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● *Original Contribution*

BROADBAND ATTENUATION MEASUREMENTS OF PHOSPHOLIPID-SHELLED ULTRASOUND CONTRAST AGENTS

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Abstract—The aim of this study was to characterize the frequency-dependent acoustic attenuation of three phospholipid-shelled ultrasound contrast agents (UCAs): Definity, MicroMarker and echogenic liposomes. A broadband through-transmission technique allowed for measurement over 2 to 25 MHz with a single pair of transducers. Viscoelastic shell parameters of the UCAs were estimated using a linearized model developed by N. de Jong, L. Hoff, T. Skotland and N. Bom (Ultrasonics 1992; 30:95–103). The effect of diluent on the attenuation of these UCA suspensions was evaluated by performing attenuation measurements in 0.5% (w/v) bovine serum albumin and whole blood. Changes in attenuation and shell parameters of the UCAs were investigated at room temperature (25°C) and physiologic temperature (37°C). The attenuation of the UCAs diluted in 0.5% (w/v) bovine serum albumin was found to be identical to the attenuation of UCAs in whole blood. For each UCA, attenuation was higher at 37°C than at 25°C, underscoring the importance of conducting characterization studies at physiologic temperature. Echogenic liposomes exhibited a larger increase in attenuation at 37°C versus 25°C than either Definity or MicroMarker. (E-mail: raymonjl@mail.uc.edu) **© 2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound contrast agents, Microbubbles, Broadband characterization, Size distribution, Polyvinylidene fluoride transducer, Echogenic liposomes, Definity, MicroMarker.**

INTRODUCTION

The use of microbubbles as ultrasound contrast agents (UCAs) in vascular imaging is well established. Contrast-enhanced ultrasound imaging of arteries has been used as a non-invasive method for screening patients at risk for cardiovascular events, identifying disease progression and monitoring the effectiveness of preventive therapies (Feinstein 2006). Techniques that use UCAs in therapeutic applications, such as drug and gene delivery (Bekeredjian et al. 2005; Laing and McPherson 2009; Sutton et al. 2013), are also under active development. Phospholipid-based UCAs are of particular interest because they can be targeted to molecular components of atherosclerotic disease by attaching specific ligands to their surfaces (Elbayoumi and Torchilin 2008; Klegerman et al. 2010; Klibanov 2006; Kornmann et al. 2010; Lindner 2004; Weissig 2010).

One such UCA under development is echogenic liposomes (ELIP) (Alkan-Onyuksel et al. 1996; Demos et al. 1999; Hitchcock et al. 2010; Huang et al. 2001; Paul et al. 2012). These agents consist of phospholipid vesicles enclosing both an aqueous space and entrapped gas. ELIP are echogenic because of the presence of air, which is entrapped and stabilized by the lipid during the rehydration process (Huang 2010). Previous studies have suggested that the freeze-drying procedure is key to the generation of defects in the lipid bilayers that, on rehydration, fuse and trap small amounts of air (Huang et al. 2001, 2002). ELIP formulations differ from other commercially available contrast agents primarily in size, shell material and gas content. Most commercially available contrast agents have mean diameters between 1 and 5 μm and consist of microbubbles encapsulated by a protein, polymer or lipid shell (Stride 2009). These agents typically contain high-molecular-weight gases, which have low solubility in blood and, thus, increase the lifetime of the microbubbles in circulation (Qin et al. 2009; Sarkar et al. 2009). ELIP have a phospholipid bilayer shell and include a small amount

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of cholesterol, which acts to increase membrane rigidity (Huang et al. 2001). ELIP range in size from ~70 nm to several microns (Kopechek et al. 2011; Paul et al. 2012). ELIP formulations contain air, which is more soluble in blood than high-molecular-weight gases. However, ELIP with optimized lipid formulations have been shown to be echogenic and stable under physiologic conditions for tens of minutes (Buchanan et al. 2008; Radhakrishnan et al. 2012). The exact location of the entrapped air pockets in ELIP has not been fully ascertained, possibly because air pockets are stabilized by lipid monolayers within the liposome or by the lipid bilayer shell (Huang 2008).

