Ultrasound Enhanced Matrix Metalloproteinase-9 Triggered Release of Contents from Echogenic Liposomes

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Abstract

The extracellular enzyme matrix metalloproteinase-9 (MMP-9) is overexpressed in atherosclerotic plaques and in metastatic cancers. The enzyme is responsible for rupture of the plaques and for the invasion and metastasis of a large number of cancers. The ability of ultrasonic excitation to induce thermal and mechanical effects has been used to release drugs from different carriers. However, majority of these studies were performed with low frequency ultrasound (LFUS) at kHz frequencies. Clinical usage of LFUS excitations will be limited due to harmful biological effects. Herein, we report our results on the release of encapsulated contents from substrate lipopeptide incorporated echogenic liposomes triggered by recombinant human MMP-9. The contents release was further enhanced by the application of diagnostic frequency (3 MHz) ultrasound. The echogenic liposomes were successfully imaged employing a medical ultrasound transducer (4 – 15 MHz). The conditioned cell culture media from cancer cells (secreting MMP-9) released the encapsulated dye from the liposomes (30 – 50%) and this release is also increased (50 – 80%) by applying diagnostic frequency ultrasound (3 MHz) for 3 minutes. With further developments, these liposomes have the potential to serve as multimodal carriers for triggered release and simultaneous ultrasound imaging.

Keywords

Echogenic liposomes; MMP-9 triggered release; Ultrasound triggered release

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1. INTRODUCTION

Liposomes are nano- to micrometer-size vesicles with a hydrated lipid bilayer encapsulating an aqueous phase. Due to their structural similarity with biological cells, liposomes show attractive features as drug carriers (e.g., lesser toxicity, increased uptake and longer circulation time). Hence, liposomes have been extensively investigated for targeted drug-delivery applications[1]. Currently, about 10 liposomal formulations are approved by the US Food and Drug Administration for human use. Conjugation with targeting ligands leads to active targeting of the liposomes to the intended sites for drug delivery and imaging[2]. However, after reaching the intended sites, most of the liposomes release the contents passively and this process is often slow[3]. The rate of release from the liposomes can be increased by the application of external triggers, e.g., temperature[4], pH[5], light[6], ultrasound[7], metal ions[8] and enzymes[9]. Recently, we have demonstrated enzymatic release of liposomal contents in the presence of recombinant as well as cancer cell secreted matrix metalloproteinase-9 (MMP-9)[10].

MMP-2 and -9 are members of Zn^{2+} and Ca^{2+} dependent family of enzymes responsible for degradation of gelatin and collagen (IV and V) in the extracellular matrix[11]. MMPs play an important role in a variety of normal physiological processes, e.g., embryonic development, tissue metamorphosis, angiogenesis, wound healing, ovulation etc. [12] Increased expression levels of MMP-9 and MMP-2 correlate with arthritis, atherosclerosis, cancer and other diseases[13]. These two enzymes hydrolyze and weaken the fibrous caps of the plaques, leading to plaque rupture[14]. MMP-9 is also involved in progression and metastasis of many cancers and are being considered as biomarkers for various types of cancers[15]. Inhibitors of these enzymes are currently in clinical trials for adjuvant therapy of various cardiovascular diseases and cancers[16].

The ability of ultrasonic excitation to induce thermal and/or mechanical effects has been used to release drugs from different carriers such as polymeric assemblies, micelles, emulsions, microcapsules, microspheres and liposomes. However, majority of these studies were performed with low frequency ultrasound (LFUS) at kHz frequencies[17]. Although, inertial/transient cavitation thresholds are much lower at such frequencies[18] favoring ultrasound mediated destruction and subsequent release from liposomes[19], clinical usage of similar excitations will be limited due to harmful biological effects. The release is considerably diminished for MHz range excitation[20]. Diagnostic frequency ultrasound has been used for drug delivery and imaging employing micron-sized bubbles[21] and microbubbles conjugated to liposomes[22].

Recently, a modified protocol for preparing acoustically reflective liposomes (echogenic liposomes or ELIPs) has been reported[23]. The preparation protocol ensures that the liposomes entrap air pockets, although exact location of the entrapped air has not yet been exactly ascertained.[21c, 23c, 24]. It strengthens the mechanical coupling with ultrasound resulting in strong ultrasound echoes. Ultrasound excitation can also be used to destabilize the bilayer membrane and release of contents from liposomes[10b]. Since ELIPs retain all the desired properties of normal liposomes, they have been extensively investigated for simultaneous imaging and drug delivery applications employing diagnostic frequency ultrasound (1 – 10 MHz)[24-25]. However, often the amount of contents released by ultrasound excitation of the ELIPs is not optimal, ranging from 20 – 50%[26]. Herein, we demonstrate that the combination of enzymatic triggering (by MMP-9) and ultrasound excitation leads to considerably higher amounts of contents release from echogenic liposomes. The echogenic liposomes were successfully imaged employing a medical ultrasound transducer (4 – 15 MHz). We also demonstrate that conditioned cell culture media from cancer cells (secreting MMP-9) released the encapsulated dye from the liposomes (30 – 50%) and this release is

also increased (50 – 80%) by applying diagnostic frequency ultrasound (3 MHz) for 3 minutes.

