthy 2010). ELIP are lipid bilayer vesicles that entrap gas within a lipid monolayer and also encapsulate an aqueous core (Huang 2008). ELIP have a broad size distribution ranging from 40 nm to 6 μ m in diameter (Kopechek et al. 2011). The encapsulated air microbubble occupies 18% of the liposome volume (Kopechek et al. 2011; Huang et al. 2002). Recently Kopechek et al. (2011) and Paul et al. (2012) acoustically characterized ELIP by measuring their frequency dependent attenuation and backscatter coefficients. Kopechek et al. (2011) found that the ELIP attenuation and backscatter coefficients were approximately constant between 7 MHz and 25 MHz because of their polydisperse size distribution. Therefore, ELIP can be imaged using both intravascular (20–40 MHz) (Buchanan et al. 2008) and transvascular diagnostic ultrasound systems (5–12 MHz) (Smith et al. 2007).

As therapeutic agents, liposomal vesicles have the ability to encapsulate therapeutics within the aqueous core or the lipid bilayer, thus preventing the therapeutics from being scavenged *in vivo* (Wright and Huang 1989; Sharma and Sharma 1997) and from bioactivity until released from the vesicle. Liposomal encapsulation can also expand the therapeutic window by minimizing the dose-limiting side-effects of certain toxic drugs (Torchilin 2005). Additionally, liposomes are ideal delivery systems because they are biocompatible and biodegradable (Al-Jamal and Kostarelos 2011). Studies have shown that drugs (Shaw et al.

2009; Tiukinhoy et al. 2004; Huang et al. 2007), genes (Buchanan et al. 2010), therapeutic gases (Britton et al. 2010; Huang et al. 2009) and stem cells (Herbst et al. 2010) can be loaded into or complexed with ELIP. Other studies have demonstrated ultrasound-mediated delivery of therapeutics into tissues and clots (Shaw et al. 2009; Britton et al. 2010; Herbst et al. 2010). Another attractive feature of liposomes is the ability to conjugate the lipid shell with specific ligands to target certain molecular receptors in tissue (Wright and Huang 1989; Torchilin 2005). This targeting strategy allows for greater localization of the drug payload at pathological sites. Targeted ELIP have also been shown to highlight atherosclerotic components on intravascular ultrasound (IVUS) images of arteries (Hamilton et al. 2004). ELIP are therefore suitable candidates for early detection and treatment of atherosclerotic plaques. Critical to the success of such multifunctional theranostic agent is their stability under physiological conditions.

The stability of microbubble-based UCAs is dependent on thermodynamic parameters of the surrounding fluid, such as temperature (Mulvana et al. 2010) the concentration gradients of gases across the microbubble shell (Kabalnov et al. 1998b) and the hydrostatic and Laplace pressures (Van Liew and Burkard 1995). Further, unlike other perfluorocarbon gas-loaded UCAs studied in the literature, ELIP encapsulate air (Huang 2008). Air-filled UCAs have been shown to be susceptible to changes in the hydrostatic pressure and dissolved gas content in the diluent fluid (Padial et al. 1995; Sboros et al. 2000a). It is therefore important to investigate the effect of thermodynamic parameters (temperature, pressure and dissolved gas concentration) on the stability of ELIP.

Gas diffusion out of UCAs is dependent on the concentration (Sboros et al. 2000a; Mulvana et al. 2012; Kabalnov et al. 1998a) and type of gas in the surrounding fluid (Mullin et al. 2009; Itani and Mattrey 2011) as well as the gas permeability of the UCA shell (Sboros et al. 2000a; Borden and Longo 2002; Kwan and Borden 2010; Sboros et al. 2000b). Smith *et al.* (2007) investigated the diffusion of gas from ELIP and Optison® diluted in air-saturated static bovine serum albumin in a static system and insonified by (4.5 MHz, 6.0 MHz and 6.9 MHz) diagnostic ultrasound pulses. The rate of decay of echogenicity on B-mode images was used to characterize the diffusion of gas from UCAs. The slow diffusion of gas from UCAs in the absence of a destructive acoustic pulse was termed static diffusion. Optison® lost all echogenicity within 4 minutes, but ELIP remained echogenic on B-mode images for 5 minutes. The effect of gas content in the surrounding fluid medium on echogenicity of ELIP was, however, not investigated.

