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● *Original Contribution*

ACOUSTIC CHARACTERIZATION OF ECHOGENIC POLYMERSOMES PREPARED FROM AMPHIPHILIC BLOCK COPOLYMERS

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Abstract—Polymersomes are a class of artificial vesicles prepared from amphiphilic polymers. Like lipid vesicles (liposomes), they too can encapsulate hydrophilic and hydrophobic drug molecules in the aqueous core and the hydrophobic bilayer respectively, but are more stable than liposomes. Although echogenic liposomes have been widely investigated for simultaneous ultrasound imaging and controlled drug delivery, the potential of the polymersomes remains unexplored. We prepared two different echogenic polymersomes from the amphiphilic co-polymers polyethylene glycol–poly-DL-lactic acid (PEG-PLA) and polyethylene glycol–poly-L-lactic acid (PEG-PLLA), incorporating multiple freeze-dry cycles in the synthesis protocol to ensure their echogenicity. We investigated acoustic behavior with potential applications in biomedical imaging. We characterized the polymeric vesicles acoustically with three different excitation frequencies of 2.25, 5 and 10 MHz at 500 kPa. The polymersomes exhibited strong echogenicity at all three excitation frequencies (about 50- and 25-dB enhancements in fundamental and subharmonic, respectively, at 5-MHz excitation from 20 μg/mL polymers in solution). Unlike echogenic liposomes, they emitted strong subharmonic responses. The scattering results indicated their potential as contrast agents, which was also confirmed by clinical ultrasound imaging. (E-mail: sarkar@gwu.edu)

Key Words: **Ultrasound imaging, Contrast agent, Microbubble, Polymersomes, Echogenic, Drug delivery.**

INTRODUCTION

Ultrasound imaging is one of the most widely employed diagnostic tools in the medical field. However, it suffers from poor contrast. To improve the contrast of ultrasound imaging, coated microbubbles are used as contrast agents (deJong et al. 1991; Goldberg et al. 2001; Paul et al. 2014). They are also being investigated as potential drug delivery agents (Bull 2007; Eisenbrey et al. 2010; Klibanov 2006; Paul et al. 2014). These microbubbles (diameter: 0.5–5 μm), although ideal as vascular agents, cannot extravasate through pores of blood vessels. The leaky vasculature of a solid tumor with pores typically 100–700 nm offers great potential for targeted delivery into tumors through enhanced permeability and retention (EPR) (Maeda et al. 2000).

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Nanometer-size lipid bilayer vesicles or liposomes are ideal extravascular drug delivery agents for tumors. Recently, we and other groups have reported that when prepared in the presence of mannitol as an excipient, liposomes can be made echogenic (echogenic liposomes [ELIPs]) (Coussios et al. 2004; Huang 2008; Huang et al. 2001; Kee et al. 2007; Kopechek et al. 2011; Nahire et al. 2012, 2013, 2014b; Paul et al. 2012, 2014). Here we report on a novel echogenic vesicle, which, unlike liposomes, is enclosed by an amphiphilic polymer bilayer.

As noted above, ELIPs have been widely studied. Huang and co-workers optimized the protocol for their preparation (Hamilton et al. 2002; Huang et al. 2001, 2002a, 2002b). The first acoustical characterization of ELIPs was performed employing a 20-MHz frequency catheter for both *in vitro* and *in vivo* experiments (Alkan-Onyuksel et al. 1996). Videodensitometry analysis was used to measure the echogenicity of liposomes (Buchanan et al. 2008; Huang 2008). Coussios et al. (2004) reported an *in vitro* characterization of ELIPs, in which they found backscattering

signals from these liposomes to be comparable to those of the microbubble-based contrast agent Optison. The echogenicity of liposomes was detected with a Philips L12-5 linear array transducer system using B-mode pulses (Smith et al. 2007). Although the exact mechanism underlying the echogenicity of ELIPs remains unknown—specifically the exact location of the gas body in the small vesicles responsible for generating the reflected signals—careful *in vitro* characterization has convincingly indicated its echogenicity for both fundamental and second harmonic signals. (Paul et al. 2012). Modeling of ELIP acoustic behaviors has also been attempted by assuming gas pockets of comparable size (Raymond et al. 2015).

Polymersomes (Discher et al. 1999) are 100- to 600-nm vesicles enclosed by bilayer membranes that are made of amphiphilic block copolymers, instead of lipids. The vesicles carry hydrophobic drugs in the polymer bilayer and hydrophilic molecules in the aqueous core. They provide unique advantages in biomedical applications (Zhang and Zhang 2017). For example, the membrane of the polymersomes is almost an order of magnitude tougher, sustains far greater areal strain before rupture, and is at least 10 times less permeable to water than phospholipid bilayers (Discher et al. 1999). Incorporation of the biocompatible polyethylene glycol (PEG) as the hydrophilic block renders the vesicles long circulating (Mohammadi et al. 2017). A targeting peptide or ligand actively transports the polymersomes to the cancer tissues and inside the cytosol (Simon-Gracia et al. 2016). We recently reported that the presence of small organic molecules can successfully target the polymeric vesicles to cancer cell nuclei (Anajafi et al. 2016) or mitochondria (Kulkarni et al. 2016b). Because of their stability, after targeting, the polymersome requires a trigger to release the encapsulated drugs (Hu et al. 2017; Thambi et al. 2016). We have used enhanced concentrations of reducing agents and hypoxia (internal triggers) of solid tumors to deliver anticancer drugs from polymersomes successfully (Anajafi et al. 2016; Karandish et al. 2016; Kulkarni et al. 2016a).

Although ultrasound is known primarily for its imaging capabilities, it is also widely used as an external trigger to release encapsulated drugs from liposomes (Moussa et al. 2015; Zhang et al. 2016). However, to date, ultrasound-sensitive polymersomes have hardly been studied. In a preliminary investigation of polymersomes prepared with PEG–poly-DL-lactic acid (PEG-PLA) block co-polymers in the presence of mannitol, Zhou et al. (2006) reported acoustic activity in a diagnostic imaging system. We recently reported the preparation of echogenic polymersomes from the reduction-sensitive, amphiphilic block copolymer PEG–S–S–PLA with 10-dB enhancement in the scattering signal for a concentration of 10 μg/mL (Nahire et al. 2014a). To our knowledge, there has not been another report on interactions between ultrasound and polymersomes. Moreover, detailed acoustic characterization of such polymersomes is lacking.

The meager literature on echogenic polymersomes and, at the same time, their potential for dual drug delivery and imaging capabilities warrant further careful investigation. Also, polymersome (as well as liposome) suspensions, although of an average size of 100–600 nm, have significant polydispersity (Nahire et al. 2014a), indicating the possibility of excitation frequency-dependent responses. The resonant frequency of an entrapped air pocket responsible for echogenicity depends on its size. As noted earlier, the exact location and size of the air pocket responsible for the echogenicity of ELIPs or echogenic polymersomes remain uncertain. It is possible that the vesicles act as cavitation nuclei that are responsible for generating air bubbles, which then determine the echogenicity. In any event, investigation of the acoustic response of echogenic polymersomes as a function of frequency is key to their desired clinical use. Such an investigation, in turn, could shed light on the mechanism of echogenicity of ELIPs or polymersomes. In the work described here, we investigated the scattered responses of echogenic polymersomes) to ultrasound waves as a function of frequency (2.25, 5 and 10 MHz. We also studied polymersomes made of two different amphiphilic polymers, PEG-PLA and PEG–poly-L-lactic acid (PEG-PLLA) (PLA and PLLA are stereoisomers) to investigate the effects of stereochemistry and how they might affect the packing of the bilayer and thereby the mechanical properties and scattered response.