2. MATERIALS AND METHODS

2.1 Synthesis of lipopeptide (LP4)

The lipopeptide LP4 was synthesized using a microwave assisted peptide synthesizer (Liberty, CEM Corporation, Matthews, SC) following a reported protocol[27]. Commercially available Fmoc-protected amino acids (0.1 mM) (Peptides International, KY, USA) were used in the synthesis employing Fmoc-Gly-CLEAR acid resin (Peptides International, KY, USA) as the solid support. A mixture of 1-hydroxybenzotriazole (HOBT, AK Scientific, CA) and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU, ChemPep, FL) was used as coupling agent in 5 fold excess. Coupling steps were performed with 20 W microwave power for 5 minutes at 50 °C except for arginine (25 °C for 25 minutes). Diisopropylethylamine was used as the activator base during the peptide coupling reactions. The Fmoc deprotection was carried out by 5% piperazine (TCI America) using N,N-dimethylformamide as the solvent (Macron Chemicals, NJ). The peptide was cleaved from resin using a mixture of trifluoroacetic acid (TCI America), triisopropyl silane and water (95:2.5:2.5) for three hours at room temperature with constant stirring. Subsequently, the reaction mixture was filtered and the filtrate was treated with cold ether to precipitate the crude peptide, dried and stored at −20 °C until use.

2.2 Purification of crude lipopeptide

Purification of the crude LP4 was conducted by reverse phase high performance liquid chromatography (Shimadzu Scientific Instruments) using a diphenyl semipreparatory column (Grace Vydac, 300 Å pore diameter silica, 5 Qm particle size, 10×250 mm) as the stationary phase. A linear gradient (0-70%) of acetonitrile in water was used at a flow rate of 8 mL/min over 45 minutes. Trifluoroacetic acid (25 mM) was added to both solvents and was monitored at 235 nm using a UV detector. After evaporating solvents from the purified product, the purity was determined using MALDI TOF mass spectrometry with an AB 4800 MALDI TOF/TOF Mass Analyzer using α-cyano-4-hydroxy-cinnamic acid as the ionizing matrix. The dried powder was stored in freezer (−20 °C) until use.

2.3 Circular Dichroism (CD) Spectroscopy

CD spectra were recorded (to ascertain the triple helical structure of LP4) using a Jasco J-815 CD spectrometer with 1 mm path length quartz cuvette. The lipopeptide LP4 (0.5 mg/mL) was dissolved in 4 mM phosphate buffer (pH = 4.0) and stored at 4°C overnight before recording the spectra. For each spectrum, 12 accumulations were carried out at scanning speed of 50 nm/min in order to obtain a good signal to noise ratio. For liposome incorporated LP4, 1 mg/mL concentration was used and 36 accumulations were performed to reduce noise. The CD spectrum of MMP-9 was subtracted from CD spectrum of ELIPs treated with MMP-9. Melting temperature of LP4 was calculated by plotting the intensity at 225 nm against temperature.

2.4 Preparation of dye encapsulated Echogenic Liposomes (ELIPs)

Stock solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Avanti Polar Lipids) was prepared (1 mg/mL) by dissolving in chloroform and stored in freezer. Solutions of POPC (2 mg) and LP4 (2.6 mg) were mixed in the molar ratio of 70:30 respectively in a 10 mL round bottom flask. A thin film at the bottom of the flask was formed by evaporating the solvent at 40 °C using a rotary evaporator. In order to remove any residual solvents, the flask was placed under high vacuum overnight. Subsequently, the dried film was hydrated

