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● *Original Contribution*

CHARACTERISATION OF LIPOSOME-LOADED MICROBUBBLE POPULATIONS FOR SUBHARMONIC IMAGING

James R. McLaughlan,*† Sevan Harput,* Radwa H. Abou-Saleh,‡§ Sally A. Peyman,‡ Stephen Evans,‡ and Steven Freear*

*School of Electronic and Electrical Engineering, University of Leeds, Leeds, UK; †Division of Biomedical Imaging, University of Leeds, Leeds, UK; ‡School of Physics and Astronomy, University of Leeds, Leeds, UK; and §Department of Physics, Faculty of Science, Mansoura University, Mansoura City, Egypt

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Abstract—Therapeutic microbubbles could make an important contribution to the diagnosis and treatment of cancer. Acoustic characterisation was performed on microfluidic generated microbubble populations that either were bare or had liposomes attached. Through the use of broadband attenuation techniques (3–8 MHz), the shell stiffness was measured to be 0.72 ± 0.01 and 0.78 ± 0.05 N/m and shell friction was 0.37 ± 0.05 and 0.74 ± 0.05 × 10^{-6} kg/s for bare and liposome-loaded microbubbles, respectively. Acoustic scatter revealed that liposome-loaded microbubbles had a lower subharmonic threshold, occurring from a peak negative pressure of 50 kPa, compared with 200 kPa for equivalent bare microbubbles. It was found that liposome loading had a negligible effect on the destruction threshold for this microbubble type, because at a mechanical index >0.4 (570 kPa), 80% of both populations were destroyed. (E-mail: j.r.mclaughlan@leeds.ac.uk)

Key Words: **Ultrasound contrast agents, Drug delivery, Subharmonic imaging, Microfluidics, Liposomes, Microbubbles.**

INTRODUCTION

Phospholipid-encapsulated microbubbles are routinely used as contrast agents for diagnostic ultrasound imaging because of their acoustic impedance mismatch with blood and their highly compressible nature in response to an ultrasound field (Claudon et al. 2013; Cosgrove 2006; Forsberg et al. 1998). Maximum scatter of an ultrasound wave by microbubbles occurs when the frequency of these waves is equal to the resonance frequency of the microbubbles. The size of encapsulated microbubbles is a key factor in their resonant frequency, and most commercial contrast agents (1–10 μm) have resonances within the range of frequencies used for diagnostic ultrasound imaging (Stride and Saffari 2003).

Microbubbles can undergo both linear and non-linear oscillations depending on the amplitude of the applied acoustic field (Emmer et al. 2007). Contrast imaging uses non-linear microbubble behaviour for a number of imaging techniques, such as pulse inversion, harmonic imaging and power modulation (Burns et al. 1994; Schrope and Newhouse 1993; Simpson et al. 1999). Coded excitation, such as chirps, are techniques used to increase the signal-to-noise ratio (SNR) for ultrasound imaging (Misaridis and Jensen 2005) by increasing the transited energy without decreasing the axial resolution or increasing the acoustic pressure. Longer-duration exposures can be used to increase the non-linear behaviour of microbubbles (Zhang et al. 2007), which can improve the contrast-to-tissue ratio (CTR) for contrast imaging (Harput et al. 2013; Sun et al. 2007). The response of a microbubble to an acoustic field depends on a number of factors, such as the frequency of excitation, microbubble size and shell composition (Sun et al. 2014). Generally, for low-amplitude excitation, microbubbles will oscillate linearly around their equilibrium radius, where the frequency content of the backscattered signal would be determined by the excitation waveform. Increasing the amplitude of excitation can result in non-linear oscillations of the

Address correspondence to: James R. McLaughlan, Electronic and Electrical Engineering, University of Leeds, Leeds, LS2 9JT, UK. E-mail: j.r.mclaughlan@leeds.ac.uk