Physiologic temperatures can also affect the stability of the encapsulated gas microbubbles (Mulvana et al. 2010) and the nonlinear acoustic emissions from UCAs (Mulvana et al. 2011; Vos et al. 2008). Buchanan *et al.* (2008) examined the echogenicity of ELIP in phosphate buffered saline and in human serum using 20-MHz intravascular ultrasound. They manipulated the lipid composition to improve the longevity of ELIP echogenicity at body temperature. They hypothesized that the incorporation of longer acyl chain phospholipids minimized the diffusion of gases out of ELIP at body temperature.

Echogenicity (Padial et al. 1995; Mor-Avi et al. 1994; Vuille et al. 1994) and nonlinear acoustic emissions (Andersen and Jensen 2010a; Forsberg et al. 2005; Adam et al. 2005; Shi

et al. 1999) from UCAs are sensitive to changes in hydrostatic pressures in the surrounding medium. Huang *et al.* (2002) observed a 50% loss of echogenicity from ELIP exposed to a hydrostatic overpressure of 380 mmHg for 60 s at room temperature. However, the echogenicity of ELIP at physiologically relevant and cyclical pressures was not studied.

Although several parameters affecting the stability of ELIP have been investigated, the effect of temperature transitions, hydrodynamic pressure variations and total dissolved gas content in biological fluids have yet to be investigated. The objective of this study is to determine the practical role each of these parameters play in the stability of ELIP echogenicity. In this study, ELIP echogenicity was quantitatively assessed in a flow phantom at room and body temperature, at total dissolved air concentrations found in arterial and venous beds and at normotensive and hypertensive pressures. To help bridge the translation to *in vivo* applications, our studies were carried out in porcine plasma and whole blood.

Materials and Methods

Preparation of echogenic liposomes

The protocol of liposome preparation by dessication, rehydration, sonication, freezing and lyophilization is described by Buchanan *et al.* (2008). The molar ratio of lipids was 27:42:8:8:15 of L- α -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-phos-phoethanolamine (DPPE) (Avanti Polar Lipids, Alabaster, AL, USA), and cholesterol (CH) (Sigma Aldrich St. Louis, MO, USA). ELIP were prepared at the University of Texas Health Science Center at Houston in 6 mg vials and shipped with refrigerant packs overnight. Each vial of lyophilized ELIP was reconstituted with 0.6 ml of 0.2 μ m filtered deionized water (NANOPure, Barnstead International, Dubuque, IA, USA) to give a lipid concentration of 10 mg/ml. Reconstituted ELIP were diluted in a biological fluid for each of the following sets of experiments to give a final lipid concentration of 0.05 mg/ml, which has been used in both *in vitro* and *in vivo* studies (Hamilton et al. 2004; Smith et al. 2007).

Physiologic flow phantom

The physiologic flow phantom (Figure 1) consisted of a 250 mL water-jacketed reservoir and 2 mm inner diameter water-jacketed tubing (Radnoti, Monrovia, CA, USA) 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 and the stroke volume was adjusted to obtain the appropriate pulse pressure, for normotensive (40 mmHg) or stage 1 hypertensive (55 mmHg) conditions (Chobanian et al. 2003). An after-load with an adjustable height was used to control the mean hydrodynamic pressure in the system (Figure 2). An inline pressure transducer (PendoTECH, Princeton, NJ, USA) was used to measure the hydrodynamic gauge pressure above atmospheric pressure. 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 water bath (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. This setup allowed the ELIP to be maintained at the desired temperature (22 °C or 37 °C) at all times. 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. The inner diameter and wall thickness of the tubing were selected to match those of small and medium arteries such as the brachial artery in humans (Holubkov et al. 2002; Weidinger et al. 2002) and the abdominal aorta in small animals (Masuo et al. 2002; Korosoglou et al. 2008).