with 100 mM carboxyfluorescein (>90% fluorescence is quenched) in HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer (25 mM, pH = 8) with added ions (Ca^{2+} and Zn^{2+}) and 2 mL of 0.64 M mannitol (final concentration 0.32 M) at 50 °C. Mannitol is a weak cryoprotectant; we have recently observed that finite amount of mannitol during preparation is critical for ensuring the echogenicity of ELIPs[23c]. The lipids were hydrated for 3 hours and the resultant multilamellar vesicles were bath sonicated for 10 minutes. The liposomal solution was exposed to three freeze (−70 °C) and thaw cycles. Subsequently, the liposomes were extruded first through 800 nm and then 200 nm polycarbonate filters (Nuclepore, Whatman) 15 times at 60 °C using a mini-extruder (Avanti Polar Lipids). To remove the unencapsulated dye, the liposomes were gel filtered using Sephadex G-100 column conditioned with HEPES buffer (25 mM, pH = 8). The osmolarity of the eluent buffer was adjusted (540 mOsm/kg with 0.17 M NaCl solution in HEPES buffer, pH = 8,) to that of the liposomal solution to ensure the minimum leakage of dye from liposomes due to osmotic shock. A similar procedure was followed to prepare the liposomes without any encapsulated dye using HEPES buffer without carboxyfluorescein. ELIPs for CD spectroscopic studies were prepared by the same method using a 4 mM phosphate buffer (pH = 4.0).

2.5 Measurement of Size Distribution

Particle size distribution (PSD) of ELIPs was measured using a Dynamic Light Scattering (DLS) instrument (Malvern Zetasizer Nano-ZS90). DTS 0012 polystyrene latex disposable sizing cuvette (RI: 1.59) was used and the measurements were conducted at a scattering angle of 90°. Samples (0.1 mg/mL in HEPES buffer) were equilibrated for 120 seconds and 12 readings were then taken for a single sample at constant temperature (25 °C). Each batch of ELIPs was tested for polydispersity and each experiment repeated three times to ensure reproducibility of the results.

2.6 Transmission Electron Microscopy (TEM)

The ELIPs samples were dispersed to 1 mg/mL concentration 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 minutes, the samples were negatively stained with 1% phosphotungstic acid for 1.5 minutes and subsequently wicked off with filter paper and allowed to dry before viewing. The samples were observed using a JEOL JEM-2100-LaB_6 transmission electron microscope operating at 200 kV.

2.7 Release studies with recombinant MMP-9

The release experiments were carried out using a microplate multidetection instrument (Spectramax M5, Molecular devices) employing the liposomes prior to freeze drying at 25 °C. Release was monitored by recording the fluorescence emission intensity at 518 nm with excitation wavelength 480 nm. All experiments were conducted in triplicate. Each well contained 20 μL of 0.1 mg/mL ELIPs in HEPES buffer (25 mM, pH = 8.0, osmolarity 540 mOsm/kg adjusted with 0.17 M NaCl in HEPES buffer, pH = 8) and 16 μL of 25 μM MMP-9 (final concentration: 2 QM). Release of dye was observed over 60 minutes. The emission intensity was recorded in one minute interval. After 1 h, 10 μL of Triton-X100 was added to disrupt all the liposomes and the emission intensity was measured (excitation: 480 nm). This fluorescence intensity was treated as total (100%) release. The percent release was calculated using the formula:

$$Release\ (\%) = \frac{Observed\quad intensity - Initial\quad intensity}{Final\quad intensity - Initial\quad intensity} \times 100$$

2.8 Measurement of Echogenicity of ELIPs

For echogenicity experiments, the freeze-dried ELIPs sample was suspended in a solution of phosphate buffered saline (PBS) with 0.5% by weight of bovine serum albumin (BSA). We observed that the liposome solution prior to freeze drying was not echogenic. Echogenicity of the freeze dried and then reconstituted ELIPs was investigated using an *in vitro* acoustic setup (Figure 1) which is capable of measuring non linear response from contrast agents. The setup employs two single element spherically focused immersion transducers (Panametrics NDT) confocally positioned at right angles. This type of transducer placement ensures the similarity of scattered signals to backscattered echoes[28] and also provides high spatial resolution[29]. Each transducer has an individual diameter of 1.27 cm and focal length of 3 cm. The sample is held in a rectangular chamber. Holes were drilled on adjacent walls for insertion of transducers. Complete immersion of the transducers required 100 mL of solution. Appropriate amount of ELIPs was dissolved in 100 mL of PBS-BSA solution to yield a final lipid concentration of 10 Qg/mL which is low enough to ensure absence of multiple scattering effects. The transmitting transducer has a nominal center frequency of 3.87 MHz with a −6 dB bandwidth of 86.4%. The receiving transducer has a center frequency of 5.54 MHz with an 85% bandwidth. The transmitting transducer was excited at 3.5 MHz with a 32 cycle sinusoidal wave. A 0.4 mm needle hydrophone (PZT-Z44-0400, Onda Corporation, CA, USA) was used to calibrate transducers. A programmable function generator was utilized (Model 33250A; Agilent, Santa Clara, CA) to generate the wave which was amplified using a power amplifier (Model A-300; ENI, Rochester, NY) and fed to the transmitting transducer. The scattered signal was received using the other transducer and a pulser/receiver (Model 5800; Panametrics-NDT, Waltham, MA) with a 20 dB gain. A digital oscilloscope (Model TDS2012; Tektronix, Beaverton, OR) was used to observe the signal in real time. Scattered voltage-time responses were acquired from the oscilloscope using LabView (Version 6.0.3; National Instruments, Austin, TX) via a GPIB IEEE 488 cable and GPIB card and saved on a PC for post experimental analysis using MATLAB (MathWorks, Natick, MA). Fast Fourier Transforms of 50 oscilloscope acquisitions were obtained and averaged in frequency domain which was then converted to dB scale using unit reference. Responses at fundamental, second and sub harmonic frequencies were extracted from the resultant data set and plotted.