METHODS

Preparation of polymersomes

Echogenic polymersomes were prepared by the solvent exchange method (Lee and Feijen 2012) using the synthesized PEG(2000)-PLA(5000) and commercially available PEG(2000)-PLLA(5000) (Akina, West Lafayette, IN, USA). Polymers (6 mg/mL) were dissolved in tetrahydrofuran (THF). Note that the choice of molecular weight of the polymers is determined by the ability to form bilayers and polymersomes. In the future, we plan to explore the effects of molecular weight variation with these or other polymersomes. Each polymersome batch was prepared by adding 2.5 mg of polymer dropwise to 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution (pH 7.4) containing 0.32 M mannitol. The solutions were stirred for 20 min at room temperature, and then air was passed through the mixture for 45 min to remove the organic solvent. The polymersomes formed were sonicated at 25 °C for 60 min (Symphony 117 V, 60 Hz, power level: 9). Subsequently, the polymersomes were freeze-thawed for 3 cycles (–80 °C and 65 °C) and then freeze-dried using a Labconco instrument for 4 d. The

reconstituted echogenic polymersomes in buffer were used to conduct the experiments.

Atomic force microscopy imaging

The two polymersome solutions (PEG–PLA and PEG–PLLA, both 20 µg/mL) were prepared by reconstituting the freeze-dried powder in an aqueous buffer (5 mM HEPES buffer, pH 7.4). The samples were prepared by incubating 10 µL of each solution on silicon substrates for 20 min in a sealed compartment to protect from evaporation at room temperature. The samples were gently dried under purified airflow. The imaging measurements were performed using a commercial atomic force microscope (NT-MDT NTEGRA AFM). Samples were imaged under ambient conditions in semi-contact mode with a resonant frequency of 190-kHz AFM probes (Budget sensors).

Ultrasound imaging

Dried polymersomes were reconstituted in 5 mM HEPES buffer, pH 7.4, at a concentration of 20 µg/mL. In a 96-well plate, 200 µL of echogenic polymersomes was dispensed into each well, and the plate was covered with parafilm. Ultrasound gel (Aquasonic 100, Parker Laboratories) was applied to a 15-MHz linear ultrasound transducer. Diagnostic ultrasound Terason t3200 (MediCorp LLC) was employed to image the polymersomes. In another set of experiment, we performed the same experiment, but with degassed HEPES buffer. Integrated density was measured with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) using the following correlation: corrected integrated density = integrated density of echogenic area – (area of echogenic polymersomes × mean intensity of control reading).

Experimental setup to measure scattering

The scattering setup (Fig. 1) employed two spherically focused transducers with the same specifications (V310-SU, Olympus NDT, Waltham, MA, USA). All transducers were calibrated using a needle hydrophone (HNC400, ONDA, Sunnyvale, CA). Each measurement was made with two transducers of the same central frequency, either 2.25 MHz (with a –6 dB: 1.48–2.90 MHz), 5 MHz (with a –6 dB: 2.95–6.77 MHz) or 10 MHz (with a –6 dB: 6.96–13.16 MHz). The transmitting and receiving transducers were fixed confocally perpendicular to each other in a 100-mL polycarbonate tank filled with deionized water, as illustrated in Figure 1. The alignment of the transducers was confirmed by checking for a strong reflection from a thin metal wire placed in the focal region. An 8-mL triangular prism was used as a sample chamber, in which the polymersome suspension was dispersed and filled. The chamber is made of a 3-D printed frame of acrylonitrile butadiene styrene (ABS) with acoustically transparent windows made of Saran Wrap. A function generator (Model AFG 3251; Tektronix, Beaverton, OR, USA) was utilized to generate a 32-cycle sinusoidal pulse of 2.25-, 5- or 10-MHz frequency at a pulse repetition frequency of 100 Hz. This signal was then amplified using a 55-dB power amplifier (Model A-300; ENI, Rochester, NY, USA) and fed to the transmitting transducer. The polymersomes at the focal volume of the transducer scattered the input signal, which was received by the receiving transducer and amplified by the pulser/receiver (Panametrics 5800, Waltham, MA USA) in through mode with a 20-dB gain. The amplified signals were then sent to the oscilloscope to be viewed in real time. Voltage-time radiofrequency signals were averaged with every 64 sequences by the oscilloscope and then were transmitted and saved onto a desktop computer using the software Signal Express Tektronix Edition (Xia et al. 2014).

Experimental procedure and data reduction

Polymersomes in the form of a dry powder were stored in the glass vial and placed in the refrigerator at 4 °C. The scattering experiment was conducted by reconstituting the dry powder in a mixture of phosphate-

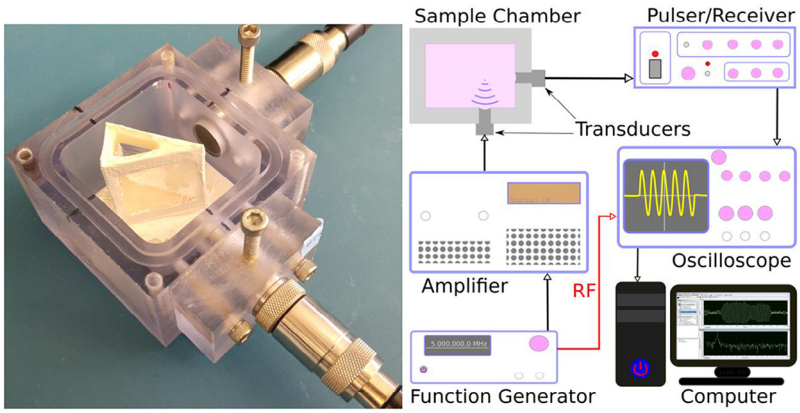

Fig. 1. Experimental setups for measuring scattering signals. RF = radiofrequency.

