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Short Communication

# Scaling of the viscoelastic shell properties of phospholipid encapsulated microbubbles with ultrasound frequency

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ABSTRACT

Phospholipid encapsulated microbubbles are widely employed as clinical diagnostic ultrasound contrast agents in the 1–5 MHz range, and are increasingly employed at higher ultrasound transmit frequencies. The stiffness and viscosity of the encapsulating "shells" have been shown to play a central role in determining both the linear and nonlinear response of microbubbles to ultrasound. At lower frequencies, recent studies have suggested that shell properties can be frequency dependent. At present, there is only limited knowledge of how the viscoelastic properties of phospholipid shells scale at higher frequencies. In this study, four batches of in-house phospholipid encapsulated microbubbles were fabricated with decreasing volume-weighted mean diameters of 3.20, 2.07, 1.82 and 1.61 μm. Attenuation experiments were conducted in order to assess the frequency-dependent response of each batch, resulting in resonant peaks in response at 4.2, 8.9, 12.6 and 19.5 MHz, respectively. With knowledge of the size measurements, the attenuation spectra were then fitted with a standard linearized bubble model in order to estimate the microbubble shell stiffness $S_p$ and shell viscosity $S_f$, resulting in a slight increase in $S_p$ (1.53–1.76 N/m) and a substantial decrease in $S_f$ ($0.29 \times 10^{-6}$–$0.08 \times 10^{-6}$ kg/s) with increasing frequency. These results performed on a single phospholipid agent show that frequency dependent shell properties persist at high frequencies (up to 19.5 MHz).



## 1. Introduction

Ultrasound contrast agents consist of encapsulated, gas-filled microbubbles that typically range from 1–10 μm in diameter. Systemically circulating microbubbles enhance the echogenicity of blood, and by exploiting their nonlinear response to ultrasound they enable the detection of microvascular perfusion. Contrast-enhanced ultrasound imaging is currently widely employed in the 1–5 MHz range for applications in the heart [1], kidney [2], and liver [3]. Contrast imaging in the 5–15 MHz range is of increasing interest for more superficial targets such as the carotid artery [4], and at frequencies above 15 MHz for applications including intravascular ultrasound [5], ocular [6] and preclinical small animal imaging [7].

There has been considerable research conducted over the past two decades directed toward the understanding of microbubble behavior at ultrasound frequencies in the 1–5 MHz range. This work has resulted in a wealth of information that has enabled the development of improved microbubble detection methods and has laid the foundation for a more quantitative interpretation of contrast images. For example, it is now well recognized that the viscoelastic properties of the encapsulating shell, the shell stiffness and friction, play a central role in influencing both linear and nonlinear microbubble behavior. While at an earlier stage of development, there has also been significant progress recently in understanding the behavior of microbubbles at ultrasound frequencies of 10 MHz and beyond. The large majority of this research has been conducted with phospholipid encapsulated agents, which have been shown to have a substantial capacity to generate acoustic emissions at higher ultrasound frequencies [8]. Both linear and nonlinear scattering and imaging have been demonstrated with a range of phospholipid agents such as Definity™ [9,10], MicroMarker™ [11,12], Targestar™ [13,14], as well as with "in-house" formulations [15,16].

Aside from imaging studies, there has also been recent work into the investigation of the basic mechanisms responsible for nonlinear scattering at higher frequencies. It has been observed that nonlinear scattering is associated with "smaller" bubbles [17–19] and that the nature of nonlinear oscillations appears to transition from compression dominated to expansion dominated as frequencies shift above 20 MHz [20]. It has also been found that the shell

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friction term appears to be lower for smaller bubbles at higher frequencies, which has been suggested to be a key factor in enabling sufficiently high bubble oscillation amplitudes to enable entry into nonlinear dynamic modes. This work was conducted with decanted populations of Definity™ bubbles in the 7–15 MHz and 12–28 MHz frequency range using attenuation measurements and a linearized bubble model [21,22]. Indeed, this finding appears to be consistent with experimental observations of individual microbubbles at frequencies below 10 MHz [23,24], which indicate that shell friction (viscosity) reduces with increasing frequency, possibly due to a shear thinning behavior of phospholipids. Due to the significance of the implications of scaling bubble shell properties with increasing frequency, it is of interest to conduct such studies on other phospholipid agents and ideally across a wide range of frequencies with the same type of phospholipid formulation.

In this study, an in-house phospholipid encapsulated agent is employed to gain insight into the frequency dependence of microbubble shell properties. Size-manipulation is conducted via differential centrifugation techniques in order to produce four distinct batches with relatively narrow size distributions and distinctly different mean bubble sizes. Attenuation experiments are then performed on each batch to obtain the frequency-dependent response of the microbubble population, and in this manner microbubble shell properties can be subsequently estimated. The purpose of this work is twofold. Firstly, it is to obtain shell property estimates at higher ultrasound frequencies for a phospholipid encapsulated agent other than Definity™, in this case with an in-house formulation that has been previously examined for use at higher ultrasound frequencies [16]. Secondly, it is to ascertain the relationship between shell properties and frequency for a single phospholipid agent. This is facilitated by the relatively narrow size distributions in each batch, which reduces the extent to which shell property averaging will occur relative to more poly-disperse populations (e.g. Definity™).

