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● *Original Contribution*

NON-LINEAR RESPONSE AND VISCOELASTIC PROPERTIES OF LIPID-COATED MICROBUBBLES: DSPC VERSUS DPPC

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Abstract—For successful *in vivo* contrast-enhanced ultrasound imaging (CEUS) and ultrasound molecular imaging, detailed knowledge of stability and acoustical properties of the microbubbles is essential. Here, we compare these aspects of lipid-coated microbubbles that have either 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as their main lipid; the other components were identical. The microbubbles were investigated *in vitro* over the frequency range 1–4 MHz at pressures between 10 and 100 kPa, and their response to the applied ultrasound was recorded using ultrahigh-speed imaging (15 Mfps). Relative to DPPC-coated microbubbles, DSPC-coated microbubbles had (i) higher acoustical stability; (ii) higher shell elasticity as derived using the Marmottant model (DSPC: 0.26 ± 0.13 N/m, DPPC: 0.06 ± 0.06 N/m); (iii) pressure amplitudes twice as high at the second harmonic frequency; and (iv) a smaller amount of microbubbles that responded at the subharmonic frequency. Because of their higher acoustical stability and higher non-linear response, DSPC-coated microbubbles may be more suitable for contrast-enhanced ultrasound. (E-mail: t.vanrooij@erasmusmc.nl) **© 2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound contrast agent, Lipid-coated microbubbles, High-speed optical imaging, Subharmonic, Second harmonic, Non-linear, Elasticity, Viscosity, Stability.**

INTRODUCTION

Ultrasound contrast agents (UCAs) consist of gas-filled coated microbubbles with diameters between 1 and 10 μm. Clinically, they have been used for several decades for contrast-enhanced ultrasound (CEUS) imaging (Cosgrove and Harvey 2009; Feinstein et al. 2010; Kaul 2008). More recent studies have documented their potential for local drug delivery and ultrasound molecular imaging (Deshpande et al. 2010; Lentacker et al. 2014; Lindner 2010).

To increase their lifetime after injection into the bloodstream, the microbubbles are stabilized with a coating. Three lipid-coated UCAs are approved for diagnostic CEUS: Definity (Lantheus Medical Imaging, North Billerica, MA, USA), Sonazoid (Daiichi Sankyo, GE Healthcare, Tokyo, Japan) and SonoVue (Bracco Imaging, Milan, Italy). A complete and updated list of approving agencies can be found elsewhere (International Contrast Ultrasound Society [ICUS] 2014). The responses to ultrasound of Definity and SonoVue have been thoroughly characterized (Chetty et al. 2008; Faez et al. 2011b; Gorce et al. 2000; Helfield and Goertz 2013), as have the responses of several lipid-coated microbubbles for research purposes (Borden and Longo 2002; Borden et al. 2005; Chomas et al. 2002; van der Meer et al. 2007). Although all of these microbubble types are coated with a combination of lipids, it is unclear how the different lipids affect their stability and their response to ultrasound. The hydrophobic chain length of the phospholipids incorporated into the microbubble shell was found to influence the dissolution of the microbubbles; passive dissolution rates decreased for chains from 16 carbon (C) atoms (DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) to 22 C atoms

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(DBPC, 1,2-dibehenoyl-*sn*-glycero-3-phosphocholine), but increased again for chains of 24 C atoms (Borden and Longo 2002; Garg et al. 2013). The influence of chain length on ultrasound-driven dissolution (*i.e.,* acoustical stability) was different from that of passive dissolution. When exposed to ultrasound, microbubbles that had lipids with 16 C atoms incorporated into their shell were still least stable, whereas those with lipids having 18 C atoms (DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine) were as stable as the microbubbles having lipids with chain lengths of 22 C atoms. For even longer chains (24 C atoms), acoustical stability decreased again (Garg et al. 2013). Next to dissolution or stability, other properties of microbubbles with different lipid coatings have been studied. Helfield et al. (2012) varied the shell microstructure using different cooling rates and observed a change in subharmonic response, but without a clear relation to the shell microstructure. Shell viscosity was found to depend on the coating composition and manufacturing method, and viscosity was lower when higher concentrations of emulsifier were used (Hosny et al. 2013). Wang and Yeh (2013) explained this by the increased movements of lipids caused by the emulsifier. The bulk linear properties of monodisperse bubbles produced using flow-focusing microfluidic devices were reported to depend on both the radius and the acoustic pressure (Gong et al. 2014; Parrales et al. 2014). However, none of these studies investigated the properties of one specific lipid on the acoustic response of the microbubbles. Understanding the influence of this replacement on microbubbles' acoustical stability and their response to ultrasound may aid the design of circulating microbubbles for CEUS and of targeted microbubbles for local drug delivery and ultrasound molecular imaging applications.