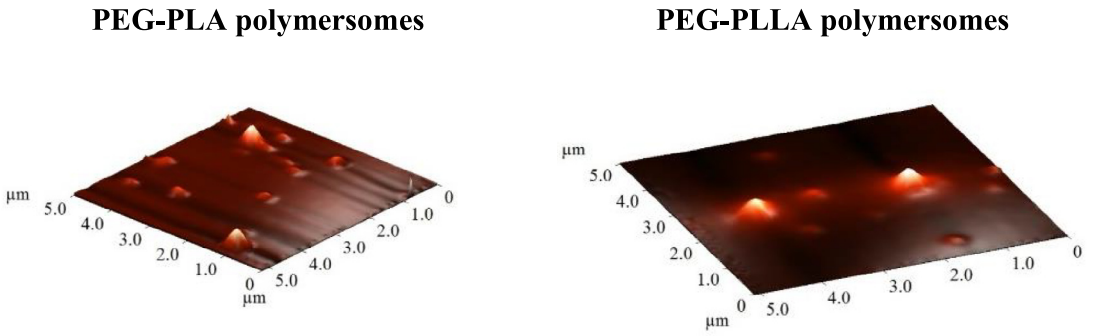

Fig. 2. Atomic force microscopic images of the echogenic polyethylene glycol–poly-L-lactic acid (PEG-PLLA) and polyethylene glycol–poly-DL-lactic acid (PEG-PLA) polymersomes.

buffered saline (PBS) and bovine serum albumin (BSA) to a concentration of 20 µg polymer/mL and injecting 8 mL into the sample chamber. For the experiments in degassed solution, the dissolved gas in the solution of PBS and BSA was removed by a vacuum pump. The measurement was repeated four times to guarantee the reliability of experimental data. The control signal, that is, without polymersomes, and the response of the polymersomes were measured as mentioned before. A MATLAB FFT program was used to obtain the average response in the frequency domain (25 voltage–time acquisitions are used). The scattered response was converted into a decibel scale by taking the reference voltage to be unity. Fundamental, second and subharmonic scattered responses were extracted from the power spectrum. The final data are reported as enhancement over the control signal (without polymersomes). Therefore, for the enhancement, effectively the reference signal of the decibel scale is the scattering from the buffer solution. Each experiment was repeated four times with fresh samples (n = 4). Data are presented as the mean ± standard error of the mean. Student's t-test was performed to determine statistical significance ($p < 0.05$), which is indicated by asterisks in figures.

RESULTS AND DISCUSSION

Size and morphology

Size and morphology of the echogenic PEG-PLA and PEG-PLLA polymersomes were characterized by atomic force microscopy. Images reveal they are spherical (Fig. 2). We observed that the average diameters of the PEG-PLA and the PEG-PLLA polymersomes were 400 and 450 nm, respectively. We also determined the hydrodynamic diameters of the two batches of polymersomes by dynamic light scattering. The average hydrodynamic diameters of the PEG-PLA and PEG-PLLA polymersomes were 407 ± 7 and 453 ± 10 nm, respectively (Fig. 3).

Echogenicity by a clinical ultrasound scanner

The scattered responses were also confirmed by using a clinical ultrasound scanner (Terason t3200) (Fig. 4). In degassed solution, however, the same setup revealed no signals because of the presence of polymersomes (Fig. 5). This indicates that the preparation protocol, which closely mimics that used for echogenic liposomes incorporating freeze-drying in the presence of mannitol, was successful in rendering the polymersomes echogenic. As noted

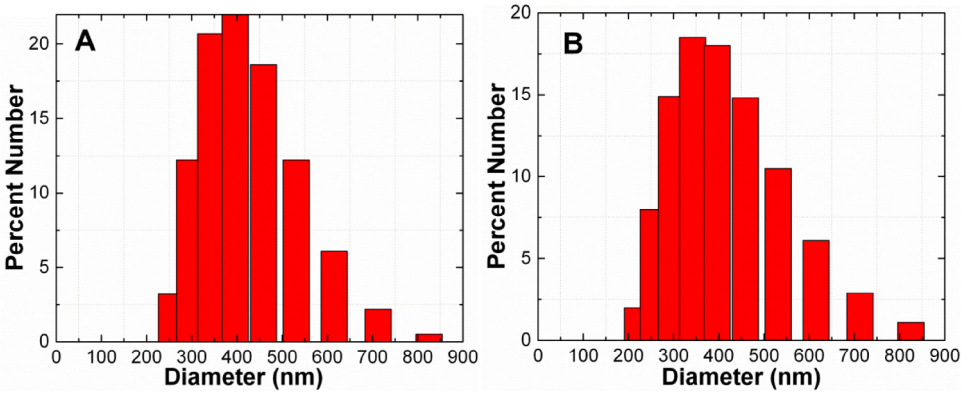

Fig. 3. Hydrodynamic diameters of the (A) polyethylene glycol–poly-DL-lactic acid and (B) polyethylene glycol–poly-L-lactic acid polymersomes, as determined by dynamic light scattering.

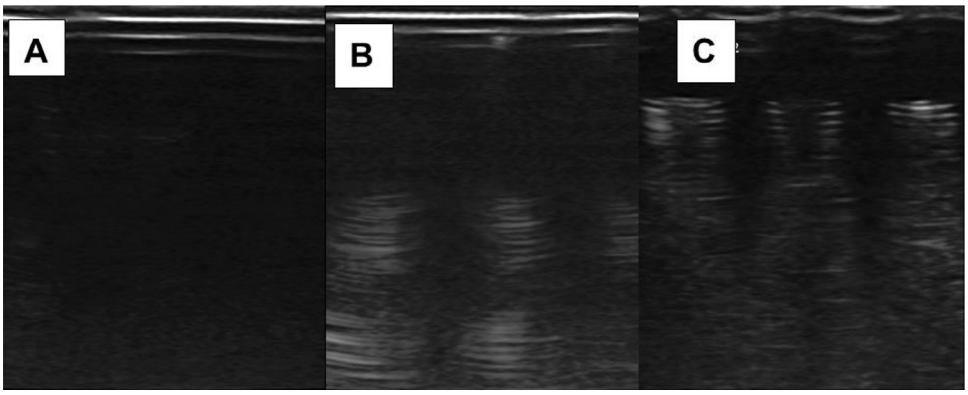

Fig. 4. Ultrasound images of (A) control (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] pH 7.4), (B) polyethylene glycol–poly-DL-lactic acid (PEG-PLA) echogenic polymersomes and (C) polyethylene glycol–poly-L-lactic acid (PEG-PLLA) echogenic polymersomes using Terason t3200.

earlier, the exact mechanism underlying echogenicity remains unknown. However, freeze-drying in the presence of mannitol is presumed to leave echo-generating air pockets trapped in the bilayer (Huang 2008; Huang et al. 2001, 2002a, 2002b; Huang and MacDonald 2004). The intensity of the ultrasound images in Figure 4 was determined with ImageJ software and is illustrated Figure 6. The integrated density of the ultrasound echo shows that the PEG-PLA polymersomes were more echogenic than the PEG-PLLA polymersomes.

Linear and non-linear scattering

Microbubbles generate non-linear signals (sub- and second harmonics) at higher excitation pressures (deJong et al. 1994; Katiyar and Sarkar 2011; Katiyar et al. 2011; Paul et al. 2010, 2013; Sarkar et al. 2005). They can be used for harmonic (imaging at twice the excitation frequency) (Chang et al. 1995) and subharmonic (imaging at half the excitation frequency) imaging (Shankar et al. 1998; Shi and Forsberg 2000). Here, we investigated the scattered responses, including non-linear activities, of the polymersome suspension at an excitation pressure amplitude of 500 kPa to obtain possible non-linear responses without substantial destruction for all reported frequencies. In Figure 7a are the frequency spectra of scattered responses from both PEG-PLA and PEG-PLLA at the excitation pressure of 500 kPa and excitation frequency of 5 MHz. The fundamental response at 5 MHz is almost 50 dB higher than that of the control (without polymersomes) for both polymersomes, which indicates that they are highly echogenic at this concentration. In contrast, Figure 7b, which is a plot of the scattering responses of both polymersomes in a degassed solution, indicates that neither of them is echogenic, a finding consistent across four repeated measurements. This means that the presence of dissolved gas is essential to the echogenicity of the polymersomes. We do find a weak second harmonic peak for PEG-PLA polymersomes (Fig. 7a).

