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IN VITRO CHARACTERIZATION OF LIPOSOMES AND OPTISON® BY ACOUSTIC SCATTERING AT 3.5 MHZ

Constantin-C. Coussios*, Christy K. Holland*, Ludwika Jakubowska*, Shao-Ling Huang†, Robert C. Macdonald†, Ashwin Nagaraj‡, and David D. McPherson‡

*Department of Biomedical Engineering, University of Cincinnati, Cincinnati, OH, USA

†Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL, USA

‡Department of Internal Medicine and the Feinberg Cardiovascular Research Institute, Northwestern University, Chicago, IL

Abstract

Liposomes are phospholipid vesicles that can encapsulate both gas and fluid. With antibody conjugation, new formulations, known as immunoliposomes, can be targeted to atheroma and other pathologic components and are, thus, being developed as novel diagnostic ultrasound (US) echo contrast agents to enhance atherosclerosis imaging. The majority of these echogenic liposomes range in diameter from 0.25 to 5.0 μm . To quantify the echogenicity of liposome suspensions of varying concentrations, the backscattering coefficient at 3.5 MHz was determined experimentally. The backscattering coefficient was also estimated theoretically as a function of air volume fraction by modeling the encapsulated air as a free air bubble and assuming single bubble scattering. For most of the liposome concentrations examined in this study (on the order of $10^8/\text{mL}$), the backscattering coefficient equals or exceeds that of Optison® at the human clinical dosage (on the order of $10^4/\text{mL}$). Experimental measurement of the decrease in backscattering coefficient shows promise as a sensitive method for determining whether liposomes are left intact or destroyed during imaging; thus, helping to explore their potential as a vehicle for targeted drug delivery. In addition, the attenuation of US through liposome suspensions is negligible at 3.5 MHz relative to the attenuation through Optison® (0.25 dB/cm), suggesting that liposomes have a much higher scatter-to-attenuation ratio and could be more efficient as contrast agents.

Keywords

Liposome; Optison®; Contrast agent; Backscattering coefficient; Acoustic scattering; Attenuation

Address correspondence to: Dr. Constantin-C. Coussios, Department of Engineering Science, University of Oxford, Parks Road, Oxford OX1 3PJ, U.K. constantin.coussios@eng.ox.ac.uk.
Dr. Coussios' current address is Department of Engineering Science, University of Oxford, Parks Road, Oxford OX1 3PJ, U.K.

INTRODUCTION

To evaluate the progression of atherosclerosis, accurate methods are necessary to identify and quantitate the extent, components and morphology of atheroma. Calcium, fibrous tissue, and fatty tissue can be separated and identified (Ng et al. 1993; Landini et al. 1986; Barzilai et al. 1987; Picano et al. 1988; Jones et al. 1989) using ultrasound (US) imaging techniques developed for plaque component characterization. Liposomes, or phospholipid bilayer vesicles enclosing gas and fluid, are novel agents that may permit evaluation of vasoactive and pathologic endothelium. Since first examined by Bangham et al. (1965), liposomes have been developed for a wide range of technical and medical applications. Echogenic liposomal dispersions can be prepared by dispersing a lipid in water, adding mannitol, and freezing and drying, or lyophilizing (Alkan-Onyuksel et al. 1996a, 1996b; Huang et al. 2001). The echogenicity of these preparations is due to the presence of gas, which is entrapped and stabilized by the lipid during the rehydration process following lyophilization (Huang et al. 2002).

Liposomes have been evaluated as drug delivery systems (Alkan-Onyuksel and Son 1992; Sejourne et al. 1997; Betageri et al. 1993) and as diagnostic contrast agents (Alkan-Onyuksel et al. 1996a, 1996b). Echogenic immunoliposomes (ELIP) are nontoxic and can carry either water-soluble compounds in the aqueous compartment or insoluble compounds within the phospholipid bilayers (both drugs and genes) (Tiukinhoy et al. 2000, 2002). Recently, these targeted ELIPs have been found to enhance left ventricular thrombus with transvascular US after IV injection (Hamilton et al. 2002a).