Image acquisition and processing

ELIP in the flow system were imaged with the L12–5 linear array transducer of the Philips HDI 5000 diagnostic ultrasound scanner (Philips, Bothell, WA, USA) which was operated in fundamental B-mode (center frequency of 6.9 MHz). The acoustic output (mechanical index or MI) during image acquisition was 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 runs. 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 post-processed using MATLAB® (Mathworks, Natick, MA, USA). Previous studies of image analysis methods (Smith et al. 2007; Porter et al. 2006) established that mean digital intensity (MDI), unlike mean gray scale value (MGSV), was directly proportional to the backscattered acoustic power and independent of the grayscale color map. A technique developed by Porter *et al.* (Porter et al. 2006) was modified to establish the relationship between the MGSV for the scanner settings used in these experiments and the MDI. 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.* (Porter et al. 2006) defined ROIs within the tissue mimicking speckle (shown as ROI₁ in Figure 3(a)) thus computing MGSV over regions of varying intensity. In the current study, ROIs were defined within the hyperechoic regions created by the wire targets (shown as ROI₂ in Figure 3(a)). These regions exhibited a more uniform intensity. The MGSV and MDI for corresponding ROIs (Figure 3(a)) were computed and a polynomial fit was found (equation 1) as shown in figure 3(b).

$$\text{MDI(dB)} = -0.0011(\text{MGSV})^2 + 0.48(\text{MGSV}) \quad (1)$$

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 3(c). The average MDI after the echogenicity arrived at a steady state was used to compute the MDI of ELIP at 120/80 mmHg.

Effect of plasma dissolved gas concentration on the echogenicity of ELIP

A dissolved oxygen (DO) meter (DO 110, Oakton Instruments, Vernon Hills, IL, USA) was used to measure the oxygen saturation in plasma. Based on the handling of the plasma *in vitro*, this value was used as a measure of total dissolved gas. 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 87–92%, respectively (Altman 1959; Aksnes and Rahn 1957).

The citrated pooled porcine plasma used in these experiments (8304906, Lampire Biological Labs, Pipersville, PA, USA) had $63\% \pm 5.5\%$ saturated dissolved oxygen when thawed from $-80\text{ }^{\circ}\text{C}$ to $22\text{ }^{\circ}\text{C}$. 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 2 atmospheres 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 $37\text{ }^{\circ}\text{C}$ for 60 min to warm the samples to body temperature, while maintaining the same dissolved gas concentration. The measured dissolved oxygen content at $37\text{ }^{\circ}\text{C}$ is shown in Table 1. Reconstituted ELIP were added to each 30 ml plasma aliquot in the water-jacketed reservoir, pumped through the flow system, and imaged 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 echogenicities at pressures above 1 mmHg at each dissolved gas concentration.

Effect of the temperature of transitions in plasma on ELIP echogenicity

Porcine plasma was warmed to room temperature, and the gas saturation increased to $91\% \pm 3.8\%$. Thirty-milliliter samples were aliquoted in syringes. Plasma aliquots were maintained at room temperature or warmed to $37\text{ }^{\circ}\text{C}$ and added to the reservoir of the flow system. Reconstituted ELIP were subsequently added to the plasma. The initial temperature of the plasma (T_p) and the temperature of the flow system (T_s) are shown in Table 2. The difference between the initial temperature of plasma (T_p) and the temperature of the flow system (T_s) is denoted by T in this table. As the ELIP flowed from the reservoir to the imaged section of the tubing (within 20 s), the temperature of the plasma and ELIP transitioned to the inline temperature indicated in the table. 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. Using Microsoft Excel® a two-way ANOVA was performed to determine if there were significant differences ($p < 0.05$) between ELIP in plasma at $22\text{ }^{\circ}\text{C}$ and ELIP in plasma at $37\text{ }^{\circ}\text{C}$ ($T = 0\text{ }^{\circ}\text{C}$) at all 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.

Effect of normotensive and hypertensive pressures on the echogenicity of ELIP

Porcine plasma at room temperature with a dissolved air content of $91.5\% \pm 2.9\%$ was aliquoted into sealed syringes and maintained at $37\text{ }^{\circ}\text{C}$. Plasma aliquots were added into the

37 °C water-jacketed reservoir along with 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 overpressures.