Figure 8 provides a detailed analysis of the acoustic scattering, illustrating responses at the fundamental and

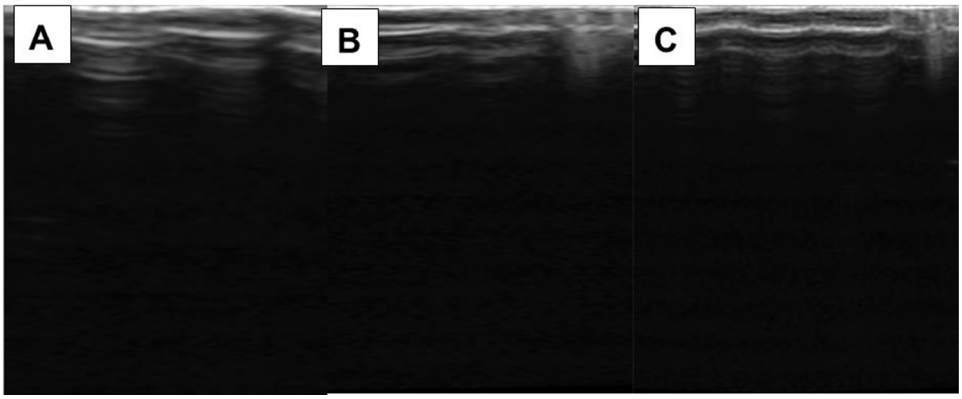

Fig. 5. Ultrasound images with degassed buffer with Terason t3200. (A) Control (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] buffer, pH 7.4). (B) Polyethylene glycol–poly-DL-lactic acid (PEG-PLA) echogenic polymersomes. (C) Polyethylene glycol–poly-L-lactic acid (PEG-PLA) echogenic polymersomes.

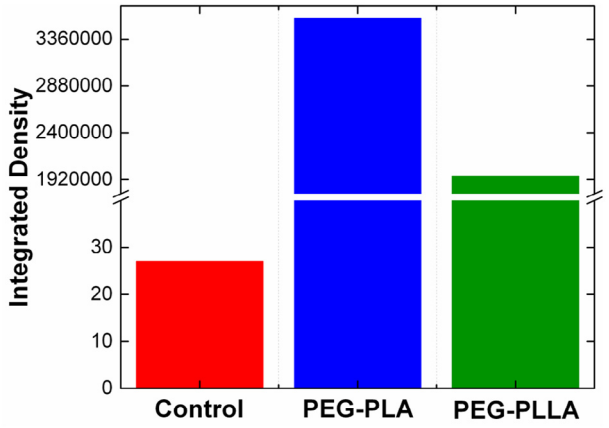

Fig. 6. Integrated density calculated with ImageJ software for control (*red bar*), polyethylene glycol–poly-L-lactic acid (PEG-PLLA) echogenic polymersomes (*blue bar*) and polyethylene glycol–poly-DL-lactic acid (PEG-PLA) echogenic polymersomes (*green bar*).

subharmonic frequencies. At the excitation frequency of 2.25 MHz, both PEG-PLA and PEG-PLLA enhance the fundamental frequency by almost 20 dB. The enhancement is similar to what we observed previously for echogenic liposomes (15- to 20-dB enhancement at a concentration of 10 μg/mL) that were obtained with a similar setup at an excitation frequency of 3.5 MHz (Paul et al. 2012). Note that comparison of the lipid (10 μg/mL) and polymer (20 μg/mL) concentrations in ELIPs and polymersomes is difficult because of the difference in their molecular weight: ~ 13 μM for lipids (molecular weight of DSPC [1,2-distearoyl-*sn*-glycero-3-phosphorylcholine] 790) and 2.5 μM for polymers (molecular weight: 8000). Note that concentration here was chosen to find sufficiently strong scattered signal. Future studies in appropriate animal models are needed to determine the proper clinical dosage. At 2.25 MHz, both polymersomes generate significant intensities at subharmonic frequency, exhibiting their potential to be used in subharmonic imaging. Note that ELIPs did not generate subharmonic responses even at 750-kPa excitation (Paul et al. 2012). The scattered responses of the two different polymersomes are statistically similar. At 5-MHz excitation frequency, the polymersomes produce an enhancement of more than 50 dB in the fundamental component, which is significant compared with conventional microbubble-based ultrasound contrast agents. Even the subharmonic response is strong (30 dB) at 5-MHz excitation. At 10-MHz excitation, these polymersomes provide an enhancement of 30 dB. Generally, for microbubble-based contrast agents far off the resonant frequency, the scattering responses are lower. Comparison of the scattered responses at these three frequencies and the observation of maximum scattering at 5 MHz suggest that 5 MHz is closer to the average resonance frequency of the gas pockets associated with the polymersome population. However, also note that the broadband signal seen in Figure 7a indicates the possibility of inertial cavitation. Polymersomes with small air pockets may act as cavitation nuclei and, under acoustic excitation, may have grown to become coated bubbles to generate the echogenicity.

From the preceding results, we see that the polymersomes are good at scattering ultrasound. However, to be a viable ultrasound contrast agent, polymersomes should be stable until reaching the target tissue and continue to scatter ultrasound for a sufficient period. To check this aspect, we performed the time-dependent scattering experiments on the polymersomes. The fundamental responses for PEG-PLLA and PEG-PLLA are compared in

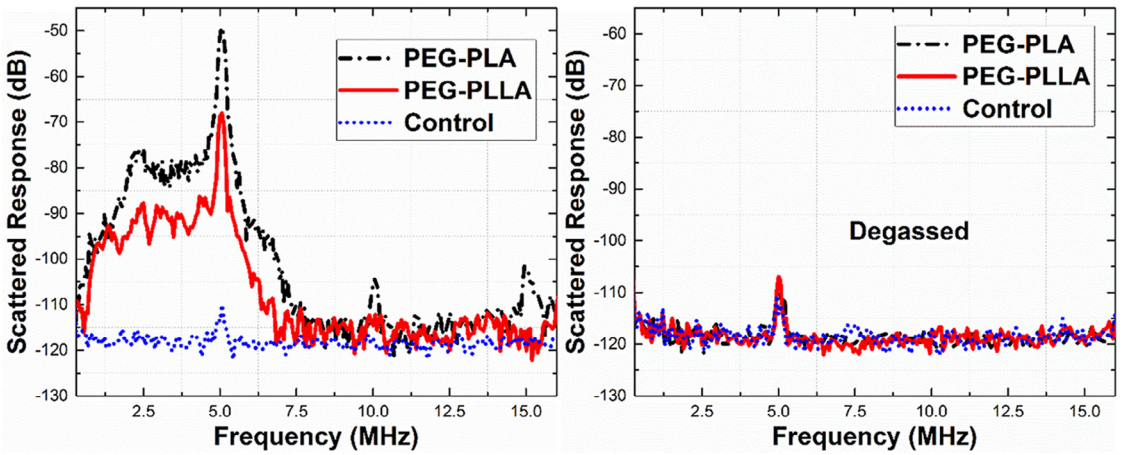

Fig. 7. Frequency spectra of polyethylene glycol–poly-L-lactic acid (PEG-PLLA) and polyethylene glycol–poly-DL-lactic acid (PEG-PLA) polymersomes and control (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] buffer, pH 7.4) at an excitation frequency of 5 MHz and excitation pressure of 500 kPa. Left: In solution with dissolved gas. Right: In degassed solution.