The objective of this study was to provide a quantitative assessment of US scattering from ELIP at 3.5 MHz relative to scattering from a US Food and Drug Administration (FDA)-approved contrast agent (Optison®). A center frequency of 3.5 MHz was chosen because it is commonly utilized in clinical diagnostic echocardiography. However, further studies are needed to elucidate the scattering properties of the liposomes over a broader frequency range. To obtain experimental results that are independent of the transducer, system and insonifying pulse characteristics, the backscattering coefficient (BSC) was used as a measure of echogenicity. This technique was originally developed by Sigelmann and Reid (1973) and first applied to the characterization of a contrast agent (Albunex®) by Bleeker et al. (1990). A significant advantage of using the BSC is that experimental results can be readily compared to theoretical estimates of the scattering cross-section. A theoretical model for scattering by Optison® and liposome suspensions is first described, yielding predictions of the BSC as a function of particle radius and concentration, and liposomal gas volume fraction. An experimental technique permitting accurate experimental measurements of the BSC and attenuation from contrast agent suspensions is also presented, and results are compared with the proposed theoretical models. Finally, the effectiveness of the ELIP as a contrast agent at 3.5 MHz, and its potential as a vehicle for targeted drug delivery are discussed.

Theory

US scattering from albumin-stabilized microbubbles, such as Albunex® or Optison®, was studied theoretically by Church (1995). These microspheres consist of a single gas bubble

encapsulated by a 15 nm-thick albumin shell and have mean radii in the range of 1.00 to 2.25 μm , with 93% less than 5 μm (Optison[®] product insert, Mallinckrodt Medical, St. Louis, MO). By modeling the shell as a continuous surface layer of incompressible solid elastic material and by accounting for damping provided by the shell viscosity, Church was able to predict that the surface layer supports a strain that counters the Laplace pressure and, thereby, stabilizes the bubble against dissolution. The elasticity of the shell was shown to increase the resonance frequency of encapsulated bubbles relative to free air bubbles of equivalent size. Church also demonstrated that the viscosity of the shell produces a notable decrease in the pulsation amplitude of encapsulated bubbles, causing a significant decrease in their total scattering cross-section relative to free air bubbles (Fig. 1). Furthermore, he indicated that damping provided by the viscosity of the shell dominates thermal effects for bubble radii of less than 10 μm . Perhaps counter-intuitively, Church reached the conclusion that the attenuation coefficient through a suspension of shelled bubbles was lower than for free air bubbles throughout the frequency range, even though he assumed a driving frequency well below resonance to reach this result.

The liposomes in the present study differ substantially from the albumin-stabilized microbubbles studied by Church. They are smaller in size, with 60% reported to have a radius smaller than 0.5 μm , 25% in the range of 0.5 to 1.5 μm , 10% in the range of 1.5 to 2.5 μm and 5% in excess of 2.5 μm (Huang et al. 2002). Although little is known of the internal structure of echogenic liposomes, it is speculated that gas entrapped either between or within the two phospholipid monolayers during lyophilization of the liposomes is primarily responsible for their echogenicity (Huang et al. 2002).

The mechanical properties of phospholipid bilayers have been extensively studied by Evans and Skalak (1980). Phospholipid bilayers with unsaturated fatty acyl chains are less rigid (Hianik et al. 1998) and less viscous than albumin (protein) shells (Evans and Hochmuth 1978). In accordance with the conclusions drawn by Church (1995), it is unlikely that the high elasticity and low viscosity of the phospholipid bilayer would play a significant role in the acoustic response of the liposomes. Furthermore, the size and distribution of the gas pockets within each liposome have yet to be determined accurately. For the purpose of a first estimate, it was assumed that the gas entrapped within each liposome behaves as a single spherical free air bubble with a volume in the range of 5 to 100% of the ELIP volume. The shape of the gas pockets within the liposome is not known, but the acoustic response of single bubbles is known to depend primarily on bubble volume, not bubble shape (Strasberg 1953). The resonant frequency, ω_0 , of the gas bubble contained in the liposome can be evaluated using the linear oscillator model and including the effect of surface tension (Devin 1959; Apfel 1981). The scattering crosssection for an individual liposome, σ_s , is defined as the acoustic power scattered in all directions per unit incident intensity and can be estimated as (Hoff 2001):