## 2. Materials and methods

### 2.1. Agent preparation

Microbubbles were prepared in-house based on previously described in-house procedures [16,25]. DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids) and Polyoxyethylene-40 stearate (PEG40S; Sigma–Aldrich) were combined at a molar ratio of 9:1 and diluted in phosphate-buffered saline (PBS) at a ratio of 2%. The lipid mixture was first sonicated at low power (7 min and 15% amplitude; Branson Digital Sonifier™) in order to mix the solution and heat the pre-microbubble suspension above the main phase transition temperature of the phospholipid (~55 °C for DSPC). Perfluorobutane gas was then introduced, after which a high power sonication (100% amplitude; 400 W) was applied to the suspension for about 6 s at the gas–liquid surface. This procedure resulted in a poly-disperse microbubble suspension from which "batches" of more narrowly distributed subpopulations were subsequently derived. Size isolation of such subpopulations was achieved using a differential centrifugation technique [26]. Following the microbubble generation process, 10 ml of agent was collected in a 20 ml syringe (length = 3.5 cm) for subsequent centrifugation. In order to obtain microbubble populations with different size distributions, the centrifugation spin speed and duration were estimated based on bubble migration in a centrifugal field. By first measuring the initial bubble number density and gas volume fraction of the native phospholipid agent, calculations similar to those performed in Ref. [26] were conducted, and through an iterative process the speeds and durations that resulted in the desired size distributions were assessed (Table 1). For each batch, two centrifugation cycles were conducted (e.g. at 55 g for 1 min and 92 g for 1 min) to provide an approximate bubble size range, first removing bubbles that are larger than a specific bubble size (e.g. 4 μm), then removing bubbles under a desired size threshold (e.g. 2 μm). After each cycle, the desired solution (which is either the supernatant or the microbubble 'cake' depending on the isolation criteria) was re-diluted in PBS and placed in a new 20 ml syringe. This process was repeated 3–5 times in order to preferentially isolate discernible size distributions while maintaining high microbubble concentrations. Finally, a centrifuge cycle at 500 g for 10 min was performed to remove excess lipids from the suspension solution.

### 2.2. Attenuation and size distribution measurements

The frequency dependent attenuation properties of diluted suspensions of the batches were measured using a narrowband pulse-echo approach, similar to the methodology employed in Refs. [22,27]. In order to cover a wide frequency range (1.5–27 MHz), two transducers (model #595396, 5 MHz, 76 mm focus, 12.7 mm diameter, Olympus NDT Canada Inc., Quebec, Canada; model #ISO2002HR, 20 MHz, 38 mm focus, 6.35 mm diameter, Valpey–Fisher, Hopkinton, MA, USA) were employed in this study. Attenuation experiments were conducted within a de-ionized and degassed water bath. The experimental apparatus consisted of the two transducers focused upon aluminum reflectors, and a sample chamber with a mylar window containing diluted agent was placed within the transducer beam path (Fig. 1). The sample chamber was placed on a magnetic stirrer in order to ensure a fresh population of microbubbles upon each insonication. An arbitrary waveform generator (model AWG5002C, Tektronix, Beaverton, OR, USA) was used to generate low amplitude, narrowband pulses with center frequencies spaced at 0.5 MHz intervals (20 cycles for the 5 MHz transducer and 40 cycles for the 20 MHz transducer) in order to extend the effective frequency range of each transducer. The inter-pulse spacing was 10 ms, and this sequence was repeated at a rate of ten times per minute. These signals were then amplified by 53 dB (model A-150, ENI, Rochester, NY, USA) and sent to one of the two transducers. The received echoes were amplified by 35 dB (model AU1583, Miteq, Hauppauge, NY, USA), band-pass filtered and then digitized (400 MHz sampling frequency, Agilent Technologies Inc., Palo Alto, CA, USA) for offline analysis. In order to minimize potential nonlinear effects, the peak negative pressure at the focus for all waveforms was limited to 25 kPa, as calibrated with a 0.075 mm diameter needle tip hydrophone (model 1544, Precision Acoustics, Dorchester, UK). Agent was diluted in gas-equilibrated saline (0.9% w/v NaCl), and the average dilution ratios employed for the attenuation experiments differed for each batch, resulting in 1:15,000, 1:6667, 1:4800 and

**Table 1**
Size characteristics of the four batches of in-house phospholipid agent. Note that the batches were differentially centrifuged in order to exhibit decreasing mean bubble diameters. Mean and standard deviations are displayed.

| Batch number | Centrifuge regimen | Num. weighted mean diameter (μm) | Vol. weighted mean diameter (μm) | Num. per ml in vial ($\times 10^9$) |
|---|---|---|---|---|
| 1 | 55 g – 1 min and 92 g – 1 min | 2.31 ± 0.05 | 3.20 ± 0.10 | 1.69 ± 0.10 |
| 2 | 125 g – 1 min and 280 g – 1 min | 1.52 ± 0.03 | 2.07 ± 0.11 | 1.62 ± 0.62 |
| 3 | 180 g – 1 min and 250 g – 2 min | 1.35 ± 0.04 | 1.82 ± 0.14 | 2.49 ± 0.53 |
| 4 | 300 g – 1 min and 400 g – 2 min | 1.02 ± 0.02 | 1.61 ± 0.23 | 0.74 ± 0.33 |