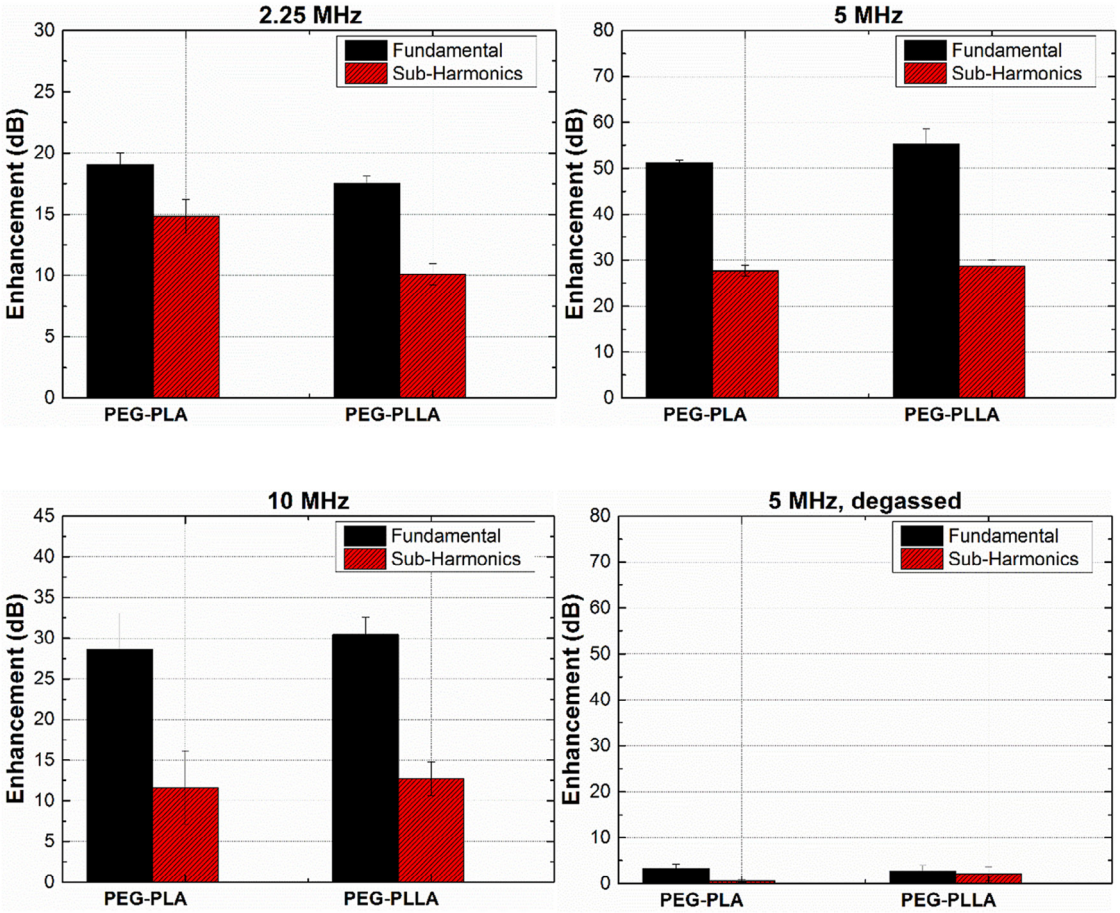

Fig. 8. Enhancement of scattering signals for the echogenic polymersomes at excitation pressure of 500 kPa excitation pressure and excitation frequencies of 2.25, 5 and 10 MHz. PEG-PLLA = polyethylene glycol–poly-L-lactic acid, PEG-PLA = polyethylene glycol–poly-DL-lactic acid.

Figure 9 as functions of time. At the excitation frequency of 2.25 MHz, PEG-PLA has a slightly higher response than PEG-PLLA, whereas at 5 MHz, the response of PEG-PLLA is higher. There is no significant difference in time-dependent scattering responses of the two polymersomes at 10 MHz. The curves all exhibit the tendency for fundamental responses to first increase with increasing time and then decrease, resulting in a peak amplitude within the time interval investigated. The eventual decrease in signal with time indicates slow destruction. Even though the signals decrease, PEG-PLLA polymersomes generate signals sufficiently long, especially at 5 MHz, to be explored for clinical use. We, however, note that their stability may differ greatly under physiologic pressure and flow conditions, a possibility that can be ascertained by further studies in animal models. We also note that the observation could also be affected by polymersome replenishment in the probe area induced by acoustic streaming. The results seen are an integrated effect of all such phenomena. As noted before, echogenicity is believed to be caused by air pockets trapped in the polymersomes, the scattering cross sections of a bubble being orders of magnitude higher than those of a solid or liquid sphere (Pierce 2007). The rise in echogenicity with time indicates growth or generation of air bubbles. Note that perfluorocarbon-based microbubbles have been reported to grow after introduction into a suspension because of the influx of dissolved air from the surrounding liquid (Chatterjee et al. 2005; Shi and Forsberg 2000). The gas diffusion and resulting growth have been mathematically modeled (Chen et al. 2002; Kabalnov et al. 1998) to account for the effects of the encapsulation (Katiyar et al. 2009; Sarkar et al. 2009). However, here for polymersomes, in the absence of a second gas, the transient increase in echogenicity is puzzling. It indicates a growth in the air pockets trapped after the introduction of polymersomes into the solution over time in the presence of ultrasound. The growth could be due to rectified diffusion—imbalance in diffusion of dissolved air in the surrounding medium into and out of the bubble during expansion and contraction because of