$$\sigma_s(a, \omega) = 4\pi a^2 \frac{\Omega^4}{(1 - \Omega^2)^2 + (\Omega\delta)^2}, \quad \Omega = \frac{\omega}{\omega_0}, \quad (1)$$

where α is the gas bubble radius, ω the angular frequency and δ the damping constant as defined by Church (1995). The scattering cross-section at 3.5 MHz is plotted as a function of liposome diameter and gas volume fraction in Fig. 2. The choice of gas volume fraction can cause as much as three orders of magnitude change in scattering cross-section for a given liposome radius below resonance, but the difference is less notable above resonance.

The issue of whether collections of liposomes can be treated as individual scatterers must also be addressed. The validity criterion for single scattering is given by $n\sigma_s/k_r < 1$, where n is the number of scatterers per unit volume and k_r is the real part of the acoustic wavenumber (Waterman and Truell 1961). At 3.5 MHz, the scattering cross-section of an average-sized liposome (0.5 μm in radius) is approximately $1 \times 10^{-14} \text{ m}^2$; the corresponding wave number is $14.7 \times 10^3 \text{ m}^{-1}$ and the number density of nontargeted liposomes used for *in vivo* experiments is on the order of $3.3 \times 10^8/\text{cm}^3$, giving $n\sigma_s/k_r = 2.2 \times 10^{-4}$. The criterion for single scattering is, therefore, well satisfied at 3.5 MHz and, unless substantially higher concentrations of liposomes are used, the single scattering approximation holds.

Theoretical estimates of the scattering cross-section are best compared to experimental measurements by use of the backscattering coefficient (BSC), which is defined as the acoustic power scattered in the backward direction per unit incident intensity, per unit solid angle, per unit interrogated volume of scattering medium (Sigelmann and Reid 1973). Assuming single scattering, the BSC is given by:

$$BSC(\omega) = n\sigma_{dbs}(\omega), \quad (2)$$

where σ_{dbs} is the differential backscattering cross-section, evaluated at 180° relative to the direction of the incident wave. The relationship between σ_{dbs} and σ_s is strongly dependent on the ratio between the driving frequency, ω , and the resonant frequency, ω_0 . At frequencies well below resonance, most of the scattering occurs at 180° relative to the incident wave, with an angular distribution pattern depending on the scatterer shape and the contrast in acoustic properties between the particle and the surrounding medium (Coussios 2002). Below and at resonance, the axisymmetrical pulsation of the particle (*i.e.*, the monopole term) dominates and the scattered power can be assumed to be evenly distributed over all solid angles, yielding:

$$BSC(\omega) = n \frac{\sigma_s(\omega)}{4\pi}, \quad (3)$$

The method used to determine the BSC of contrast agent suspensions experimentally is described in detail in the next section.

MATERIALS AND METHODS

Experimental apparatus

The experimental apparatus used for this investigation was a modified version of the arrangement originally proposed by Shung et al. (1976) and Shung and Reid (1976) to

measure the backscattering coefficient of blood. The apparatus consisted of a 3.5-MHz lightly-focused immersion transducer (Picker International, New York, NY, 595831A;), a custom-built sample holder and a polyvinylidene difluoride (PVDF) bilaminar hydrophone (Sonic Industries, Halboro, PA, 805-214;) submerged in a rigidly supported, water-filled Lucite tank at 23° C (Fig. 3). In this setup, the transducer acted both as a transmitter and a receiver. The hydrophone was mounted on a computer-controlled three-axis positioner (Velmex Industries) and was used for the purpose of alignment, calibration and attenuation measurements. The sample holder was a rectangular stainless-steel frame (34.8 mm × 35.8 mm), over which a latex membrane was stretched (approximate thickness of 65 μm); thus, creating two planar acoustic windows. The acoustic properties of latex ($c \approx 1600$ m/s, $\rho \approx 0.91 \times 10^{-3}$ kg/m³) are extremely similar to those of water, so that 90% of the free-field acoustic intensity was effectively transmitted to the sample.

