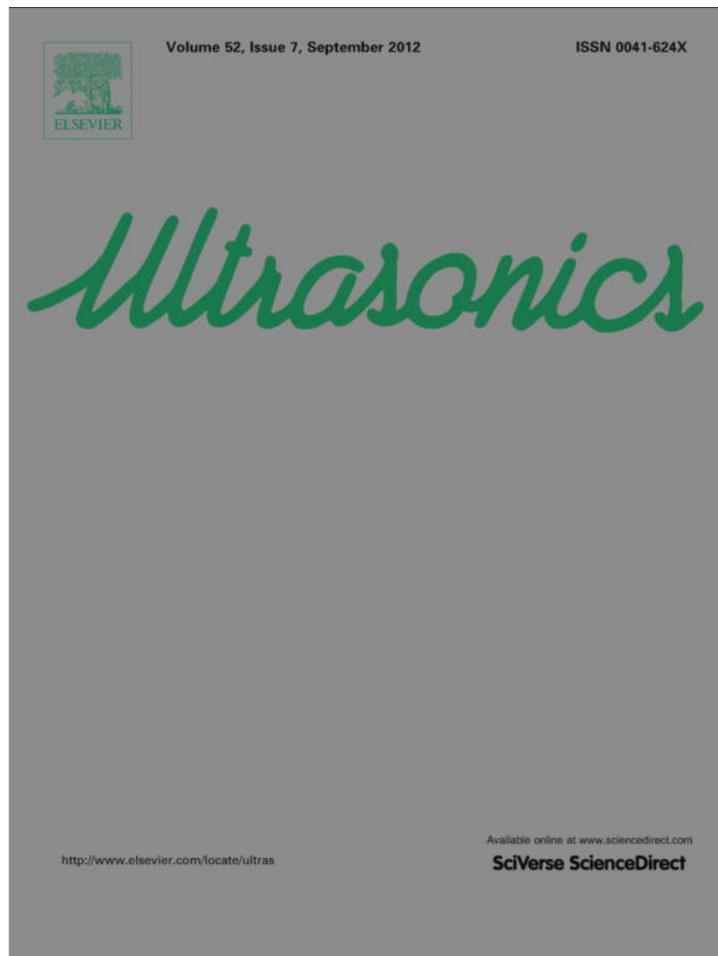


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## Ultrasonics

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## *In vitro* measurement of attenuation and nonlinear scattering from echogenic liposomes

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## ARTICLE INFO

## Article history:

Received 5 August 2011

Received in revised form 18 January 2012

Accepted 16 March 2012

Available online 29 March 2012

## Keywords:

Ultrasound imaging

Contrast agents

Echogenic liposomes

Scattering

Attenuation

## ABSTRACT

Echogenic liposomes (ELIP) are an excellent candidate for concurrent imaging and drug delivery applications. They combine the advantages of liposomes—biocompatibility and ability to encapsulate both hydrophobic and hydrophilic drugs—with strong reflections of ultrasound. The objective of this study is to perform a detailed *in vitro* acoustic characterization – including nonlinear scattering that has not been studied before – along with an investigation of the primary mechanism of echogenicity. Both components are critical for developing viable clinical applications of ELIP. Mannitol, a cryoprotectant, added during the preparation of ELIP is commonly believed to be critical in making them echogenic. Accordingly, here ELIP prepared with varying amount of mannitol concentration are investigated for their pressure dependent linear and non-linear scattered responses. The average diameter of these liposomes is measured to be 125–185 nm. But they have a broad size distribution including liposomes with diameters over a micrometer as observed by TEM and AFM. These larger liposomes are critical for the overall echogenicity. Attenuation through liposomal solution is measured with four different transducers (central frequencies 2.25, 3.5, 5, 10 MHz). Measured attenuation increases linearly with liposome concentration indicating absence of acoustic interactions between liposomes. Due to the broad size distribution, the attenuation shows a flat response without a distinct peak in the range of frequencies (1–12 MHz) investigated. A 15–20 dB enhancement with 1.67 µg/ml of lipids is observed both for the scattered fundamental and the second harmonic responses at 3.5 MHz excitation frequency and 50–800 kPa amplitude. It demonstrates the efficacy of ELIP for fundamental as well as harmonic ultrasound imaging. The scattered response however does not show any distinct subharmonic peak for the acoustic excitation parameters studied. Small amount of mannitol proves critical for echogenicity. However, mannitol concentration above 100 mM shows no effect.

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### 1. Introduction

Commercially available ultrasound contrast agents are micron sized gas bubbles (1–10 µm in diameter) with a stabilizing encapsulation made of molecules of protein/lipids/surfactants. These microbubble-based agents have also been investigated for drug delivery applications [1–5]. In the past few years, specialized echogenic liposomes (ELIP) have been developed for concurrent imaging and drug delivery [6–9]. However, thorough acoustic investigations to quantify their effectiveness as ultrasound contrast agents have so far been limited. Here, we report an *in vitro* investigation of attenuation characteristics as well as linear and

nonlinear scattered responses of echogenic liposomes prepared following a previously described protocol. Note that nonlinear scattered responses of ELIP have not been studied before; they are critical for harmonic and subharmonic imaging modalities. These modalities promise better contrast-to-tissue ratios than those obtained by conventional imaging in the fundamental mode.