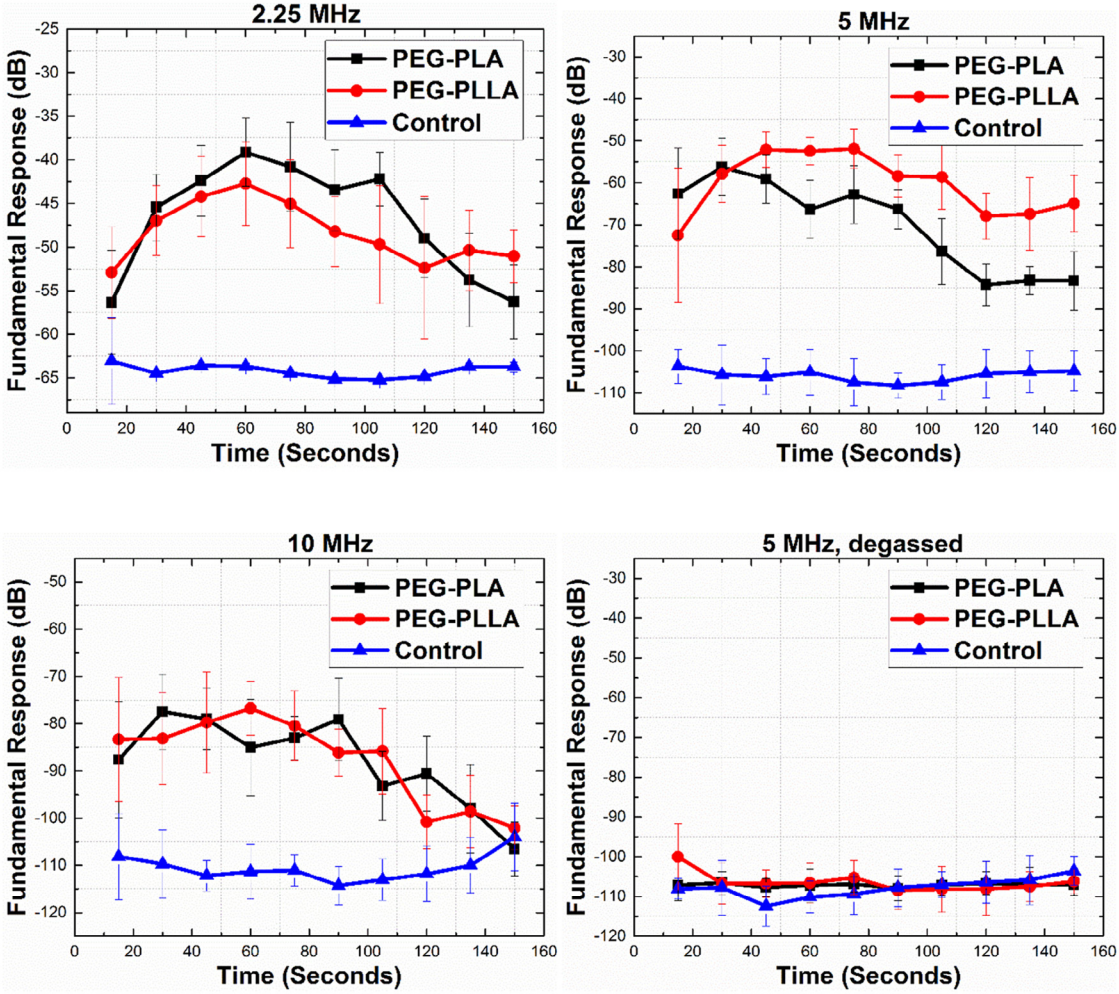

Fig. 9. Evolution of fundamental responses from echogenic polymersomes with respective to time. PEG-PLLA = polyethylene glycol–poly-L-lactic acid, PEG-PLA = polyethylene glycol–poly-DL-lactic acid.

differences in surface area (Crum 1984; Eller and Flynn 1965). Using the Gilmore–Akulichev equation, Church (1988) computed the threshold pressure for rectified diffusion as ~300 kPa in the range 1–5 MHz for an initial bubble radius of ~250 nm. The results are for clean air–water surface tension values. For any coating, the surface tension will be reduced, facilitating growth, but the coating also impedes air transport.

The non-linear signals—sub- and superharmonics—scattered by contrast microbubbles offer opportunities for novel non-linear imaging modalities. Time-dependent subharmonic responses of both polymersomes, plotted in Figure 10, reveal trends similar to those of the fundamental responses in Figure 9. The degradation with time is maximum at 10 MHz, but at 5 MHz, the signal from PEG-PLLA retains its echogenicity for a long period, a scenario similar to what was seen for fundamental responses (Fig. 9). In view of the results, we conclude that the difference in stereochemistry between the two polymers, PEG-PLLA and PEG-PLA, does not lead to large differences in the scattering properties of the corresponding polymersomes. One would have expected differences because of better packing in the homogeneous PEG-PLLA bilayer, in contrast to PEG-PLA, which consists of a racemic mixture of two PEG-conjugated stereochemical forms: poly (L-lactide) (PLLA) and poly (D-lactide) (PDLA).

CONCLUSIONS

We prepared two different echogenic polymersomes from the amphiphilic polymers PEG-PLA and PEG-PLLA, respectively, and performed acoustic investigations of their dynamic behaviors. The strong acoustic responses, fundamental as well as subharmonic, of both polymersome suspensions indicated their ability to be used as contrast agents. Both samples produce about 20-dB fundamental

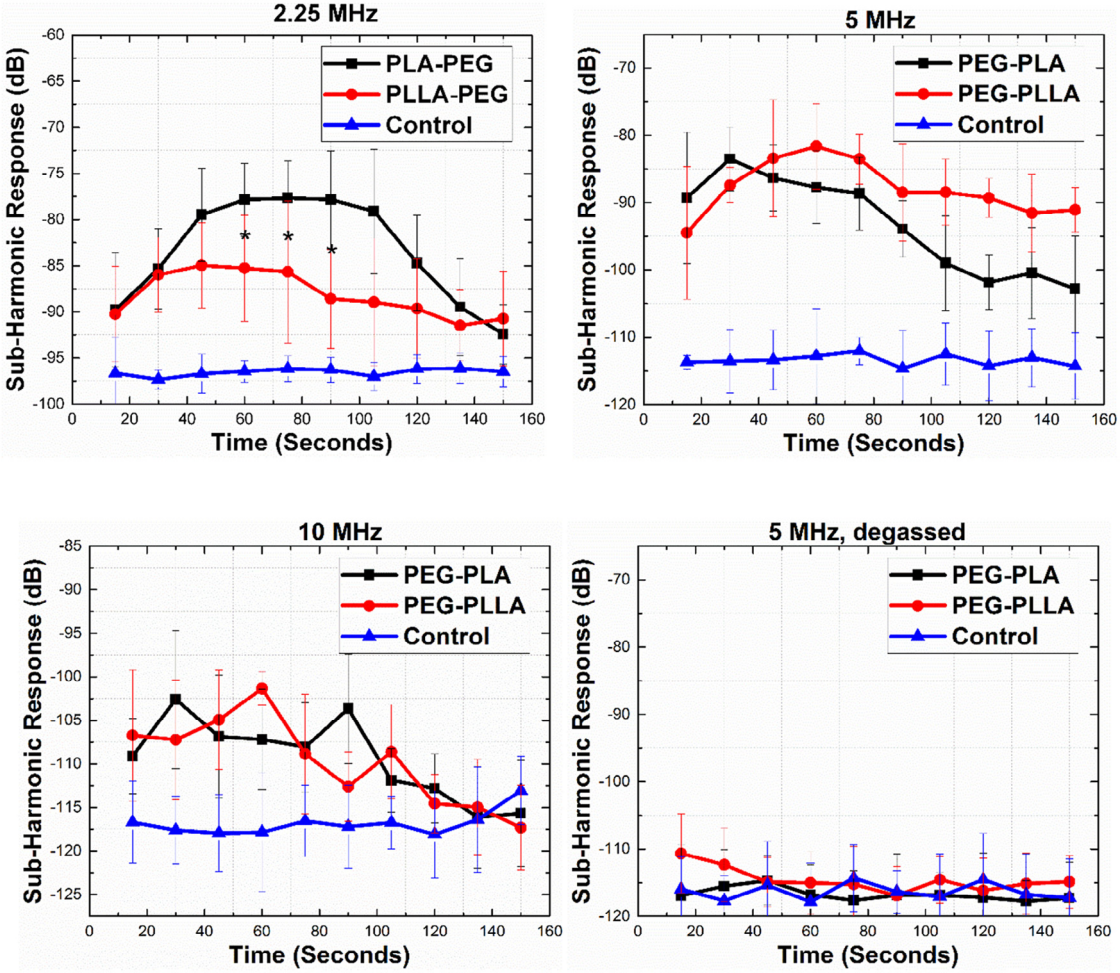

Fig. 10. Evolution of subharmonic responses from echogenic polymersomes with respect to time. *$p < 0.05$, Student's *t*-test for differences between the responses of glycol–poly-L-lactic acid (PEG-PLLA) and polyethylene glycol–poly-DL-lactic acid (PEG-PLA) polymersomes.