2.9 Culture of cancer cells and harvesting of conditioned media

All cell lines were obtained from American Type Culture Collection (Manassas, VA). PANC-1 (Human pancreatic cancer), PC-3 (Human prostate cancer), MCF-7 (Human breast cancer), 22Rv-1 (Human prostate cancer), HeLa (Human cervical cancer) and bEnd-3 (immortalized rat brain endothelial cells) were cultured in clear (without added phenol red) RPMI media supplemented with 5% antibiotics (penicillin, streptomycin), 10% (by volume) fetal bovine serum and grown in incubator at 37 °C in humidified atmosphere containing 5% CO_2. After three generations, confluent cells were centrifuged (200 g) and the supernatant media collected and frozen until use.

2.10 Ultrasound mediated release studies

The *in vitro* setup for ultrasound enhanced release studies employed a single element unfocused immersion transducer (Model IP301HP; Valpey Fisher Corporation, Hopkinton, MA). The active element of the transducer has a diameter of 0.3175 mm and a nominal center frequency of 3.5 MHz with a −6 dB bandwidth of 85%. An arbitrary function generator was used to generate a continuous sinusoidal wave with desired parameters (Model 33250A; Agilent, Santa Clara, CA) which was then amplified with a power amplifier (Model A-150; ENI, Rochester, NY) and fed to the transducer. The release studies were carried out in a 48 well plate filled with 500 μL sample with a total lipid concentration of 0.02 mg/mL. The transducer face was always kept immersed in the sample volume at a

distance of 20 mm from the base (Figure 2). The homogeneity of the sample was maintained using a small magnetic stirrer. The entire plate was kept on an ice bath to minimize the temperature changes. Most release studies were performed with 3 MHz continuous wave at 1 MPa pressure for an exposure period of 180 seconds. We note that although the current setup has often been used to subject biological cells or liposomes to ultrasound [30], it allows reflections from air water interface. This effect has been studied in detail recently [31] to show that the reflection creates a standing wave pattern giving rise to a spatially varying acoustic field. Following these studies, we are currently developing a setup that would address this effect, and will be used to determine optimal ultrasound excitation parameters. For the limited goal of demonstrating ultrasound mediated release here, current setup is adequate. Also note that less than 1% energy transfer due to stimulation from a transducer positioned in one well was measured at a neighboring well indicating negligible inter well interference. All experiments were repeated three times to ensure the reproducibility of results obtained.

2.11 Ultrasound enhanced MMP-9 triggered release

This study was carried out in a 48 well plate, with 500 µL of 0.02 mg/mL of liposomes (prior to freeze drying) in each well using an ice bath. Three different procedures were followed for these experiments. In one set of experiments, we incubated 0.02 mg/mL of ELIPs with 2 QM MMP-9 for an hour and then applied the ultrasound (3 MHz, 1 MPa) for 180 seconds. In second set, we applied the ultrasound first and then allowed ELIPs to incubate with MMP-9. In third set we added MMP-9 to ELIPs and immediately applied the ultrasound to get the simultaneous exposure result. Control samples were also acquired for each of these set of experiments. All the experiments were conducted in triplicates in order to ensure repeatability of the results obtained.

For studies with conditioned media harvested from different metastatic cancer cell lines, we added 50 µL of media to 450 µL of ELIPs (lipid concentration: 0.02 mg/mL) and after one hour we applied the ultrasound. Two different negative control measurements were performed: one without the cell culture media (RPMI) and ultrasound and other with RPMI media as well as ultrasound whereas bEnd 3 cell media was treated as a positive control.