The transducer was excited by broadband pulses generated by a UTA-3 pulser-receiver (Aerotech Laboratories, Phoenix, AZ). The pulse emitted by the transducer was 634 ns long, corresponding to two full cycles at 3.5 MHz, and the pulse-repetition period (PRPe) was 2.1 ms (PRF = 476 Hz). The signal received by the transducer was amplified by 35 dB by the pulser-receiver and displayed on the digital oscilloscope (LeCroy Corp., Chestnut Ridge, NY, 9350 CL; 500 MHz, 500 Ms/s). The time scale and delay of the oscilloscope were adjusted to display the signal backscattered by the walls of the sample holder and intervening sample. A typical trace is shown in Fig. 4.

Calibration and sample positioning

The US field produced by the 3.5-MHz Picker transducer was characterized in 3-D with the 0.4 mm PVDF bilaminar membrane hydrophone. An axial beam profile is shown in Fig. 4, indicating that the focal length is 75 mm. Also shown in Fig. 4 is a typical backscattered signal superimposed on the transducer axial beam profile. By placing the near wall of the chamber 75 mm from the transducer and by choosing a 10-μs gate duration, the interrogated sample volume was exposed to uniform peak-to-peak pressure levels in the range of 1.5 to 1.6 MPa. For this reason, the portion of the trace outlined in Fig. 4 was used for the evaluation of the BSC, as described below. The peak rarefaction pressure was 0.47 MPa, corresponding to a mechanical index (MI) (without deration) of 0.25 (Apfel and Holland 1991, AIUM/NEMA 1992). Note that the MI is based on calculations of the inertial cavitation threshold within one acoustic cycle, which relies on the demand of extremely high collapse temperatures (on the order of 5000 K) and should not be expected to predict contrast agent destruction.

To prevent cross-contamination between samples of different contrast agent concentrations, the sample holder was removed prior to each experiment and the latex membrane was replaced. Correct alignment of the sample holder with the transducer was achieved by filling it with 30 mL of phosphate-buffered saline (PBS) (Baxter International, Deerfield, IL) and by rotating the holder to minimize the time between the two wall reflections (42.5 μs); thus, ensuring normal incidence of the US beam. The alignment was further verified by ensuring that the pressure amplitude received by the hydrophone, placed directly behind the far wall of the container, was identical from experiment to experiment.

Preparation of contrast agent suspensions

Lyophilized liposomes were manufactured by mixing component phospholipids of phosphatidylcholine (PhC), phosphatidylglycerol (PhG), maleimido-4(p-henylbutyrate)-phosphatidylethanolamine (MPB-PhE), and cholesterol (CHOL) (30 mg total) in the mole ratio of 69:8:8:15 in a round-bottomed flask. The chloroform solvent was removed by evaporation under argon in a 50°C water bath to form a thin film of lipid on the flask walls. The resulting lipid film was then placed under high vacuum (< 0.1 mmHg) for 2 to 8 h to complete removal of the solvent. The dry lipid film was rehydrated with deionized water to yield a concentration of 10 mg lipid/mL. The rehydrated mixture was then sonicated in a water bath until the mean size of the liposomes was about 90 nm (60 to 100 nm) as assessed via laser light scattering (Pozharski et al. 2001). To increase the multilamellar structure, an equal volume of 0.2 M mannitol was added and the lipid mixture was then frozen for 30 min at -70°C . The frozen samples were immediately lyophilized under a vacuum of 30 mTorr with a condensation chamber temperature of -50°C for 24 h.

Both Optison[®] microspheres and appropriate amounts of lyophilized liposomes were suspended in 20 mL of PBS and added to the 30 mL of PBS already placed inside the sample holder for alignment purposes, to achieve the desired concentration in 50 mL. All samples were stirred by hand and left to settle for 5 s, providing sufficient time for any large entrained bubbles to rise to the surface prior to insonification.