Liposomes are vesicles with a hydrated lipid bilayer encapsulating an aqueous phase. They are spontaneously formed when phospholipids are dispersed in water. The bilayer membrane is formed when the hydrophobic portions of the lipids interact with one another leaving the hydrophilic group directed towards the inner and the outer aqueous phases. Due to their structural similarity with biological cells liposomes have lesser toxicity, longer circulation time in the blood stream and greater uptake by target organs/tissues. These properties make liposomes an ideal candidate for use as drug delivery agents. And since their discovery by Bangham in [10], they have been extensively studied as agents for delivering

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drugs and genes to specific sites/organs of the human body. Due to the presence of both hydrophilic and hydrophobic ends of the lipids, liposomes can be simultaneously loaded with both water-soluble and water-insoluble drugs. The water-soluble drugs can be loaded in the inside aqueous phase and water-insoluble drugs in the lipid bilayer [11,12]. Ultrasound mediated drug release from liposomes has also been studied recently [13–16]. Currently, about 10 liposomal drug formulations are approved by the US Food and Drug Administration for human use [13,17].

A modified preparation protocol has been developed by Huang et al. to render a liposome echogenic. It involves a number of freeze–thaw cycles in presence of a cryoprotectant, mannitol, followed by freeze-drying (lyophilization) and reconstitution [9,18]. During the freeze–thaw cycles and lyophilization the lipid bilayer develops defects, which later during rehydration traps air [19,20]. The presence of air inside gives rise to a mismatch in the acoustic impedance, and the air pocket can oscillate under acoustic excitation. Both effects enable these liposomes to generate an echo under acoustic excitation [19]. Therefore, mannitol is believed to play a critical role in ensuring echogenicity of these liposomes. Even though echogenicity of these liposomes is related to the existence of these trapped air pockets, their exact location is not fully ascertained. From energetic considerations, Huang et al. hypothesized that these air pockets should be formed within the lipid bilayer near the hydrophobic tails of the lipid molecules [17,18,21]. However, they also suggested that air can also be entrapped as a lipid monolayer coated bubble encapsulated within the aqueous core of the liposomes [18]. Huang and co-workers have also measured the total amount of air entrapped in the liposomal solution; it accounts for 10–33% of the liposome volume [21,22]. Recent TEM images of ELIP prepared by Kopechek et al. using the same protocol shows existence of entrapped air pockets [20]. In fact the preparation protocol has also been utilized to encapsulate bioactive gases like nitric oxide [23] and xenon [24]. Echogenic liposomes were found to retain all the properties of normal liposomes [17]. They can be loaded with various therapeutic agents similar to conventional liposomes and used for simultaneous imaging and targeted drug delivery [25–28]. Furthermore, Hitchcock et al. demonstrated that echogenic liposomes can nucleate cavitation by lowering the thresholds for both stable and inertial cavitation [29]. Cavitation has been hypothesized as a cause for ultrasound induced increased permeability of the biological membranes [30] that can enhance the drug uptake by tissues [31–33]. Therefore, it is possible to use echogenic liposomes for ultrasound mediated controlled drug and gene delivery. Effects of drug-loading on echogenicity and efficiency of drug delivery by ELIP have been extensively studied [12,21,28,29,34,35]. Note that in order to suitably optimize echogenic liposomes based drug-delivery, one would need to carefully investigate the role of ELIP induced cavitation and destruction, as was recently done for lipid coated microbubbles [36].

There have not been many studies of the acoustic behaviors of ELIP, especially their nonlinear scattered response. During design and development of ELIP, echogenicity was tested using a 20 MHz high frequency intravascular US (IVUS) imaging catheter [9,19,37,38]. The mean gray scale values for the region of interest (ROI) were obtained from the videodensitometric analysis of the images. This was used as a measure of echogenicity of the liposomes prepared. However, detailed characterization and understanding of the mechanism of echogenicity can only be achieved through controlled *in vitro* experiments. *In vitro* tests so far have been performed by Coussios et al. [39] with echogenic liposomes suspended in a solution of PBS mixed with 0.5% bovine serum albumin. They measured both backscattering and attenuation coefficients using a 3.5 MHz lightly focused immersion transducer and compared them with measurements from Optison® bubbles. More recently, the same group has extended the investigation to a

broadband frequency dependent attenuation study in the range 3–25 MHz. They also reported backscatter coefficient of  $0.011–0.023 \text{ (cm str)}^{-1}$  in the frequency range of 6–30 MHz [20]. Acoustic destruction thresholds of ELIP were also studied *in vitro* using L12-5 linear array transducer [40].

Our aim here is to understand the linear and nonlinear acoustic responses from these liposomes including the effects of components in the preparation protocol that are believed to be critical for echogenicity. Towards that goal, here we report the measurement of frequency dependent attenuation coefficient and linear and nonlinear scattered responses of ELIP prepared with varying concentrations of mannitol. Note that nonlinear responses from contrast agents are utilized for harmonic [41,42] and subharmonic imaging [43–47]. Hence, characterization of nonlinear responses can help in appraising the effectiveness of ELIP for such nonlinear imaging modalities with potentials for higher contrast-to-tissue ratio. Acoustic responses also help in determining the material properties of the encapsulating shells [48,49], and this approach has recently been extended to ELIP [20].