2.12 Ultrasonic imaging of the echogenic liposomes

The Terason t3200™ Diagnostic Ultrasound (MedCorp LLC., Tampa, FL) was utilized to image the reflection of the echogenic liposomes. A layer of Aquasonic® 100 (Parker Laboratories, Inc., Fairfield, New Jersey) ultrasound gel was applied to the 15L4 Linear (4.0-15.0 MHz) (MedCorp LLC., Tampa, FL) ultrasound transducer sound plate. The transducer with gel was placed over the parafilm covering the wells containing ELIPs (0.2 mg/mL in all four wells) in a 96 well plate. The ultrasound scan properties of the echogenic liposomes were set at 0.7 Mechanical Index (MI), 0.6 Thermal Index (TIS), Omni Beam activated, level C Image Map, level 3 Persistence, high (H) frequency, level 3 TeraVision, level 51 2D Gain, level 60 Dynamic Range (DR), 3 cm scan depth, and 22 Hz frame rate. The images were labeled and saved.

3. RESULTS AND DISCUSSION

Nonfibrillar collagens (types IV and V) are the principal substrates of MMP-9[32]. The collagens contain high amounts of the amino acid triad Glycine-Proline-Hydroxyproline (GPO) and this contributes to the triplehelical structure[33]. We have previously synthesized triple helical substrate lipopeptides for MMP-9 and optimized the contents release from liposomes in the presence of this enzyme[10a]. For these studies, we decided to synthesize the same lipopeptide [LP4, amino acid sequence: $CH_3(CH_2)_{16}CONH$-GPQGIAGQR(GPO)$_4$GG-COOH, where MMP-9 cleavage site is between Glycine and

Isoleucine] for preparing the ELIPs. The lipopeptide LP4 was synthesized employing a microwave assisted peptide synthesizer, purified by reverse phase HPLC and the purity was confirmed by MALDI-TOF mass spectrometry (Calculated MH$^+$: 2333.26; Observed: 2333.28). Circular dichorism (CD) spectroscopic studies confirmed triple helical structure for LP4 in phosphate buffer (pH = 4.0; Figure 3A). Poor solubility of the lipopeptide in buffer of higher pH prevented us from conducting these studies at physiological pH (7.4). Temperature-dependent CD spectra (5 – 60 °C) indicated the presence of an isosbestic point (Figure 3A) and the melting temperature was determined to be 49 °C (Figure 3B). This melting temperature is similar to that observed for human collagen (48 °C)[34]. The isosbestic point in Figure 3A indicates that the triple helical lipopeptide is melting to monomeric species without going through any intermediates[35].

After confirming triple helical structure of lipopeptide LP4 in solution, we prepared ELIPs incorporating 30 mol% of LP4 and 70 mol% of POPC in phosphate buffer (pH = 8.0) employing a reported protocol[21b, c]. This liposomal composition was based on our previous mechanistic and optimization studies on MMP-9 triggered release of liposomal contents[10]. We observed that LP4 retains the triple helical structure when incorporated into the liposomes (Figure 4, black trace). It is hypothesized that the hydrolysis of triple helical substrate peptides by MMP-9 requires unwinding of the triple helix by the enzyme followed by hydrolysis[36]. We observed that upon incubation of these liposomes with 2 μM recombinant human MMP-9, the triple helicity decreased substantially (Figure 4, red trace). These results demonstrate that MMP-9 is able to unwind and cleave the triple helical LP4 even when incorporated in the lipid bilayer of the ELIPs. We also observed that 2 μM of MMP-7, MMP-10 or Trypsin released less than 5% of the encapsulated contents from the liposomes. This is likely due to the inability of these enzymes to unwind the triple helix[2b, 37].

Next, we prepared ELIPs with carboxyfluorescein encapsulated and the liposomes were subsequently freeze-dried for long-term storage and shipment[38]. We determined the average diameters of the ELIPs before and after freeze-drying employing dynamic light scattering. We observed that average diameter of the liposomes in the reconstituted powder was larger (190 ± 35 nm; Figure 5D) compared to that prior to freeze-drying (116 ± 22 nm, Figure 5C). The polydispersity index also increased to 0.85 from 0.3 – indicating a large distribution of size in the reconstituted liposomes[39]. Transmission electron microscopy corroborated these observations. TEM images show that the reconstituted ELIPs were heterogeneous (50 nm – 1 Qm) with median size between 100 – 200 nm (Figures 5A and B). Using an optical microscope, we observed micron sized liposomes also (Supporting Information, Figure S1). During the freeze drying and subsequent reconstitution steps, some of the liposomes fuse with each other – leading to a more heterogeneous size distribution. We have used mannitol[40] during the ELIPs preparation which plays a crucial role in ensuring echogenicity by aiding air entrapment.[23c] Also note that the relatively high polydispersity indicates presence of larger liposomes (also seen in the inset of Figure 5D and Supporting Information Figure S1). These larger liposomes with air pockets of size around a micrometer are primarily responsible for the echogenicity of ELIPs. Consistent with this, we also observed that the liposome solution prior to freeze drying was not echogenic.