The Optison[®] concentration chosen for our studies (5 to 8×10^4 microspheres/mL) was determined by dividing the recommended clinical dose of Optison[®] (0.5 mL) by the total blood volume for a human adult, 5000 mL. Approximately 1 mL of Optison[®] was aspirated from its original container using a needle and syringe, as well as a venting needle to prevent damage to the microspheres. a total of 5 mL were directly aspirated from the syringe using a micropipette and gently released into 20 mL of PBS, yielding a concentration of 1 μL Optison[®]/mL in 50 mL. This corresponds to a number density in the range of 5 to 8×10^4 microspheres/mL (Optison[®] product insert).

The range of liposome concentrations investigated in this study was chosen on the basis of *in vivo* animal studies (Hamilton et al. 2002a, 2002b). Liposome suspensions were prepared by reconstituting appropriate amounts of the same batch of lyophilized liposomes in PBS. The four suspensions were prepared in this manner, rather than by successive dilutions of a single liposome suspension, to prevent potential bubble dissolution prior to exposure of each sample to US. Flow cytometry data indicated that a 0.2 mg lipid/mL suspension contained approximately 3.3×10^8 liposomes/mL. Based on this result, the lipid concentration and estimated liposome number density for each of the four liposome suspensions examined in this study are shown in Table 1.

Estimation of the backscattering coefficient

A variety of data-reduction techniques for the experimental determination of the BSC have been described previously. Siegelmann and Reid (1973) first proposed a substitution method that consists of comparing the power backscattered from the sample under examination to that reflected from a target of known reflection coefficient. However, their technique was

strictly limited to insonification by nondecaying sine-wave bursts, using an unfocused transducer and a cylindrical sample holder. A more practical generalized approach that accounted for narrowband and broadband pulses was later proposed by Madsen et al. (1984), and was further developed to encompass the case of focused transducers by Hall et al. (1989). A similar technique was used in the present study that relied, not only on backscattering measurements but, also, on direct hydrophone measurements to determine the beam characteristics and transducer transfer function.

To evaluate the BSC, the acoustic power scattered in the backward direction, the solid angle spanned by the receiving aperture of the transducer, the acoustic intensity incident onto the region containing the scatterers and the extent of the interrogated volume must be known.

Determination of the backscattered acoustic power requires knowledge of the receiving transfer function of the transducer. This was evaluated by positioning the transducer vertically inside the tank and at a distance, L , of 75 mm away from the water surface, and by measuring the received signal corresponding to the wave reflected from the air-water interface. This target was chosen over more commonly used planar steel reflectors due to the much higher acoustic impedance mismatch between air and water, giving a reflection coefficient that is almost equal to -1 . It can be further assumed that the reflected acoustic field at the transducer face is 180° out of phase with the field that would be produced by an identical transducer radiating from a distance $2L = 150$ mm (Madsen et al. 1984). A transverse beam profile at distance $2L$ from the transducer was obtained and was integrated over the beam width, giving the acoustic power of the reflected field. The factor $\chi(\omega)$ relating the acoustic power scattered in the backward direction to the electrical intensity of the signal received by the transducer can, thus, be evaluated.

The mean acoustic intensity incident on the sample was obtained in a similar fashion, by integrating the transverse beam profile at an axial distance $z = 75$ mm (Fig. 5) over the -3 -dB beam width. The interrogated volume, v_i , was evaluated by obtaining two transverse beam profiles at positions corresponding to the near and far side of the gated region ($z = 71$ mm and $z = 79$ mm). The -3 -dB beam width (1.0 mm) at these axial distances indicated that there was no substantial beam divergence and that the interrogated region could safely be considered to be cylindrical. The interrogated volume was, therefore, estimated by taking the product of the -3 -dB beam area with the depth of the gated region (8 mm). The solid angle spanned by the receiving aperture of the transducer was obtained by dividing its receiving aperture, A_r (12.7 mm element diameter), by the square of the distance L to the center of the gated region (75 mm). Finally, the backscattering coefficient could be estimated as:

$$BSC(\omega) = \frac{\chi(\omega) \overline{V_s(\omega) V_s^*(\omega)}}{\left(\frac{A_r}{L^2}\right) I_i v_i}, \quad (4)$$

where V_s is the received voltage amplitude and the over-bar indicates spatial averaging over the gated region. To ensure optimal noise rejection, the mean squared voltage amplitude was estimated by averaging 100 waveforms, corresponding to the responses to 100 consecutive pulses.