Previous acoustic characterization studies by Kopechek et al. (2011) and Paul et al. (2012) revealed that the scattering properties of ELIP are suitable for various ultrasound imaging applications including intravascular ultrasound (20 MHz or higher), as well as fundamental and harmonic imaging (3–12 MHz). In both of these studies, several transducers were used to cover the frequency range for attenuation measurements. Furthermore, both studies were conducted at room temperature. Recent work by Mulvana et al. (2010) indicates that the acoustic characteristics of the phospholipid-based UCA SonoVue are affected by temperature in the range 20°C–40°C for transvascular diagnostic frequencies (1–6 MHz). Vos et al. (2008) also investigated the influence of temperature (22°C vs. 37°C) on ultrasound excitation of SonoVue and Definity using optical techniques. However, the effect of temperature on the acoustic characteristics of phospholipid-based UCAs over both transvascular and intravascular frequencies has not been fully determined.

The objective of the present study was to investigate the shell properties of phospholipid-based UCAs over a broad frequency range at room temperature and also under physiologic conditions. We hypothesize that the temperature dependence noted by Mulvana et al. (2010) will be evident in other phospholipid-based UCAs and may also depend on frequency. These differences may affect the optimal insonation parameters for diagnostic as well as therapeutic applications (Laing and McPherson 2009; Qin et al. 2009). Lipid shells have a wide variety of material properties. Therefore, three formulations of ELIP, as well as two commercially available phospholipid-based UCAs, Definity and MicroMarker, were characterized in this study. Definity is a commercially available UCA that has been approved by the U.S. Food and Drug Administration for left ventricular opacification in patients with sub-optimal echocardiograms (Patil and Main 2012). MicroMarker is a contrast agent developed specifically for pre-clinical high-frequency (>20 MHz) ultrasound imaging (Bracco Research, Geneva, Switzerland). According to the manufacturer, this agent was developed on the same principles as the second-generation clinical contrast agent SonoVue (VisualSonics Rev 1.4). We compared the measured attenuation of the UCAs at 25°C and 37°C. The following sections provide background on the methodology used to assess shell parameters. Finally, the results of attenuation measurements and estimated values for shell parameters are discussed.

METHODS

Agent handling and preparation

Definity. Definity (perflutren lipid microspheres; Lantheus Medical Imaging, North Billerica, MA, USA) consists of octofluoropropane (C_3F_8) microbubbles encapsulated by a lipid shell monolayer composed of three phospholipids. Vials of Definity were activated according to the manufacturer's instructions. Briefly, a vial was removed from refrigeration and allowed to warm to room temperature (20°C–24°C) before activation by shaking for 45 s using a Vial-Mix (Lantheus Medical Imaging). All measurements were performed within 1 h of activation, and the agent was resuspended by hand agitation (inverting the vial) for 10 s before each withdrawal. A 20-gauge needle was used to withdraw the agent from the middle of the vial.

MicroMarker. Vevo MicroMarker (VisualSonics, Toronto, ON, Canada; Bracco Research, Geneva, Switzerland) consists of a mixture of nitrogen and perfluorobutane gas (C_4F_{10}) encapsulated by a monolayer shell composed of polyethylene glycol, phospholipids and fatty acids (VisualSonics PN11691). The agent was prepared according to the manufacturer's directions by injecting 0.7 mL of saline (0.9% w/v) into the vial using a 21-gauge needle. The syringe was detached from the needle, which was left in the vial to vent to atmospheric pressure for a few seconds and then removed. The vial was agitated by hand for 1 min and allowed to rest for 10 min at room temperature before the sample was withdrawn using a 20-gauge needle.

Echogenic liposomes. Three formulations of ELIP, each previously described (Buchanan et al. 2008; Huang et al. 2002; Tiukinhoy-Laing et al. 2007), were evaluated in this study. The original formulation described by Huang et al. (2002) consists of L-α-phosphatidylcholine (EggPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-[1′-rac-glycerol] (DPPG) and cholesterol (CH) in a molar ratio of 69:8:8:15. This formulation, which is referred to as ELIP, resulted in an echogenic dispersion after preparation using a process involving sonication of the lipid in water,