Echogenicity of ELIP in whole blood at multiple hydrodynamic pressures

Porcine whole blood samples with sodium citrate (citrated) or with citrate-phosphate-dextrose (CPD) were shipped overnight from Lampire Biological Labs at 4 °C to the University of Cincinnati. The whole blood samples were gently mixed and brought to room temperature using a laboratory rocker. 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. Dissolved gas levels were not measured in the blood samples. The blood samples were stored and transported at 4 °C, unlike the plasma samples that were frozen and stored at -80 °C. Freezing causes degassing of the fluid (Francis and Pashley 2006). Therefore the pressurization procedure is necessary to increase the dissolved gas levels in plasma samples, but not in blood samples.

Results

Effect of plasma dissolved gas concentration on the echogenicity of ELIP

At arterial and venous dissolved gas concentrations and physiologic hydrodynamic pressure (120/80 mmHg), ELIP remained echogenic (Figure 4a). When dissolved gas concentrations in plasma were well below physiologic levels ($DO \sim 67\%$), the ELIP remained echogenic at 1 mmHg and 60/20 mmHg hydrodynamic pressures but rapidly lost echogenicity (within 25 s) at physiologic (120/80 mmHg) pressure (Figure 4b). All subsequent experiments were done 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.

Effect of the temperature of plasma containing ELIP on echogenicity

Figure 5b shows the effect of temperature transitions on the echogenicity of ELIP in plasma. In 37 °C plasma, without a temperature transition ($T = 0$ °C), ELIP echogenicity was not different at different hydrodynamic pressures. In plasma at 22 °C (both $T = 0$ °C and $T = 15$ °C) ELIP echogenicity at 120/80 mmHg was lower than echogenicity at 1 mmHg ($p = 0.04$ and $p = 0.02$ respectively). ELIP in plasma undergoing a temperature transition of $T = 15$ °C appeared to develop a hyperechoic line of scatterers on the upper wall of the tubing at 120/80 mmHg (Figure 5a). However, good intra-luminal contrast (Shi et al. 2004; Okada et al. 2005) (19.8 ± 0.6 dB) was maintained despite the reduced MDI. When comparing all

three pressures for both $T = 0^{\circ}\text{C}$ (22°C and 37°C) there was no difference in echogenicity ($p=0.056$). A loss of echogenicity was noticed in ELIP at 120/80 mmHg in plasma cooled from 37°C to 22°C ($T = -15^{\circ}\text{C}$) ($p=0.002$). For ELIP in plasma with a -15°C temperature transition ($T = -15^{\circ}\text{C}$), most of the ELIP lost echogenicity within 10 seconds of increasing the overpressure to 120/80 mmHg. Therefore, a decrease in temperature tends to cause liberation of the gas from the ELIP. Isolated acoustic scatterers apparently result from coalescence and aggregation of liberated gas. These large bubbles floated to the upper wall of the tubing.

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 6). However, despite the lowered echogenicity (3 dB), the ELIP provided good intra-luminal contrast relative to just plasma (18.6 ± 1 dB) as a blood pool agent. As well, 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 changes.

Echogenicity of ELIP in whole blood at multiple hydrodynamic pressures

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 7). The complete blood count (CBC) assays of the whole blood indicated abnormal red blood cell (RBC) morphology by the presence of poikilocytes and spherocytes in some samples of citrated whole blood used for ELIP dilution. Citrated whole blood samples containing abnormal RBCs were analyzed as a separate group from citrated whole blood containing normal RBCs. 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 7). These findings indicate that ELIP are echogenic in whole blood at physiologic pressure and body temperature.

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.

Effect of plasma dissolved gas concentration on the echogenicity of ELIP

The main dissolved gases *in vivo* include nitrogen, oxygen and carbon dioxide. Nitrogen and oxygen compose 95% of the total dissolved gases (Altman 1959; Mines 1986). Therefore the

relative partial pressures of nitrogen and oxygen, both inside and outside the ELIP affect in-gassing and out-gassing of the air encapsulated in ELIP. This, in turn, affects the echogenicity of ELIP. 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. While the amount of oxygen bound to hemoglobin is 64 times the amount of oxygen dissolved in plasma, this bound oxygen is unavailable to contribute to in-gassing or out-gassing of microbubbles due to the oxygen dissociation curve (Mines 1986). The oxygen dissolved in plasma is under-saturated (94%) relative to alveolar levels (Altman 1959) due to ventilation perfusion inequalities in the lungs and oxygen consumption in the tissue (Mines 1986).