Equation (4) is very similar to the expression for the BSC obtained by Marsh et al. (1998). Their expression also includes a correction for the attenuation of the incident and scattered waves as they propagate through the contrast agent suspension. Such a correction was not deemed necessary in the present experimental context, due to the low contrast agent concentrations and narrow gated volume (see Fig. 4) used in this study. The validity of this assumption is investigated further in the next section, where experimental measurements of the attenuation are presented.

Attenuation measurements

Attenuation measurements were obtained by placing the hydrophone behind the sample holder, as shown in Fig. 3. The voltage amplitude received when the sample holder contained only PBS, $V_{\text{ref}}(\omega)$, was compared to the voltage amplitude received with a contrast agent suspension, $V(\omega)$. The attenuation coefficient, α , is given in dB/cm by:

$$\alpha = \frac{20 \log_{10} \left(\frac{V_{\text{ref}}}{V} \right)}{D}$$

where D is the width of the sample holder (3.48 cm) in the direction of sound propagation (Herman et al. 2000).

RESULTS

The BSC of the four liposome suspensions described in Table 1 is plotted as a function of liposome concentration in Fig. 6. The BSC ($0.037 \text{ cm}^{-1} \text{ sr}^{-1}$) obtained for a suspension of Optison[®] microbubbles at the clinical concentration ($5 \text{ to } 8 \times 10^4/\text{mL}$) is provided for comparison. The three liposome suspensions of highest concentrations are shown to scatter more than Optison[®]. Furthermore, the BSC of liposome suspensions increases linearly with liposome concentration. This linear-dependence is in excellent agreement with theoretical predictions and confirms that single scattering holds.

To investigate liposome stability, another liposome suspension at the highest concentration (9.9×10^8 liposomes/ mL) was continuously exposed to pulsed US (634-ns pulse duration, 2.1-ms PRPe) for 400 s. The BSC was measured every 2 s (without averaging 100 waveforms), with results shown in Fig. 7. The BSC decays exponentially and drops to less than half of its initial value after 50 s. To ensure that this decay is not caused by the liposomes moving out of the US beam, the US was turned off after 200 s. The sample was removed from the sample holder using a pipette and returned to the sample holder 200 s later. The sample was remixed and allowed to settle for 5 s before the US was turned back on. A small increase in the BSC is observed after remixing, but it is not significant relative to the overall decrease in scattering levels. In addition, the BSC did not decrease over the resting period of 200 s, indicating that no gas dissolution occurs over this time scale. This suggests that the observed decay in BSC is directly related to liposome destruction. The results of Fig. 7 also indicate that evaluating the mean squared voltage by averaging over 100 consecutive pulses is not unreasonable because no significant change in the BSC is observed over a time interval (0.21 s) corresponding to 100 times the PRPe (2.1 ms).

The attenuation through Optison[®] and liposome suspensions was also investigated, by observing the variation in pressure amplitude recorded by the hydrophone on the far side of the sample holder (see Fig. 3) over a period of 120 s. Results for Optison[®] at the clinical dose are plotted in Fig. 8, indicating that the attenuation, initially equal to 0.25 dB/cm, decays exponentially and drops to half its initial value after 50 s. This suggests that Optison[®] microspheres undergo destruction, or possibly gas dissolution (Podell et al. 1999), when exposed to 3.5-MHz US at a peak rarefaction pressure of 0.47 MPa. Interestingly, in spite of repeated experiments using the highest liposome concentration (9.9×10^8 /mL), no measurable attenuation was observed through liposome suspensions (see Fig. 8). This feature is further addressed in the Discussion section.