Echogenicity of the ELIPs (reconstituted from freeze dried powders) with and without encapsulated carboxyfluorescein was investigated using the acoustic setup for scattering measurements shown in Figure 1. We observed that the liposomes showed echogenicity only when freeze dried and subsequently reconstituted. However, this leads to some loss of the encapsulated dye. As mentioned above, the echogenicity is likely due to the presence of larger size liposomes with encapsulated gas in the reconstituted samples (see Figures 5B and 5D). The ELIPs were excited at an acoustic pressure amplitude of 500 kPa. Figure 6A shows

the FFT of the scattered response from suspension with and without any ELIPs. At an acoustic pressure of 500 kPa, we observed distinct peaks at both fundamental frequency (i.e., at excitation frequency of 3.5 MHz) and second harmonic frequency (i.e., at twice the excitation frequency, 7 MHz) in presence of the ELIPs. However, no distinct subharmonic peak (i.e. response at half the excitation frequency, 1.75 MHz) was observed. The nominal central frequency (5.54 MHz) of the receiving transducer is rather high compared to the subharmonic frequency (1.75 MHz). However, using a receiving transducer with a lower central frequency (2.25 MHz) did not change the result. We conclude that these ELIPs do not generate any significant subharmonic response at this excitation frequency. Figure 6B shows the experimentally measured echogenic response from the ELIPs (the scattered fundamental and second harmonic components). Control measurements are acquired without any ELIPs in the sample solution. Each set of experiments was repeated five times. The mean of the five different runs is plotted along with the corresponding standard deviation. We also imaged the ELIPs (in a well plate) employing a Terason t3200 ultrasonic medical imaging system using a 4 – 15 MHz transducer (Figure 6C, D).

We observed that both ELIP formulations with and without encapsulated dye show echogenicity with nearly 20 dB enhancement of the fundamental response when reconstituted in PBS-BSA. The preparation procedure includes addition of mannitol, a weak cryoprotectant. It has been hypothesized that lyophilization in presence of mannitol creates defects in the bilayer that serves as nucleation sites for air entrapment [23a, b, 25a, 41]. The presence of air creates a mismatch in acoustic impedance rendering the liposomes echogenic i.e., capable of reflecting ultrasound waves [42]. It has been reported that these air pockets are created during the rehydration step in the PBS-BSA/PBS solution[42a]. Creation of air pockets is facilitated by the use of mannitol during the lyophilization [23a, 25a, 42a]. Mannitol thus plays a critical role in the echogenicity of the ELIPs. Recently, we have shown that a small but finite amount of mannitol is required for echogenicity. [23c] Although echogenicity of the ELIPs have been attributed to the presence of these air pockets, the exact location of these air pockets still remains uncertain[43]. Hypothesized structure of ELIPs considers existence of trapped air within the bilayer in between hydrophobic tails of the lipid molecules[24, 44]. Air can also be trapped as a small lipid monolayer coated bubble freely floating within the aqueous core of the liposomes[23b, 24]. Recently, TEM image have shown the existence of such individual lipid coated bubbles[45]. The lipid coating in both situations—in the bilayer or for individual bubbles—can render the air pocket stable against Laplace pressure driven dissolution by drastically decreasing the effective surface tension at the air liquid interface[46]. The lipid shell also modifies the echogenicity of the air pocket and thereby of the emulsion[29, 47]. Notably, there is no difference in the echogenicity of the ELIPs due to dye-loading. The ELIPs also show non linear response with around 10 dB enhancement for second harmonic response.

We conducted the enzymatic release studies employing an optimized concentration of freshly-prepared liposomes (0.01 mg/mL of total lipid). We used the self-quenching property of the encapsulated dye (carboxyfluorescein) in determining the contents release from the liposomes[10b]. In order to determine if the presence of the entrapped air pockets in the echogenic emulsion has any effect in the fluorescence of the dye, we recorded the emission spectra of carboxyfluorescein (5 µM in 25 mM HEPES buffer, pH = 8.0) in the presence of ELIPs (without any encapsulated dye) and regular liposomes. We observed that these emission spectra were superimposable – indicating that the entrapped air in the ELIPs is not affecting the emission from carboxyfluorescein (Supporting Information, Figures S2 – S4).