## 2. Materials and methods

### 2.1. Preparation of echogenic liposomes and reconstitution procedure

Stock solutions of lipids are prepared by dissolving the lipid powders in chloroform–methanol (9:1) mixture and stored at  $-20^\circ\text{C}$ . The concentrations are 10 mg/ml for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1 mg/ml for 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), cholesterol (CH) (Avanti Polar Lipids, Alabaster, AL, USA). The lipids in desired lipid molar ratio (DPPC: DDPG: DPPE: CH in 69:8:8:15) are taken in a 50 ml round bottom flask. The flask is gently shaken to form a uniform solution. A thin lipid film is obtained by evaporating this mixture in a rotary evaporator at  $40^\circ\text{C}$  for about 5–10 min. The thin film is then dried in a vacuum desiccator overnight to remove all residual organic solvents. The dry lipid film is hydrated with 3 ml of 0.32 M mannitol (Alfa Aesar, MA, USA) solution. The solution is then sonicated for 10 min using a bath sonicator, then frozen at  $-70^\circ\text{C}$  for 30 min followed by thawing the frozen liposomes to room temperature. This freeze–thaw cycle is repeated five times. The frozen liposomes are subsequently lyophilized using a freeze-drying apparatus (Labconco, MO, USA) for 24 h. The lyophilized dry cake of echogenic liposomes is stored at  $4^\circ\text{C}$  until use, when it is reconstituted at desired concentration before an experiment. They are reconstituted in a phosphate buffered saline (PBS) with 0.5% by weight bovine serum albumin (BSA). Appropriate amounts of powder are measured for each experiment and added to 150 ml of the PBS–BSA solution, already poured in the sample chamber to have the desired lipid concentration. Liposomes prepared using 320 mM mannitol has 1 mg of lipids in every 6 mg of lyophilized powder. The PBS–BSA solution is prepared by adding 2.5 g of BSA powder to 500 ml of PBS buffer. The mixture is then thoroughly shaken and kept refrigerated for a minimum of 48 h before use.

### 2.2. Measurement of size distribution

Particle size distribution (PSD) of ELIP is measured using a dynamic light scattering (DLS) instrument (Malvern Zetasizer Nano-ZS90) controlled with the Zetasizer software (version 6.20). DTS 0012 polystyrene latex disposable sizing cuvettes (RI: 1.59) are used and measurements are performed at a scattering angle of  $90^\circ$ . The lyophilized liposome powder is reconstituted in PBS to give a final concentration of 0.1 mg/ml of liposomes. The cuvette is equilibrated for 120 s and 12 readings are then taken for a single

measurement at a constant temperature of 25 °C. Each batch of liposome is tested for PSD and each experiment repeated three times to ensure reproducibility of the results obtained.

### 2.3. Transmission electron microscopy

The liposome samples are diluted to 1 mg/mL (total lipid) and dropped onto 300 mesh Formvar coated copper grids previously coated with 0.01% poly-L-lysine and allowed to stand for 1 min before wicking off with filter paper. After air drying for 2 min, the samples are stained with 1% phosphotungstic acid for 1.5 min and subsequently wicked off with filter paper and allowed to dry before viewing. The samples are observed using a JEOL JEM-100CX-II transmission electron microscope operating at 80 kV.

### 2.4. Atomic force microscopy

The samples are prepared by depositing 200  $\mu$ L of the solution of ELIP in distilled water followed by air drying. Samples deposited on mica substrates are used for performing the AFM experiments. AFM images are obtained by using a MultiMode™ atomic force microscope equipped with a Nanoscope III a controller and a J-type piezo scanner from Veeco Metrology Group, Santa Barbara, CA. AFM images are taken in Tapping Mode™. Tips made from anti-mony(n) doped Si are used for obtaining the images under laboratory conditions.

### 2.5. Experimental setup to measure attenuation

The attenuation setup employs a pulse-echo system (Fig. 1a). A pulser/receiver (Model 5800; Panametrics-NDT, Waltham, MA) is

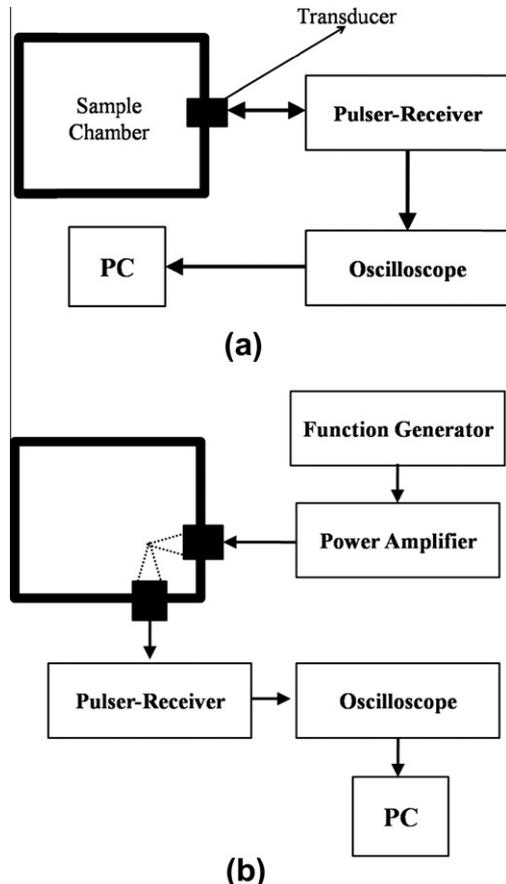


Fig. 1. Schematic of the experimental setup for *in vitro* measurement of: (a) attenuation and (b) scattering.

used to excite an unfocused broadband transducer at a PRF of 100 Hz and a pulse duration of 440 ns. Four different broadband transducers are used in transmit–receive mode with center frequencies of 2.25, 3.5, 5 and 10 MHz. The  $-6$  dB bandwidths for these transducers are 1.178–3.32 MHz, 2.5–4.99 MHz, 3.13–6.19 MHz and 6.78–12.4 MHz. The ultrasound pulse travels through the contrast agent suspension and is reflected back by the back-wall of the chamber. The reflected pulse is received by the same transducer. The total distance travelled by the pulse before being received by the transducer is 12 cm. The received signal is amplified by the pulser/receiver and fed into an oscilloscope (TDS2012, Tektronix, and Beaverton, OR, USA) and saved on a desktop computer using LabView (Version 6.0.3; National Instruments, Austin, TX) via a GPIB IEEE 488 cable and a GPIB card. Matlab® (Mathworks Inc., Natick, MA, USA) is used for post-processing of the data. 20 voltage–time RF traces acquired in an averaging mode (64 sequences) are saved for the post-processing.