In the absence of measurable attenuation for liposome suspensions, no attenuation correction is required when evaluating the BSC, and eqn (4) holds. Based on an attenuation value of 0.25 dB/cm for Optison[®], the incident and scattered waves are attenuated by less than 2% over the depth of the gated volume (see Fig. 7). The error introduced by neglecting attenuation when evaluating the BSC is, therefore, minimal.

DISCUSSION

To test the validity of the model previously presented for Optison[®], a theoretical estimate of the BSC that best fits the experimental data is presented in Fig. 9. By using eqn (3) and assuming a monodisperse distribution, the *mean* scattering cross-section was calculated. According to the data presented in Fig. 1 for Optison[®], this mean scattering cross-section corresponds to a microbubble radius of 3.12 μm . This radius is very close to the resonant microbubble radius at 3.5 MHz, as shown in Fig. 1. This agreement strongly suggests that resonant microbubbles make the strongest contribution to scattering at 3.5 MHz. Furthermore, this result indicates that the model proposed by Church (1995) for Albunex[®] provides a good description of scattering by Optison[®] microbubbles.

A similar procedure was followed to validate the theory proposed for scattering from liposomes. The theoretical prediction of the BSC that best fits our experimental data was found to correspond to a mean gas bubble radius of 0.62 μm and is plotted in Fig. 10. Only liposomes with radii larger than 0.62 μm can encapsulate this amount of gas. We deduce that the larger liposomes ($> 0.62 \mu\text{m}$ in radius) account for most of the scattering at 3.5 MHz. Based on the liposome size distribution described earlier, approximately 40% of liposomes are in this size range. Huang et al. (2002) measured the amount of gas released by the liposomes when exposed to the vapor pressure of water, and found it to be approximately 100 μL of air for every 10 mg of lipid.

Flow cytometry measurements of the liposome number density indicated that every 0.2 mg of lipid yield 3.3×10^8 liposomes which, therefore, contain about 2 μL of air. Assuming that each liposome contains the same volume fraction of air and taking into account the volume distribution of liposomes, the mean gas volume fraction of the liposomes can be estimated to be on the order of 60%.

Figure 10 also shows that the data points corresponding to the two liposome suspensions of lowest concentration appear to deviate from the trendline. This discrepancy could be due to the method by which lyophilized liposomes were re-constituted. Because they are in powder form, a small quantity of lyophilized liposomes is inevitably lost by adhesion to the walls of the mixing beaker. This small quantity represents an increasingly high percentage of the total at the lower concentrations, implying that a smaller quantity of liposomes than those weighed are effectively re-constituted.

Interestingly, the gas bubble radius providing the best fit in Fig. 10 ($0.62\ \mu\text{m}$) is substantially smaller than the resonant radius depicted in Fig. 1 ($1.08\ \mu\text{m}$). Unlike Optison[®], it would, therefore, seem that the biggest contribution to scattering at 3.5 MHz is made by subresonant gas bubbles entrapped within the liposomes. This may provide an explanation as to why liposome suspensions cause little attenuation at 3.5 MHz, even though they scatter substantially. Attenuation of the incident wave is the result of both scattering and absorption. The latter is the result of viscous losses mostly confined to the boundary layers at the interface between the scatterer and the host, within which the velocity gradients can be expected to be large. Even though resonant bubbles scatter more, they also exhibit the largest wall displacements and the steepest velocity gradients and, therefore, cause more absorption. Thus, resonant bubbles attenuate more than bubbles scattering sound below resonance. For Optison[®] microspheres, the high viscosity of the shell itself would also tend to generate substantially more absorption than the less viscous phospholipid bilayer of the liposomes.

De Jong (1993) was first to propose the ratio of scattering cross-section to attenuation as a measure of contrast agent efficiency. This concept was later formalized by Bouakaz et al. (1998) as the scatter-to-attenuation ratio (STAR). Our experimental results indicate that, even though the BSC of liposome suspensions equals or exceeds that of Optison[®] at the clinical dose, the attenuation of liposome suspensions is negligible (see Fig. 8). Based on the definition of STAR, in liposome suspensions, it tends to infinity at 3.5 MHz, which strongly suggests that liposomes are substantially more efficient than Optison[®] microbubbles as a contrast agent *in vitro* at 3.5 MHz.