In the absence of any added enzyme, less than 10% of the encapsulated dye was released in 60 minutes (Figure 7, magenta spheres). Upon incubation with 1 µM of recombinant human

MMP-9, we observed 25% release of the encapsulated dye in 20 minutes (Figure 7A, blue spheres). After this time, no further release was observed from the liposomes (Figure 7A, blue spheres). Increasing the concentration of MMP-9 to 2 μM leads to 62% release in 50 minutes (Figure 7A, green spheres). These release profiles can be fitted with a single exponential rate equation (Figure 7A, red lines through the observed data points) with rate constants of $(12.9 \pm 0.4) \times 10^{-2}$ s^{-1} (1 μM MMP-9) and $(15.1 \pm 0.1) \times 10^{-2}$ s^{-1} (2 μM MMP-9).

MMP-9 cleaves the triple helical peptide exposed on the liposome surface, leading to destabilization of the lipid bilayer and release of the encapsulated content[48]. As the bilayer "heals" itself, the release from the liposomes slows down. We also observed that the recombinant MMP-9 used in these studies undergoes self-hydrolysis and resulting in the formation of catalytically inactive enzyme. The reduction in the concentration of the active enzyme will also contribute to reducing the rate of release from the liposomes. It should be noted that for healthy individuals, the serum concentration of MMP-9 is less than 10 nM. For gastric, colorectal and pancreatic cancer patients the serum concentration of MMP-9 can be as high as 1 μM[2a]. We also observed that exposing the liposomes to diagnostic frequency ultrasound (3 MHz, 1 MPa) for 3 minutes led to around 25% release of the encapsulated dye (Figure 7B); ultrasound destabilizes the ELIPs[49]

The lipopeptide LP4 adopts triple helical conformation in the liposomes (Figure 4). The fibronectin domain of MMP-9 unwinds the triple helix and subsequently, the catalytic domain hydrolyzes the peptide bond[50]. We hypothesized that enzymes incapable of unwinding the triple helix should not release the encapsulated dye from the liposomes. In accordance with this, we observed that MMP-7, MMP-10 and trypsin (2 μM each) failed to release the encapsulated dye from the liposomes (data not shown).

After ensuring release by MMP-9 and diagnostic frequency ultrasound separately, we proceeded to determine the combined effect of these two triggers on contents release from the ELIPs. In this endeavor, we first incubated the ELIPs with MMP-9 (2 μM) for an hour and observed 62% release of the encapsulated dye. Subsequent application of ultrasound (3 MHz, 3 minutes) increased the release to 71% (Table 1). As MMP-9 releases the encapsulated dye by disturbing the lipid bilayer of the liposomes, it is likely that the entrapped air is also escaping – making the resultant liposomes less responsive to ultrasound trigger. When we applied the ultrasound first for 3 minutes, we observed 15% release of the contents from the liposomes. This indicates that a majority of the liposomes are not releasing the contents in the presence of ultrasound alone. However, upon subsequent treatment with MMP-9 (2 μM) for an hour, the release increased to 65%. When we incubated the liposomes with MMP-9 (2 μM) and applied ultrasound at the same time, the release decreased to 30%. A probable reason for this may be the local increase in temperature during the ultrasonic excitation[51]. The local heating will deactivate MMP-9, leading to a decrease in the contents release from the liposomes. The control in Table 1 represents release when the transducer was inserted in the liposomal solution without sending any ultrasound waves (for 3 minutes).

Various cancer cells are known to secrete varying amounts of MMP-9 in the extracellular matrix[52]. We decided to determine if the conditioned media from cancer cells can release the encapsulated dye from the ELIPs and if this release can be further enhanced by the application of diagnostic frequency ultrasound. In this endeavor, we cultured the cells HeLa (cervical cancer), PC 3 (prostate cancer), 22Rv1 (prostate cancer), MCF-7 (breast cancer) and PANC 1 (pancreatic cancer) in dye-free RPMI. After reaching confluency, the cells were pelleted and the media was harvested. The immortalized mouse brain endothelial cell line bEnd-3 (which does not secrete MMP-9) was taken as control[53]. Based on our results

with recombinant MMP-9, we incubated the ELIPs with the conditioned media for an hour, followed by the application of ultrasound pulses (3 MHz) for 3 minutes.