### 2.6. Experimental setup to measure scattering

The acoustic setup used for the current investigation follows the one used previously by us and others to study non-linear scattered responses from contrast microbubbles [49,50] for the characterization of Sonazoid bubbles (Fig. 1b). Two single element spherically focused transducers with individual diameter of 1.27 cm and focal length of 3 cm are employed. The transmitting transducer is confocally positioned at right angle to the receiving transducer. This arrangement ensures that scattered signals are very similar to backscattered echoes [50] and also gives high spatial resolution [49]. The solution is held in a rectangular chamber with drilled holes on adjacent sides where the transducers are inserted. The chamber requires 150 ml of solution for complete immersion of the transducers. 1.5 mg of lyophilized ELIP powder is weighed and added to the sample chamber containing PBS + BSA solution. The resulting concentration (1.67  $\mu$ g/ml) is low enough to avoid multiple scattering effects. The transmitting transducers employed have a nominal center frequency of 3.87 MHz (Panametrics-NDT) with a  $-6$  dB bandwidth of 86.4%. The receiving transducer (Panametrics-NDT) is reported to have a center frequency of 5.54 MHz and  $-6$  dB bandwidth of 85%. A programmable function generator (Model 33250A; Agilent, Santa Clara, CA) is used to generate sinusoidal waves of varying amplitudes with 32 cycles at 3.5 MHz frequency and at a PRF of 100 Hz. The signal is then amplified by a 55 dB RF power amplifier (Model A-300; ENI, Rochester, NY) before being transmitted to the transmitting receiver. The scattered ultrasound is received by the receiving transducer and sent to a pulser/receiver (Model 5800; Panametrics-NDT, Waltham, MA) in receiving mode with a 20 dB gain. Signals are then sent to a digital oscilloscope (Model TDS2012; Tektronix, Beaverton, OR) where they are observed in real time. A sample averaging mode is employed to reduce the noise in both the time and frequency domains. The oscilloscope is also connected to a computer with LabView (Version 6.0.3; National Instruments, Austin, TX) via a GPIB IEEE 488 cable and a GPIB card. Voltage signals are acquired from the oscilloscope by LabView and saved for post-experimental analysis using Matlab® (MathWorks, Natick, MA). For analysis, fast Fourier Transforms (FFTs) of 50 oscilloscope acquisitions (Hamming-windowed) are averaged in the frequency domain.

### 2.7. Experimental procedure and data reduction

The buffer (150 ml of PBS + BSA) is introduced into the sample chamber with care so as to avoid formation of air bubbles. The solution is left for 5–10 min so that air bubbles can either dissolve (small bubbles) or escape (larger bubbles) to the atmosphere. Control measurements without liposomes are then acquired. An

**Table 1**

Average diameter and the polydispersity index of ELIP (as measured by DLS) as a function of mannitol concentration.

Mannitol conc. (mM)	Averaged diameter (nm)		Polydispersity index
	Intensity	Number	
No mannitol	65 ± 7	64 ± 7	1.00 ± 0.00
5	1293 ± 474	125 ± 14	0.63 ± 0.03
10	733 ± 400	134 ± 15	1.00 ± 0.00
15	640 ± 466	122 ± 11	1.00 ± 0.00
50	500 ± 65	173 ± 31	0.72 ± 0.07
100	336 ± 7	171 ± 2	0.63 ± 0.04
150	512 ± 87	185 ± 8	0.73 ± 0.09
200	972 ± 129	180 ± 14	0.86 ± 0.01
250	374 ± 18	170 ± 34	0.63 ± 0.01
320	459 ± 35	152 ± 15	0.83 ± 0.07
350	623 ± 12	181 ± 14	0.85 ± 0.02

appropriate amount of liposome powder is weighed and added to the solution directly and gently stirred so as to create a homogeneous solution. The solution is then excited with ultrasound pulses and the responses are acquired and saved in the computer.

For attenuation, 20 voltage–time acquisitions are obtained with and without liposomes. A Matlab® code is used to take FFT (Fast Fourier Transform) of each of the voltage time response acquired and then averaged for 20 acquisitions. The attenuation coefficient is then calculated using the following expression

$$\alpha(\omega) = 20 \log_{10} \left( \frac{\bar{V}_{ref}}{\bar{V}_{sig}} \right) / d, \quad (1)$$

where  $\bar{V}_{ref}$  is the averaged response in frequency domain without ELIP in the medium,  $\bar{V}_{sig}$  is the averaged response in the frequency domain with ELIP suspended in the medium, and  $d$  is the total path travelled by the pulse before it is being received by the transducer.

For scattering a similar technique is used to get the average response in frequency domain (50 V time acquisitions are used). The scattered response is converted into a dB scale by taking a unit reference. Responses at frequencies of interest are then appropriately extracted from the resultant data set to find the fundamental, second and sub-harmonic scattered responses.

### 3. Results and discussion

#### 3.1. Size distribution

Table 1 shows the intensity averaged diameter, obtained by averaging the radius distribution weighted with the intensity of the scattered light, and the number averaged diameter for the ELIP prepared with varying amounts of mannitol (measured with DLS). The polydispersity from the DLS measurements are also reported in Table 1. The average diameter is 125–185 nm depending on the mannitol concentration. The polydispersity indices are observed to be high (0.63–1.0) indicating that the liposomal formulations have a large range of sizes. In order to corroborate these observations, ELIPs prepared with 320 mM mannitol are imaged employing a transmission electron microscope (TEM) and an atomic force microscope (AFM). Although some changes to the vesicular structure are expected under the sample preparation conditions [51], the TEM image (Fig. 2a, magnification: 7900) indicates considerable variations in the size of the liposomes. Similar results were also observed by the AFM imaging studies (Fig. 2b). Both show liposomes with diameters of 1 micron and above. We believe that these larger liposomes (although far less in number compared to those with diameters in the nanometer range) are crucial for the echogenicity observed below. They can contain a large enough air pocket inside the bilayer that oscillates while excited to generate the linear and nonlinear scattered responses.