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, echogenicities of ELIP at arterial and venous dissolved gas levels were not different. Also, Van Liew *et al.* showed that the changes in microbubble radii at the hydrodynamic pressures and dissolved gas concentrations of systemic veins and systemic arteries are not different (Van Liew and Burkard 1995). However, in under-saturated plasma ($DO < 75\%$), our studies showed that overpressure 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 (Sboros *et al.* 2000a; Duncan and Needham 2004). Duncan *et al.* found that distearoyl-phosphocholine (DSPC) lipid-coated air microbubbles dissolved within 20 s in fluids with a DO of 84%. Sboros *et al.* (Sboros *et al.* 2000a; Kwan and Borden 2010) 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 gas filled contrast agents in under-saturated fluids. It is possible that, like the stiff DSPC-shelled air microbubbles, the rigidity provided by DPPC in the lipid shell limits the rapid dissolution of gas from ELIP. 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 need to be carried out in the future to determine whether the stability of ELIP echogenicity is affected.

Effect of the temperature transitions of plasma on echogenicity of ELIP

At body temperature (37 °C), ELIP were echogenic at 1 mmHg, 60/20 mmHg, and 120/80 mmHg. Temperature transitions may occur during clinical applications. For example, ELIP may be prepared at room temperature (22 °C) and thereafter injected into the vascular system at 37 °C. A drop in echogenicity was observed at 120/80 mmHg compared to the echogenicity at 1 mmHg pressure ($T = 15\text{ }^{\circ}\text{C}$). However, even with this decrease in echogenicity, good intra-luminal contrast was maintained ($19.8 \pm 0.6\text{ dB}$) (Shi *et al.* 2004; Okada *et al.* 2005). One likely explanation is that in a temperature transition of $T = 15\text{ }^{\circ}\text{C}$, the gas is less soluble in the surrounding media. As a result, gas 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 edge of

the upper wall of the tubing. The shadowing artifact from the hyperechoic line along the upper wall of the tubing could have lowered the echogenicity measured inside the lumen.

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 of SonoVue® at 40 °C compared to that at 22 °C. This observation was also supported by a higher scattered acoustic signal from SonoVue® at 40 °C which decayed significantly over 10 minutes. 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 ($T = 0$ °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). Further, Buchanan *et al.* (2008) showed that ELIP formulated with highly saturated lipids (DPPC), which were also used in our study, were more robust at body temperature (37 °C) than those without DPPC. They hypothesized that, lipids with longer acyl chains reduced the surface tension and curtailed the diffusion of gas out of the shell.

We observed that ELIP diluted in plasma at 37 °C and subsequently cooled to room temperature, 22 °C ($T = -15$ °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 (Altman 1959). 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 further accelerate the diffusion of gas out of the ELIP by increasing the partial pressures within the ELIP. This result reiterated the need for temperature-controlled water jacketing of the tubing and reservoir to maintain the echogenicity of ELIP. In certain clinical applications, fluids such as saline are warmed to 37 °C prior to intravenous injection (Faries et al. 1991). The importance of this finding is that ELIP premixed in fluid at 37 °C should not be allowed to cool down to room temperature prior to intravenous injection.

Effect of normotensive and hypertensive pressures on the echogenicity of ELIP

There was no difference in the echogenicity of ELIP in stage 1 hypertensive arterial pressures (145/90 mmHg) compared to that at normotensive (120/80 mmHg) pressure. Other studies (Andersen and Jensen 2010a; Frinking et al. 2010) showed that at low acoustic pressures (<500kPa), the fundamental response of UCAs was independent of the overpressure in the ambient fluid. Frinking *et al.* (2010) found that there was no change in the fundamental response of phospholipid coated microbubbles at overpressures up to 120 mmHg. Similarly, Andersen *et al.* (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 investigation are consistent with our observation that there is no difference in echogenicity of ELIP at 120/80 mmHg and 140/95 mmHg as measured using fundamental B-mode imaging.

