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● *Original Contribution*

LOSS OF ECHOGENICITY AND ONSET OF CAVITATION FROM ECHOGENIC LIPOSOMES: PULSE REPETITION FREQUENCY INDEPENDENCE

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Abstract—Echogenic liposomes (ELIP) are being developed for the early detection and treatment of atherosclerotic lesions. An 80% loss of echogenicity of ELIP has been found to be concomitant with the onset of stable and inertial cavitation. The ultrasound pressure amplitude at which this occurs is weakly dependent on pulse duration. It has been reported that the rapid fragmentation threshold of ELIP (based on changes in echogenicity) is dependent on the insonation pulse repetition frequency (PRF). The study described here evaluates the relationship between loss of echogenicity and cavitation emissions from ELIP insonified by duplex Doppler pulses at four PRFs (1.25, 2.5, 5 and 8.33 kHz). Loss of echogenicity was evaluated on B-mode images of ELIP. Cavitation emissions from ELIP were recorded passively on a focused single-element transducer and a linear array. Emissions recorded by the linear array were beamformed, and the spatial widths of stable and inertial cavitation emissions were compared with the calibrated azimuthal beamwidth of the Doppler pulse exceeding the stable and inertial cavitation thresholds. The inertial cavitation thresholds had a very weak dependence on PRF, and stable cavitation thresholds were independent of PRF. The spatial widths of the cavitation emissions recorded by the passive cavitation imaging system agreed with the calibrated Doppler beamwidths. The results also indicate that 64%–79% loss of echogenicity can be used to classify the presence or absence of cavitation emissions with greater than 80% accuracy. (E-mail: radhakki@mail.uc.edu) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Stable cavitation, Inertial cavitation, Passive cavitation imaging, Doppler ultrasound, Echogenic liposomes.**

INTRODUCTION

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

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

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of UCAs has also been reported to cause cell damage, apoptosis, hemolysis and petechial hemorrhage (Chen et al. 2003; Miller and Dou 2004; Miller et al. 2008; Samuel et al. 2009; Skyba et al. 1998).

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

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

Using echogenicity measurements, Smith et al. (2007) ascertained that the acoustically driven diffusion threshold was weakly dependent on the 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 of UCAs has been investigated in several studies (Chen et al. 2003; Karshafian et al. 2009; Miller and Gies 2000; ter Haar 2002). Other studies have reported conflicting results regarding the lack of PRF dependence for inertial cavitation and cavitation-induced bioeffects (Child et al. 1990; McDannold et al. 2008; Miller and Quddus 2000).

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 affect the loss of echogenicity resulting from rapid destruction of contrast, the cavitation thresholds and the spatial distribution of cavitation emissions from UCAs.

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

METHODS

Preparation of echogenic liposomes

Echogenic liposomes were prepared and shipped overnight on ice packs from the University of Texas Health Science Center, Houston, to the University of Cincinnati. The lipid formulation as described by Buchanan et al. (2008) consisted of L-α-phosphatidylcholine (chicken egg), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-[phosphor-rac-1-glycerol], 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Alabaster, AL, USA) and cholesterol (CH) (Sigma–Aldrich, St. Louis, MO, USA) in the molar ratio 27:42:8:8:15. Each 6-mg ELIP vial was reconstituted with 0.6 mL of 0.2-μm-filtered de-ionized water (NANO-Pure, Barnstead International, Dubuque, IA, USA). ELIP were diluted in porcine plasma (Lampire Biologicals, Pipersville, PA, USA) at 37°C and 93 ± 2% dissolved oxygen to yield a final lipid concentration of 0.05 mg/mL (6.4×10^8 liposomes/mL) (Hamilton et al. 2004; Kopechek et al. 2011; Raymond et al. 2014; Smith et al. 2007).

Experimental setup

The *in vitro* flow system illustrated in Figure 1 was used to determine the cavitation thresholds and loss of echogenicity from ELIP. A peristaltic pump (Mettler Toledo, Columbus, OH, USA) was used to convect the