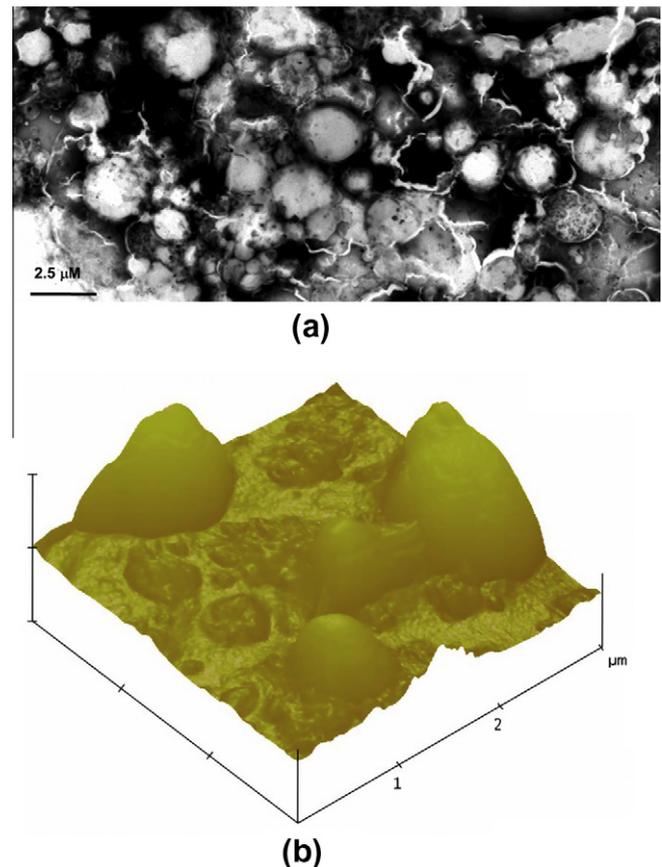
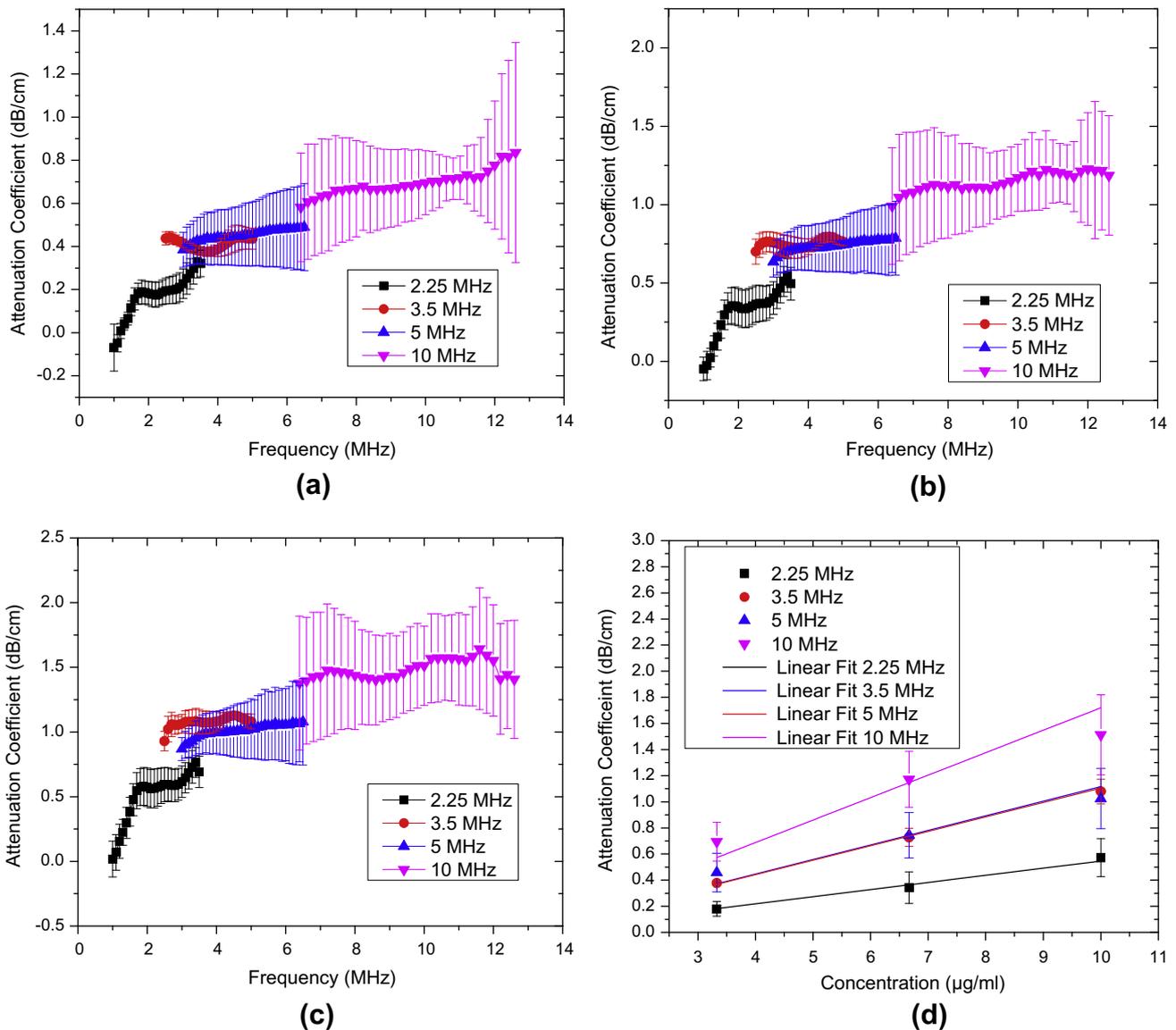


Fig. 2. (a) TEM and (b) AFM images of echogenic liposomes.