enhancement and 15-dB subharmonic enhancement at the excitation frequency of 2.25 MHz. The corresponding enhancements at 5-MHz excitation frequency are stronger—50 dB for fundamental and 25 dB for subharmonic. Finally, at 10 MHz, enhancements are 30 dB (fundamental) and 10 dB (subharmonic). The strong acoustic emission at subharmonic frequency is in sharp contrast to observation of no such emission from echogenic liposomes. PEG-PLA and PEG-PLLA, despite the differences in their stereochemistry, exhibited similar behavior at the excitation frequencies examined. Both polymersome suspensions had relatively consistent signals in the time-dependent scattering experiments. Experiments performed in degassed solutions revealed no echogenicity, confirming the key role played by the dissolved gas in ensuring the echogenicity of the polymersomes. Meanwhile, emission of subharmonics suggests the presence of air-filled microbubbles that are responsible for the echogenicity as the subharmonic signal is the unique signature of microbubbles. Polymersome echogenicity was found to increase with time initially, similar to what has been observed for perfluorocarbon gas-filled microbubbles. Future studies should include attenuation through a polymersome suspension, which can provide additional important information critical to ultrasound imaging. Furthermore, investigation should be directed to the *in vivo* stability of polymersomes, as well as the mechanism of microbubble generation in the polymersome suspension.

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REFERENCES

Alkan-Onyuksel H, Demos SM, Lanza GM, Vonesh MJ, Klegerman ME, Kane BJ, Kuszak J, McPherson DD. Development of inherently echogenic liposomes as an ultrasonic contrast agent. J Pharm Sci 1996; 85:486–490.

Anajafi T, Scott MD, You S, Yang X, Choi Y, Qian SY, Mallik S. Acridine orange conjugated polymersomes for simultaneous nuclear delivery of gemcitabine and doxorubicin to pancreatic cancer cells. Bioconjug Chem 2016;27:762–771.

Buchanan KD, Huang S, Kim H, MacDonald RC, McPherson DD. Echogenic liposome compositions for increased retention of ultrasound reflectivity at physiologic temperature. J Pharm Sci 2008;97:2242–2249.

Bull JL. The application of microbubbles for targeted drug delivery. Expert Opin Drug Deliv 2007;4:475–493.

Chang PH, Shun K, Wu SJ, Levene HB. Second harmonic imaging and harmonic Doppler measurements with Albunex. IEEE Trans Ultrason Ferroelectr Freq Control 1995;42:1020–1027.

Chatterjee D, Jain P, Sarkar K. Ultrasound-mediated destruction of contrast microbubbles used for medical imaging and drug delivery. Phys Fluids 2005;17:100603.

Chen WS, Matula TJ, Crum LA. The disappearance of ultrasound contrast bubbles: Observations of bubble dissolution and cavitation nucleation. Ultrasound Med Biol 2002;28:793–803.

Church CC. Prediction of rectified diffusion during nonlinear bubble pulsations at biomedical frequencies. J Acoust Soc Am 1988;83:2210–2217.

Coussios CC, Holland CK, Jakubowska L, Huang SL, MacDonald RC, Nagaraj A, McPherson DD. In vitro characterization of liposomes and Optison (R) by acoustic scattering at 3.5 MHz. Ultrasound Med Biol 2004;30:181–190.

Crum LA. Acoustic cavitation series: 5. Rectified diffusion. Ultrasonics 1984;22:215–223.

deJong N, Cornet R, Lancee CT. Higher harmonics of vibrating gas-filled microspheres: 1. Simulations. Ultrasonics 1994;32:447–453.

deJong N, Tencate FJ, Lancee CT, Roelandt JRTC, Bom N. Principles and recent developments in ultrasound contrast agents. Ultrasonics 1991;29:324–330.

Discher BM, Won Y-Y, Ege DS, Lee JC, Bates FS, Discher DE, Hammer DA. Polymersomes: Tough vesicles made from diblock copolymers. Science 1999;284:1143–1146.

Eisenbrey JR, Burstein OM, Kambhampati R, Forsberg F, Liu JB, Wheatley MA. Development and optimization of a doxorubicin loaded poly(lactic acid) contrast agent for ultrasound directed drug delivery. J Control Release 2010;143:38–44.

Eller A, Flynn HG. Rectified diffusion during nonlinear pulsations of cavitation bubbles. J Acoust Soc Am 1965;37:493.

Goldberg BB, Raichlen JS, Forsberg F. Ultrasound contrast agents: Basic principles and clinical applications. London: Martin Dunitz; 2001.

Hamilton A, Huang SL, Warnick D, Stein A, Rabbat M, Madhav T, Kane B, Nagaraj A, Klegerman M, MacDonald R, McPherson D. Left ventricular thrombus enhancement after intravenous injection of echogenic immunoliposomes: Studies in a new experimental model. Circulation 2002;105:2772–2778.

Hu X, Zhang Y, Xie Z, Jing X, Bellotti A, Gu Z. Stimuli-responsive polymersomes for biomedical applications. Biomacromolecules 2017;18:649–673.

Huang SL. Liposomes in ultrasonic drug and gene delivery. Adv Drug Deliv Rev 2008;60:1167–1176.

Huang SL, Hamilton AJ, Nagaraj A, Tiukinhoy SD, Klegerman ME, McPherson DD, MacDonald RC. Improving ultrasound reflectivity and stability of echogenic liposomal dispersions for use as targeted ultrasound contrast agents. J Pharm Sci 2001;90:1917–1926.

Huang SL, Hamilton AJ, Pozharski E, Nagaraj A, Klegerman ME, McPherson DD, MacDonald RC. Physical correlates of the ultrasonic reflectivity of lipid dispersions suitable as diagnostic contrast agents. Ultrasound Med Biol 2002a;28:339–348.

Huang SL, Hamilton AJ, Tiukinhoy SD, Nagaraj A, Kane BJ, Klegerman M, McPherson DD, MacDonald RC. Liposomes as ultrasound imaging contrast agents and as ultrasound-sensitive drug delivery agents. Cell Mol Biol Lett 2002b;7:233–235.

Huang SL, MacDonald RC. Acoustically active liposomes for drug encapsulation and ultrasound-triggered release. Biochim Biophys Acta 2004;1665:134–141.

Kabalnov A, Klein D, Pelura T, Schutt E, Weers J. Dissolution of multicomponent microbubbles in the bloodstream: 1. Theory. Ultrasound Med Biol 1998;24:739–749.

Karandish F, Haldar MK, You S, Brooks AE, Brooks BD, Guo B, Choi Y, Mallik S. Prostate-specific membrane antigen targeted polymersomes for delivering mocetinostat and docetaxel to prostate cancer cell spheroids. ACS Omega 2016;1:952–962.