microbubble, which can generate harmonics of the fundamental drive frequency (f_0) (de Jong et al. 2002). Superharmonics (nf_0) and ultraharmonics ($nf_0/2$) can be generated by non-linear oscillations, which are used to enhance ultrasound contrast imaging (de Jong et al. 2009; Maresca et al. 2013). Second harmonic ($2f_0$) emissions from microbubbles have been reported to improve the resolution of contrast-enhanced ultrasound imaging (Forsberg et al. 1997). Nevertheless, because of non-linear propagation of the ultrasound wave (Leighton 2007), a second harmonic component can be generated in tissue, which can also be used for imaging (Tranquart et al. 1999), but can reduce the performance of contrast imaging (Goertz et al. 2005; Tang et al. 2010; Yildiz et al. 2015). As subharmonic ($f_0/2$) emissions are unique to microbubble activity, they can be used to improve the CTR (Goertz et al. 2007; Shankar et al. 1998). However, it has been found that the CTR can be reduced in the region beyond a microbubble population because of the generation of the non-linear harmonics by the microbubbles that then propagate into the tissue (Tang et al. 2010). At higher acoustic amplitudes, other microbubble phenomena may occur, such as surface mode oscillations (Dollet et al. 2008) and lipid shedding. The latter could have implications for drug delivery using microbubbles (Borden et al. 2005). Further increases in the acoustic amplitude can result in the rapid expansion and collapse associated with inertial cavitation (Neppiras 1980; Prentice et al. 2005) and the destruction of the microbubble. In addition to the generation of non-linear harmonics, high-amplitude excitation can result in the generation of broadband emissions that can also be used for ultrasound contrast imaging (Gessner et al. 2010; Kruse and Ferrara 2005).

The Rayleigh–Plesset–Noltingk–Neppiras–Poritsky (RPNNP) equation is commonly used to simulate the dynamics of a free gas bubble in a liquid medium (Neppiras and Noltingk 1951; Noltingk and Neppiras 1950; Plesset 1949; Poritsky 1951; Rayleigh 1917). This equation, including its limitations (Leighton 1994), forms the basis for most theoretical models of encapsulated microbubbles (Doinikov and Bouakaz 2011). A commonly used modification of this equation for phospholipid-shelled microbubbles is the Marmottant model (Marmottant et al. 2005). This model introduces a term for effective surface tension that is dependent on the instantaneous microbubble radius, which results in three regimes for shell motion: buckled, elastic and ruptured. A consequence of this modification is the ability to then simulate large-amplitude oscillations, which can lead to non-linear behaviour in the microbubble. Such an effect has been predicted by this model and observed using high-speed imaging (de Jong et al. 2007); it is "compression-only" behaviour, which is when a microbubble, typically in a buckled state, undergoes compression in response to an ultrasound field, but very limited expansion. These non-linear oscillations can give rise to harmonic emissions from microbubbles at low acoustic pressures (Sijl et al. 2011). Subharmonic emissions generated by non-linear oscillations of phospholipid-encapsulated microbubbles are used for *in vivo* and clinical applications of diagnostic ultrasound imaging (Eisenbrey et al. 2015). These emissions required a threshold acoustic pressure to be exceeded to be generated, which is true for both free gas bubbles (Prosperetti 1976) and coated microbubbles (Sijl et al. 2010). Prosperetti described a general derivation for the acoustic pressure thresholds required for subharmonic components to be present in the acoustic emissions generated from acoustically driven gas bubbles, applied to coated bubbles (Prosperetti 2013). In this derivation it is noted that the subharmonic threshold can be lowered when compared with that of an uncoated bubble, because of the presence of discontinuities or near discontinuities in the shell of the microbubble, such as buckling.

The therapeutic application of ultrasound for cancer therapy has been widely investigated (Wood and Sehgal 2015), and the use of therapeutic microbubbles that can be targeted to specific cancerous cells holds particular promise for diagnosis and therapy (Klibanov and Hossack 2015). A common approach for the loading of therapeutics onto a microbubble is through the attachment of drug-filled liposomes to the shell of a microbubble (Geers et al. 2011; Kheirolomoom et al. 2007; Lentacker et al. 2010; Peyman et al. 2012). The ultrasonic release of a therapeutic payload from a microbubble can be achieved through its destruction (Christiansen et al. 2003; Ferrara et al. 2007; Korpanty et al. 2005; Lindner 2004; Mayer et al. 2008; Schlegel et al. 2016; Zhu et al. 2015) or through a controlled release mechanism such as phospholipid shedding (Luan et al. 2014). Nevertheless, should the microbubbles or free gas bubbles (generated from microbubble rupture) (de Jong et al. 2002; Postema et al. 2005) emit and/or re-radiate acoustic pressure in response to being driven by an acoustic field during this process, this could help increase target cell permeability through sonoporation, improving the therapeutic outcome (Delalande et al. 2013; Greenleaf et al. 1998; Kooiman et al. 2014; McLaughlan et al. 2013).

The aim of this study was to investigate the effect of liposome loading on the acoustic response of microbubble populations that were produced using a microfluidic manufacturing process (Peyman et al. 2012). The microbubble shell parameters, acoustic response and destruction thresholds were measured. In this article, the term *destruction* refers to the fragmentation of the microbubble and dissolution of the gas core (Christiansen et al. 2003; Ferrara et al. 2007).