#### 3.2. Attenuation

The attenuation measurements are conducted for echogenic liposomes using four different transducers (2.25, 3.5 5 and 10 MHz). The data reduction technique described above is used to generate the frequency dependent attenuation coefficient (Fig. 3). The attenuation coefficient is plotted for each transducer within its bandwidth. Attenuation coefficients are measured for three different concentrations of 3.33 μg/ml, 6.67 μg/ml and 10 μg/ml of lipids in the solution (Fig. 3a–c). The data obtained with different transducers match in the region of overlapping frequencies. The frequency dependent attenuation coefficient shows a continuous increase with increasing frequency for frequencies lower than 5 MHz. Beyond 5 MHz the frequency dependent attenuation curves show a flat response. For the entire range of frequencies (1–12 MHz) the attenuation curve does not show any peak. A peak in attenuation for a suspension of conventional contrast agents indicates the resonance frequency for the encapsulated contrast microbubbles. Note that a free bubble with a diameter of 150 nm (average diameter of these liposomes) has a resonance frequency ~40 MHz. Typically an air pocket of this size within a liposome would have an even larger resonance frequency because of the increased elasticity of the part of the bilayer. Therefore it would lead to very little acoustic response in the range of frequencies investigated here. However, note that the large polydispersity indices reported above indicate a broad size distribution including diameters over a micron (Fig. 2 clearly shows liposomes of diameter ~2 μm). We believe that the attenuation and scattered responses from the liposomal solution are primarily due to the air pockets entrapped in these larger liposomes. Note also that, for a broad distribution with sizes predominantly at the sub-micron level, attenuation is



**Fig. 3.** Frequency dependent attenuation coefficient of echogenic liposomes measured with four different transducers (2.25, 3.5, 5, 10 MHz) and plotted within their respective  $-6$  dB bandwidth for lipid concentrations of (a)  $3.33 \mu\text{g/ml}$  (b)  $6.67 \mu\text{g/ml}$  and (c)  $10 \mu\text{g/ml}$ . Data averaged for five different samples. (d) Attenuation coefficient at the central frequencies of the four transducers as a function of lipid concentration.

expected to show increase and then gradual flattening at higher frequencies. Experimental measurements of attenuation coefficients in Definity™ by Goertz et al. [52] showed that for a broader size distribution, the attenuation curve is flatter in comparison to the response from a manipulated bubble population with a sharper cut-off in size distribution. Experimental observations by Gong et al. [53] using lipid coated microbubbles also showed that as the size distribution becomes broader, the attenuation curve tends to be wider and flatter, with a less distinct peak. Fig. 3d shows that the attenuation at the central frequency for each transducer increases linearly with concentration. This indicates that for the lipid concentrations used, resulting liposome concentration is dilute enough, that multiple scattering effects are negligible. Therefore, the analysis employed to obtain the attenuation data is correct. We conclude that the attenuation is primarily due to the larger liposomes, and lack of a peak in the spectra is due to the broad size distribution.

### 3.3. Scattering

Scattering measurements are acquired for an excitation frequency of 3.5 MHz using the setup described. The scattered response from contrast microbubbles depends strongly on the acoustic pressure amplitude, indicating a non-linear response [49,50,54]. Five sets of measurements are acquired for each of the acoustic pressure amplitudes. In Fig. 4, we show the FFT of the scattered response from ELIP for two different acoustic excitations – 50 kPa and 600 kPa. Note that only the data corresponding to the higher pressure has a distinct third harmonic contribution. However, even at the higher pressure, we do not see a distinct sub-harmonic peak, in contrast to what has been observed for conventional contrast agents used in sub-harmonic imaging [49,54,55]. The mean of five data sets and the corresponding standard deviations are then plotted in Fig. 5. The fundamental response shows around 15–20 dB enhancement over the data without liposomes.

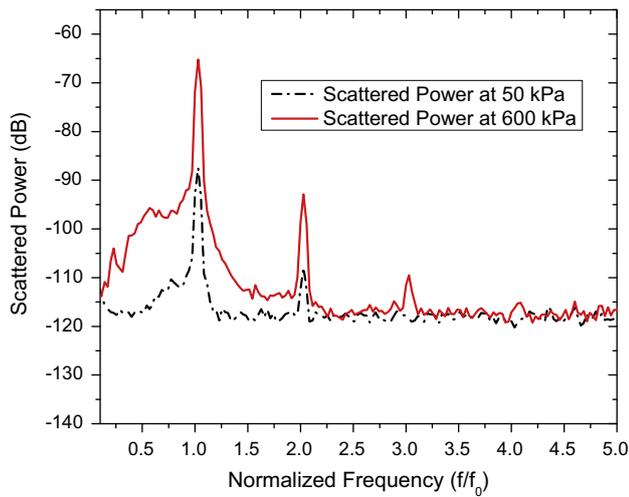


Fig. 4. FFT of the scattered signal from liposomes for acoustic pressure amplitudes 50 kPa and 600 kPa.