Katiyar A, Sarkar K. Excitation threshold for subharmonic generation from contrast microbubbles. J Acoust Soc Am 2011;130:3137–3147.

Katiyar A, Sarkar K, Forsberg F. Modeling subharmonic response from contrast microbubbles as a function of ambient static pressure. J Acoust Soc Am 2011;129:2325–2335.

Katiyar A, Sarkar K, Jain P. Effects of encapsulation elasticity on the stability of an encapsulated microbubble. J Colloid Interface Sci 2009;336:519–525.

Kee P, Abruzzo T, Smith DAB, Wang B, Huang SL, MacDonald RC, Holland CK, McPherson DD. Synthesis and acoustic characterization of a novel ultrasound controlled drug delivery system based on echogenic liposomes [Abstract]. J Am Coll Cardiol 2007;49:120a.

Klibanov AL. Microbubble contrast agents: Targeted ultrasound imaging and ultrasound-assisted drug-delivery applications. Invest Radiol 2006;41:354–362.

Kopechek JA, Haworth KJ, Raymond JL, Mast TD, Perrin SR, Klegerman ME, Huang SL, Porter TM, McPherson DD, Holland CK. Acoustic characterization of echogenic liposomes: Frequency-dependent attenuation and backscatter. J Acoust Soc Am 2011;130:3472–3481.

Kulkarni P, Haldar MK, You S, Choi Y, Mallik S. Hypoxia-responsive polymersomes for drug delivery to hypoxic pancreatic cancer cells. Biomacromolecules 2016a;17:2507–2513.

Kulkarni PS, Haldar MK, Confeld MI, Langaas CJ, Yang X, Qian SY, Mallik S. Mitochondria-targeted fluorescent polymersomes for drug delivery to cancer cells. Polym Chem 2016b;7:4151–4154.

Lee JS, Feijen J. Polymersomes for drug delivery: Design, formation and characterization. J Control Release 2012;161:473–483.

Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review. J Control Release 2000;65:271–284.

Mohammadi M, Ramezani M, Abnous K, Alibolandi M. Biocompatible polymersomes-based cancer theranostics: Towards multifunctional nanomedicine. Int J Pharm 2017;519:287–303.

Moussa HG, Martins AM, Husseini GA. Review on triggered liposomal drug delivery with a focus on ultrasound. Curr Cancer Drug Targets 2015;15:282–313.

Nahire R, Haldar MK, Paul S, Ambre AH, Meghnani V, Layek B, Katti KS, Gange KN, Singh J, Sarkar K, Mallik S. Multifunctional polymersomes for cytosolic delivery of gemcitabine and doxorubicin to cancer cells. Biomaterials 2014a;35:6482–6497.

Nahire R, Haldar MK, Paul S, Mergoum A, Ambre AH, Katti KS, Gange KN, Srivastava DK, Sarkar K, Mallik S. Polymer-coated echogenic lipid nanoparticles with dual release triggers. Biomacromolecules 2013;14:841–853.

Nahire R, Hossain R, Patel R, Paul S, Meghnani V, Ambre AH, Gange KN, Katti KS, Leclerc E, Srivastava DK, Sarkar K, Mallik S. pH-triggered echogenicity and contents release from liposomes. Mol Pharm 2014b;11:4059–4068.

Nahire R, Paul S, Scott MD, Singh RK, Muhonen WW, Shabb J, Gange KN, Srivastava DK, Sarkar K, Mallik S. Ultrasound enhanced matrix metalloproteinase-9 triggered release of contents from echogenic liposomes. Mol Pharm 2012;9:2554–2564.

Paul S, Katiyar A, Sarkar K, Chatterjee D, Shi WT, Forsberg F. Material characterization of the encapsulation of an ultrasound contrast microbubble and its subharmonic response: Strain-softening interfacial elasticity model. J Acoust Soc Am 2010;127:3846–3857.

Paul S, Nahire R, Mallik S, Sarkar K. Encapsulated microbubbles and echogenic liposomes for contrast ultrasound imaging and targeted drug delivery. Comput Mech 2014;53:413–435.

Paul S, Russakow D, Nahire R, Nandy T, Ambre AH, Katti K, Mallik S, Sarkar K. In vitro measurement of attenuation and nonlinear scattering from echogenic liposomes. Ultrasonics 2012;52: 962–969.

Paul S, Russakow D, Rodgers T, Sarkar K, Cochran M, Wheatley MA. Determination of the interfacial rheological properties of a poly(DL-lactic acid)-encapsulated contrast agent uning in vitro attenuation and scattering. Ultrasound Med Biol 2013;39:1277–1291.

Pierce A. Basic linear acoustics. In: Rossing TD, (ed). Springer handbook of acoustics. Heidelberg: Springer; 2007. p. 25–111.

Raymond JL, Luan Y, van Rooij T, Kooiman K, Huang SL, McPherson DD, Versluis M, de Jong N, Holland CK. Impulse response method for characterization of echogenic liposomesa). J Acoust Soc Am 2015; 137:1693–1703.

Sarkar K, Katiyar A, Jain P. Growth and dissolution of an encapsulated contrast microbubble. Ultrasound Med Biol 2009;35:1385–1396.

Sarkar K, Shi WT, Chatterjee D, Forsberg F. Characterization of ultrasound contrast microbubbles using in vitro experiments and viscous and viscoelastic interface models for encapsulation. J Acoust Soc Am 2005;118:539–550.

Shankar P, Krishna PD, Newhouse V. Advantages of subharmonic over second harmonic backscatter for contrast-to-tissue echo enhancement. Ultrasound Med Biol 1998;24:395–399.

Shi WT, Forsberg F. Ultrasonic characterization of the nonlinear properties of contrast microbubbles. Ultrasound Med Biol 2000;26:93–104.

Simon-Gracia L, Hunt H, Scodeller P, Gaitzsch J, Kotamraju VR, Sugahara KN, Tammik O, Ruoslahti E, Battaglia G, Teesalu T. iRGD peptide conjugation potentiates intraperitoneal tumor delivery of paclitaxel with polymersomes. Biomaterials 2016;104:247–257.

Smith DA, Porter TM, Martinez J, Huang S, MacDonald RC, McPherson DD, Holland CK. Destruction thresholds of echogenic liposomes with clinical diagnostic ultrasound. Ultrasound Med Biol 2007;33:797–809.

Thambi T, Park JH, Lee DS. Stimuli-responsive polymersomes for cancer therapy. Biomater Sci 2016;4:55–69.

Xia L, Paul S, Chitnis PV, Ketterling J, Sheeran P, Dayton P, Sarkar K. Acoustic characterization of polymer-encapsulated microbubbles with different shell-thickness-to-radius ratios using in vitro attenuation and scattering: Comparison between different rheological models. J Acoust Soc Am 2014;135:2309.

Zhang XY, Zhang PY. Polymersomes in nanomedicine: A review. Curr Nanosci 2017;13:124–129.

Zhang Y, Yu J, Bomba HN, Zhu Y, Gu Z. Mechanical force-triggered drug delivery. Chem Rev 2016;116:12536–12563.

Zhou W, Meng F, Engbers G, Feijen J. Biodegradable polymersomes for targeted ultrasound imaging. J Control Release 2006;116:e62–e64.