Echogenicity of ELIP in whole blood

The interaction of liposomes with plasma proteins is well documented. Many studies have elucidated mechanisms of adsorption and intercalation of proteins 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. Note that our ELIP formulation contains cholesterol to hinder plasma protein adsorption. The results of the current study show that ELIP were echogenic in whole blood at physiologic overpressures for 75 s.

Citrate-phosphate-dextrose (CPD) whole blood, unlike sodium citrated whole blood, maintains the pH, osmolality and ATP supply to the RBCs and mimics *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. Intravital images and micro-particle image velocimetry studies indicated the tendency for liposomes in mesenteric arteries to be pushed away from the center of the lumen towards the vessel wall due to interactions with RBCs (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).

Clinically, a contrast enhancement of 8–16 dB above the background is required for unambiguous delineation of myocardial perfusion (Shi et al. 2004). Other applications employing contrast enhanced ultrasound to detect lesions in the liver, require at least a 3 dB enhancement over normal liver parenchyma for visual detection of lesions (Okada et al. 2005). ELIP showed 14–17 dB enhancement above background in CPD whole blood and 10–13 dB enhancement above background in citrated whole blood with normal RBCs after 75 s. However, in citrated blood containing abnormal RBCs, ELIP showed only 5–10 dB enhancement above background after 75 s. 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) similarly observed that abnormal surface morphology of hypoxia-induced sickled RBCs stimulated a greater extent of liposome-RBC adhesion and fusion. Future *in vivo* and

in vitro studies are needed to determine if ELIP can be used to detect poikilocytosis and other abnormal RBC morphologies and pathologies.

There were some limitations noted in this study. The studies focused on short term 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). Dissolved gas levels were not measured in whole blood samples. However, the persistence of ELIP echogenicity in the whole blood samples indicates that the whole blood samples were not degassed. Additional *in vivo* effects also remain to be studied, including filtration due to passage through the lung (Hogg 1987), the dynamic gas exchange that occurs in the lung, and the effect of opsonization and phagocytosis of ELIP. These studies are necessary for all contrast agents as characteristics in one physiological setting may not translate to other settings.

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 will be carried out to evaluate the optimal usage of ELIP as blood pool agents.

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Figure 1.

Schematic of temperature controlled physiologic flow phantom with adjustable pulsatile pressure. Hydrodynamic pressure and temperature in the system were monitored throughout the experiment using inline sensors. L12-5 linear array transducer of the Philips HDI 5000 clinical scanner was used for B-mode imaging.

Figure 2.

Example of measured pressure waveforms in the physiologic flow phantom: hypertensive pressure 145/90 mmHg (— —), normotensive pressure 120/80 mmHg (—), 60/20 mmHg (— · —) and atmospheric pressure 1 mmHg (···).

Figure 3a

Figure 3b

Figure 3c

Figure 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_1) defined within the tissue mimicking speckle was used by Porter *et al.* (Porter et al. 2006). ROI_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.

Figure 4a

Figure 4b

Figure 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).

Figure 5a

Figure 5b

Figure 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 ($p=0.002$ ($\dagger$)). Significant decreases in ELIP echogenicity at 120/80 mmHg were observed at room temperature ($p<0.05$ ($*$)) ($N=5$).

Figure 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).

Figure 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).

Table 1

Dissolved oxygen content in plasma measured at 37 °C. Numbers represent the mean and standard deviation of the dissolved oxygen measurements (N=5).

	Degassed plasma	Plasma simulating arterial dissolved gas content	Plasma simulating venous dissolved gas content
Measured dissolved oxygen saturation at 37 °C	67 ± 5.6%	97.4 ± 1.5%	91.1 ± 3.1%

Table 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).

Temperature of plasma (T_p) °C	Temperature of flow system (T_s) °C	$T = (T_s - T_p)$ °C	Measured inline temperature °C
22	22	0	22 ± 1.2
37	37	0	36 ± 0.2
22	37	15	34.7 ± 1.1
37	22	-15	24.7 ± 0.5