It also shows a consistent increase (linear in the log–log plot) with increasing acoustic excitation amplitude until 400 kPa. Beyond this pressure, the response starts to saturate, indicating possible liposomal destruction at these higher acoustic excitations [56]. The second harmonic response also shows similar enhancement, increasing linearly till acoustic pressure amplitude of 400 kPa, and then saturation. In view of the absence of subharmonic peak, ELIP might not be suitable for non-destructive subharmonic imaging applications [57,58]. However, these liposomes are clearly echogenic with a 15–20 dB enhancement in signal over control (see also Fig. 6 below). Ordinary liposomes with an aqueous interior are not echogenic. We believe that the echogenicity of these liposomes is primarily due to the air entrapped in the bilayer of the liposomes with diameters larger than one micrometer (Fig. 2).

Mannitol and lyophilization are reported to play critical roles in the echogenicity of these liposomes [9,18,19]. In an effort to have a better understanding of their role and to determine the optimal concentration of mannitol, liposomes are prepared without lyophilization and with varying amounts of mannitol (0 mM–350 mM), and then tested for echogenicity. Note that such studies have been performed before for echogenic liposomes, with a different chemical composition, using an IVUS catheter [7,9,21]. While one of the previous studies showed existence of an optimal mannitol

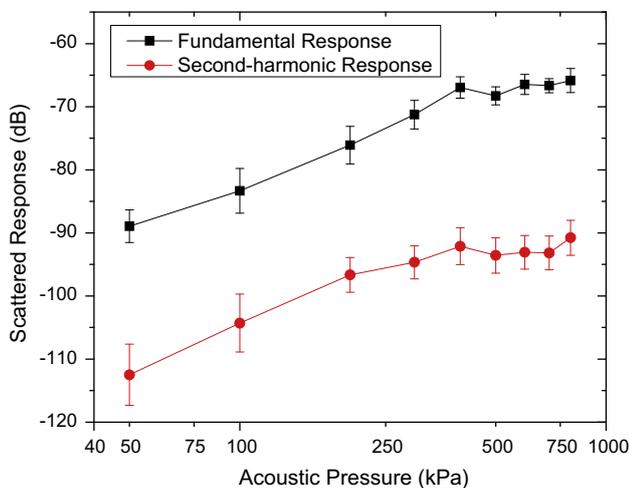


Fig. 5. Measured scattered response from echogenic liposomes at fundamental and second harmonic frequencies.

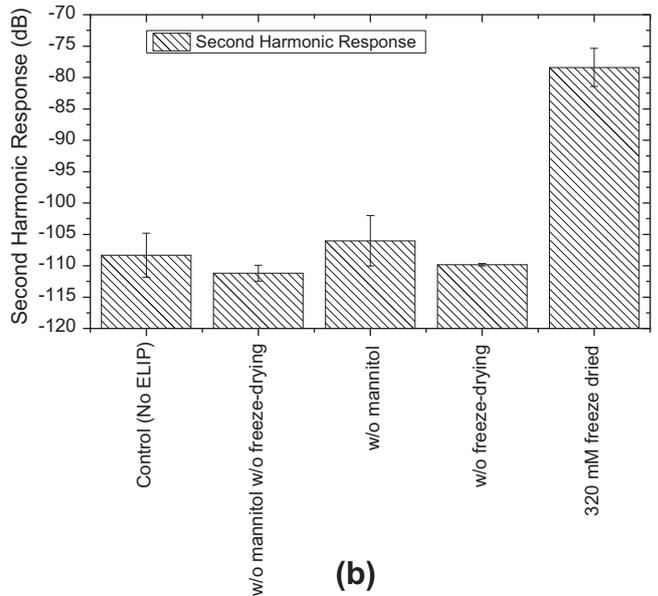
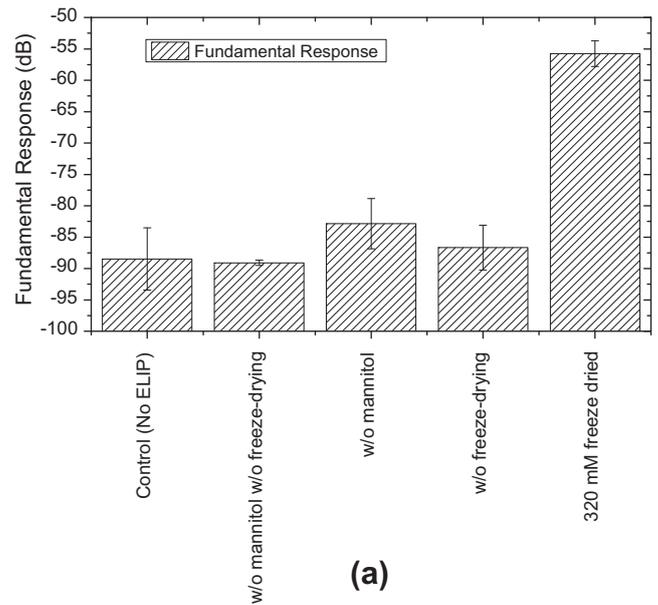
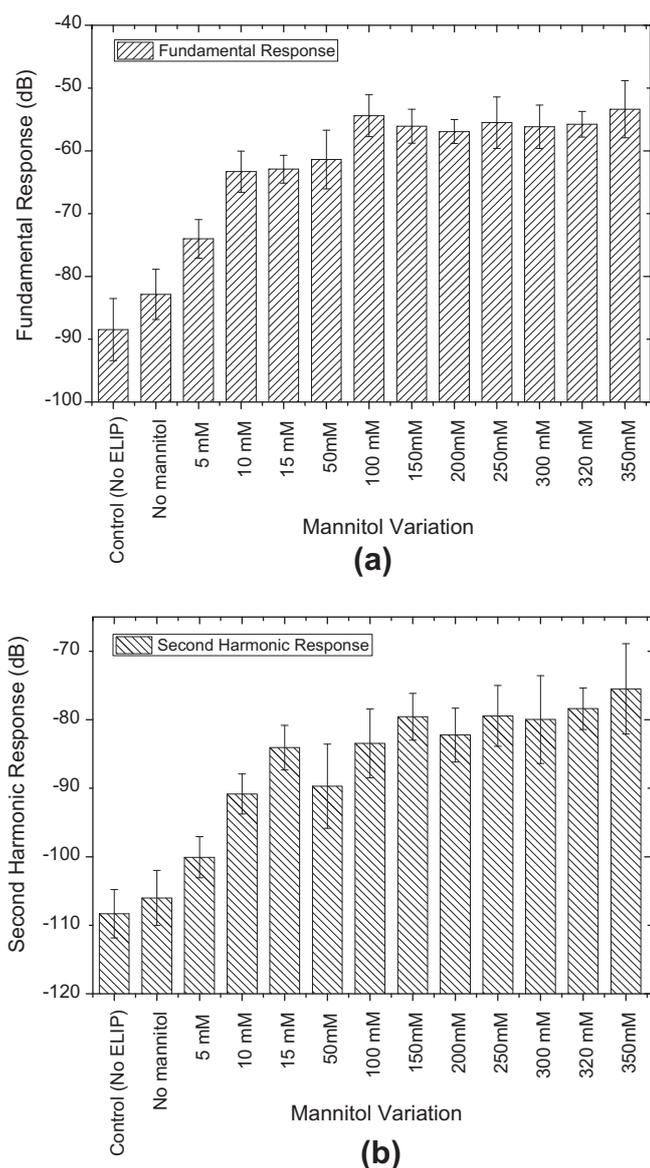