Super-resolution microscopy was used before to study the distribution of lipids in the coating of two types of microbubbles, after changing only the main shell component (Kooiman et al. 2014a). That particular study chose to use DPPC (C16:0), which is the main coating component for Definity (Lantheus Medical Imaging 2011), or DSPC (C18:0), which is the main constituent of the coating of SonoVue (Schneider et al. 1995), and the experimental agent BR14 (Krause 2002; Schneider et al. 1997). The microbubbles with DSPC as the main lipid had a heterogeneous lipid distribution throughout the shell, whereas the DPPC microbubbles had a more homogeneous lipid distribution. Similar results were also reported for mixtures of two of three of the components (Borden et al. 2006; Kim et al. 2003; Lozano and Longo 2009a, 2009b) that were used for the microbubble coating in the study by Kooiman et al. (2014a).

In the present study we used the Brandaris 128 ultrahigh-speed camera (Chin et al. 2003) to compare the responses to ultrasound of microbubbles with either DSPC or DPPC as the main lipid in the coating. More specifically, we focused on their acoustical stability, their non-linear responses at the subharmonic and second harmonic frequencies and their shell elasticity and viscosity.

METHODS

Microbubble preparation

Biotinylated lipid-coated microbubbles with a C_4F_{10} gas core (F2 Chemicals, Preston, UK) were made by sonication for 10 s as described previously (Klibanov et al. 2004; Kooiman et al. 2014a). The lipid-coating was composed of 59.4 mol% DSPC (P6517, Sigma-Aldrich, Zwijndrecht, Netherlands) or DPPC (850355, Avanti Polar Lipids, Alabaster, AL, USA); 35.7 mol% polyoxyethylene-40-stearate (PEG40 stearate) (P3440, Sigma-Aldrich); 4.1 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol) 2000 (DSPE-PEG2000) (880125, Avanti Polar Lipids); and 0.8 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG2000-biotin) (880129, Avanti Polar Lipids). The coating components were dissolved in phosphate-buffered saline (PBS), and the final concentrations were 2.5 mg/mL DSPC or DPPC, 0.625 mg/mL PEG40 stearate, 0.625 mg/mL DSPE-PEG2000 and 0.125 mg/mL DSPE-PEG2000-biotin.

Microbubble spectroscopy

The acoustical behavior of the microbubbles was studied using the Brandaris 128 ultrahigh-speed camera (Chin et al. 2003) operated in ROI mode (Gelderblom et al. 2012) at a frame rate of ~15 million frames per second. The camera was connected to a microscope (BX-FM, Olympus, Tokyo, Japan) with a 40× water-immersion objective (Olympus) and a 2× magnifier (U-CA, Olympus). We used the microbubble spectroscopy technique to characterize single microbubbles as described previously (Luan et al. 2012; van der Meer et al. 2007). An OptiCell was incubated for 1 h with 10 mL 1 vol% bovine serum albumin in PBS to prevent unspecific binding. After washing with PBS (3×), we added 10 mL of PBS and 3 μL of microbubble suspension to the OptiCell, so the concentration was ~1× 10^5 microbubbles/mL as measured with a Multisizer 3 Coulter counter (n = 3, Beckman Coulter, Mijdrecht, Netherlands).

The ultrasound signal was a 10-cycle Gaussian tapered sine wave burst generated by a Tabor 8026 arbitrary waveform generator (AWG, Tabor Electronics, Tel Hanan, Israel). This signal was then attenuated by a 20-dB attenuator (Mini-Circuits, Brooklyn, NY, USA), amplified by a broadband amplifier (ENI A-500,