CONCLUSIONS

The backscattering coefficient and attenuation of liposome suspensions were measured experimentally at 3.5 MHz as a function of liposome concentration and compared to those of Optison[®]. The echogenicity of liposomes at concentrations in excess of $1.15 \times 10^8/\text{mL}$ exceeds that of Optison[®] at the recommended clinical dose (5.0 to $8.0 \times 10^4/\text{mL}$). It has been shown, in porcine studies, that liposome concentrations as high as $1.65 \times 10^9/\text{mL}$ are well tolerated physiologically (Hamilton et al. 2002b), suggesting that liposomes might perform better *in vivo* than Optison[®]. The attenuation of Optison[®] was found to be 0.25 dB/cm at the clinical dose, but no attenuation from liposomes was measured, even at the highest concentration used in this study ($9.9 \times 10^8/\text{mL}$). If the STAR is considered to be a good measure of contrast agent efficiency, then liposomes have the potential to outperform current FDA-approved contrast agents at 3.5 MHz *in vivo*. US images produced with the use of liposomes as an echo contrast agent would not exhibit distal shadowing due to the presence of the agent in the organ of interest. In addition, a theoretical model has been

presented that treats the gas within the liposomes as a free air bubble. This model correlated well with experimental results and provided insight as to the low attenuation of liposome suspensions at 3.5 MHz. Measuring the variation of the backscattering coefficient with insonication time shows promise as a sensitive method for determining the threshold of destruction of these liposomes. This technique will aid in ascertaining the stability of these agents, as well as the optimal US pulse characteristics for either targeted imaging or drug delivery.

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Fig. 1. Total scattering cross-section of a free air bubble and an Optison[®] microsphere as a function of particle radius (after Church 1995). A shell rigidity of 88.8 MPa and a shell shear viscosity of 0.177 N s / m² are assumed for Optison[®].

Fig. 2.

Total scattering cross-section of a liposome as a function of air volume fraction and liposome radius. It is assumed that the gas entrapped within each liposome behaves as a single spherical free air bubble with a volume in the range 5 – 100% of the liposome volume.

Fig. 3.
Apparatus used for measuring the backscattering and attenuation coefficients of liposome and Optison® suspensions.

Fig. 4. Trace analysis, determination of the gate length and comparison with the transducer axial beam profile.

Fig. 5.
Transverse profile of the 3.5-MHz Picker transducer at $z = 75$ mm.

Fig. 6. Measured BSC from liposome suspensions as a function of liposome concentration and comparison with the BSC of an Optison[®] suspension ($5-8 \times 10^4/\text{mL}$).

Fig. 7. Variation in BSC during exposure of a liposome suspension (9×10^8 liposomes/mL) to pulsed 3.5 MHz US (PD = 634 ns, PRPe = 2.1 ms, MI = 0.25) for 400 s. The sample was removed from the holder and remixed after 200 s to ensure resuspension of the liposomes.

Fig. 8.

Variation in attenuation coefficient during exposure of an Optison® suspension ($5-8 \times 10^4$ microbubbles/mL) to pulsed 3.5 MHz US (PD = 634 ns, PRPe = 2.1 ms, MI = 0.25) for 120 s.

Fig. 9.

Comparison between experimental measurements (circles, bars indicate the SD) and theoretical predictions (—) of the BSC of Optison® suspensions as a function of microsphere concentration. The theoretical prediction assumes a monodisperse distribution with radius $3.12 \mu\text{m}$.

Fig. 10. Comparison between experimental measurements (circles, bars indicate the SD) and theoretical predictions (—) of the BSC of liposome suspensions as a function of liposome concentration. The mean scattering cross-sections are deduced, assuming a monodisperse distribution with radius 0.62 μm . study.

Table 1

Lipid concentration and liposome number density of the four liposome suspensions examined in this study

Lipid concentration (mg/mL)	Liposome number density (liposomes/mL)
0.05	0.83×10^8
0.10	1.65×10^8
0.20	3.30×10^8
0.60	9.90×10^8