Fig. 6. Scattered response from liposomes prepared with and without mannitol and with and without freeze-drying (lyophilization) at: (a) fundamental and (b) second harmonic frequencies and at an acoustic excitation of 500 kPa and 3.5 MHz.

concentration [9], another showed consistent monotonic increase of echogenicity with increasing mannitol content [21]. It has been reported that mannitol content also affects the encapsulation efficiency [21]. The adopted mannitol concentration of 320 mM for most of our studies was reported to be the optimal concentration for both echogenicity and encapsulation efficiency [18,37]. Also note that here we investigate the effects of mannitol concentration variation on the nonlinear (second harmonic) response that has not been investigated before. Fig. 6 plots the fundamental and second harmonic responses from liposomes prepared with four different formulations: with and without freeze-drying (lyophilization), and with and without mannitol added during preparation. It shows that without freeze-drying and mannitol addition, liposomes are not echogenic. In Fig. 7, responses from liposomes prepared with different mannitol concentrations are shown. Lipid concentration used in all cases presented in Figs. 6 and 7 is 10  $\mu$ g/ml. Both fundamental and second harmonic responses from ELIP prepared with



**Fig. 7.** Comparison of scattered response from echogenic liposomes prepared with different amounts of mannitol at: (a) fundamental and (b) second harmonic frequencies and at an acoustic excitation of 500 kPa and 3.5 MHz.

increasing concentrations of mannitol show increasing response till 50 mM, but above 100 mM, they show very little variation. The response from liposomes prepared without mannitol is the same as the control. Therefore, we conclude that a finite nonzero amount (~100 mM) of mannitol is required for ensuring sufficient echogenicity. Note that lack of echogenicity without the lyophilization/reconstitution step in the preparation protocol has also been observed previously [19].

#### 4. Summary

Echogenic liposomes prepared using a previously published technique are experimentally examined. The average diameter of these liposomes, measured using dynamic light scattering, is found to be 125–185 nm. However, the large polydispersity also indicates a broad size distribution. More specifically, TEM and AFM studies indicate many liposomes with diameters of 1–2  $\mu\text{m}$ . Both frequency dependent attenuation and excitation dependent nonlinear scattered responses are measured. Attenuation of 0.1–0.7 dB/cm is

measured in a liposomal solution containing 3.33  $\mu\text{g}/\text{mL}$  of lipids using four transducers with central frequencies 2.25, 3.5, 5, 10 MHz. The data show an increase and later saturation with frequency but no clear peak. Such a data is consistent with the broad size distribution of these liposomes. The scattered response shows a 15–20 dB enhancement with 1.67  $\mu\text{g}/\text{ml}$  of lipids of fundamental and second harmonic responses demonstrating conclusively that the liposomes are echogenic. Therefore, they are suitable for fundamental as well as harmonic imaging applications. However, no subharmonic response is found. We believe that the attenuation and the fundamental and harmonic responses are generated by larger liposomes (diameter >1  $\mu\text{m}$ ) that are shown to be present in the size distribution. They entrap air in the lipid bilayer during the specialized preparation protocol.

Mannitol is thought to be of critical importance as a weak cryoprotectant to ensure rupture in the lipid film entrapping air and thereby making liposomes echogenic [19]. Here, by measuring scattered responses from liposomes prepared with varying mannitol concentrations (0–350 mM), we demonstrate that a low but finite amount of mannitol (~100 mM) is critical for ensuring echogenicity. Lyophilization is also critical since without it liposomes are found to be nonechogenic.

#### Acknowledgements

KS and SP acknowledge helpful discussions about the preparation of echogenic liposomes with Shao-Ling Huang of University of Texas Health Center. They have also immensely benefitted by close interactions with Dr. Christy Holland and her group at University of Cincinnati, especially Jonathan Kopechek. The authors thank the comments of one of the reviewers for significantly sharpening the focus of the paper. The research was partially supported by NSF Grants DMR-1005283, CBET-1033256, and NIH Grant No. P2ORR016472 to KS and NIH Grant 1R01 CA 132034 and NSF Grant DMR 1005011 to SM.

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