We observed that the culture media (RPMI containing 10% by volume of fetal bovine serum) released 16% of the liposome encapsulated dye (Figure 8A). As cells become confluent and consume fetal bovine serum, the conditioned media from bEnd-3 cells showed less release (<5%) compared to fresh RPMI (Figure 8A). The release also decreases slightly over time. Since the release was very low (< 5%), we did not conduct any further studies to determine the reasons for this time-dependent decrease. The conditioned media from the cancer cells released varying amounts of encapsulated dye (16 – 50%, Figure 8A). We observed that the release was highest in the presence of the conditioned media from the PC-3 cells (47%, Figure 8A and 8B). The time course of the dye release in the presence of conditioned media from PC 3 cells can be fitted with a single exponential rate equation with rate constant of $(57.4 \pm 1.6) \times 10^{-2} \, \text{s}^{-1}$. However, we observed that the amount of contents release does not correlate with the total amount of MMP-9 present in the conditioned media (as determined by ELISA). ELISA determines the total amount of MMP-9, which includes both catalytically active and inactive enzymes. It is also possible that besides MMP-9, other triple helicase secreted in the conditioned media (i.e., MMP-2, MMP-14, ADAM-10 etc.) are contributing to the release of the dye[13b, 54]. However, we were pleased to find that upon application of ultrasound for 3 minutes, the amounts of dye release increased to 25 – 75% (Figure 8A). Currently we are testing the effects of ultrasound frequency, incorporation of PEG lipid and cholesterol on the contents release from these liposomes and these results will be reported later.

4. CONCLUSION

We have demonstrated that echogenic liposomes can be prepared incorporating a substrate lipopeptide for MMP-9 in the lipid bilayer. The liposomes retain echogenicity after encapsulation of a hydrophilic dye and can be imaged by ultrasound. The encapsulated contents from the liposomes are released in presence of recombinant as well as cancer cell secreted MMP-9. The contents release can be further increased by the application of diagnostic frequency ultrasound pulses. This study demonstrates that diagnostic frequency ultrasound can be used simultaneously with enzymes as an additional method to trigger contents release from suitably constructed liposomes. We employed continuous excitations for the release study, which deliver more energy than a pulsed excitation used in ultrasound imaging. Future studies are required systematically varying the intensity, frequency and duty cycles of the ultrasound stimulation to determine the optimum excitation parameters. With further developments, these liposomes have the potential to serve as multimodal carriers for triggered release and simultaneous ultrasound imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS

ELIPs	Echogenic Liposomes
MMP-9	Matrix metalloproteinase-9
LP4	Lipopeptide 4

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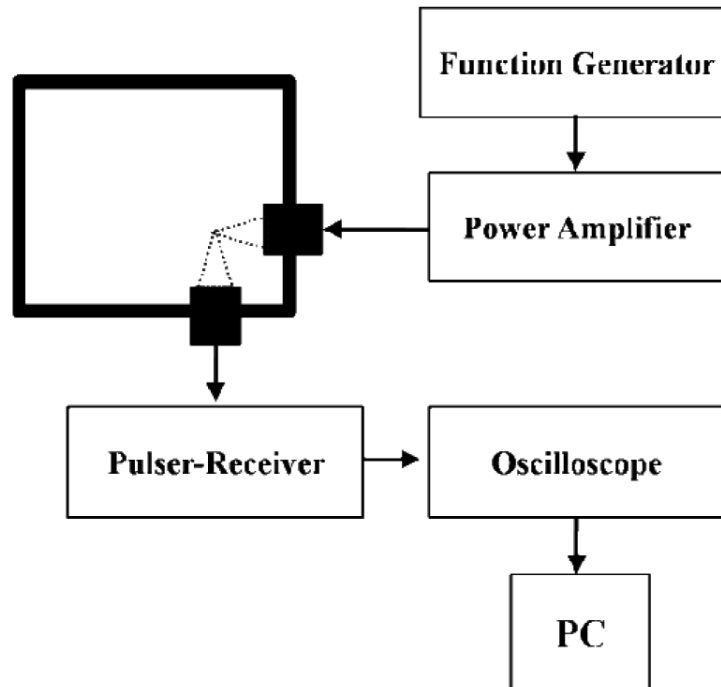
Figure 1.
Schematic of the experimental setup for *in vitro* measurement of scattering
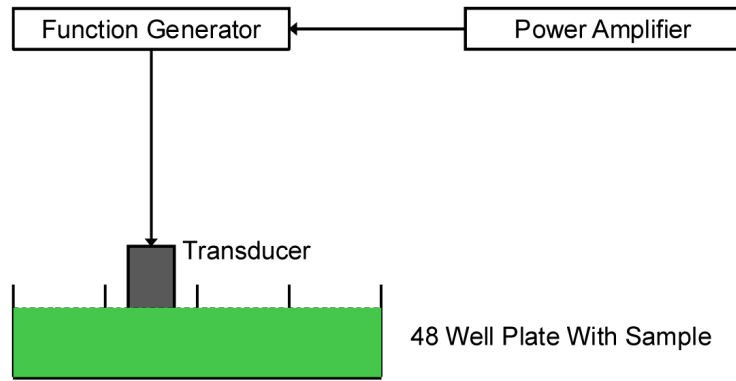
Figure 2.
Schematic of the *in vitro* experimental setup for ultrasound mediated release studies
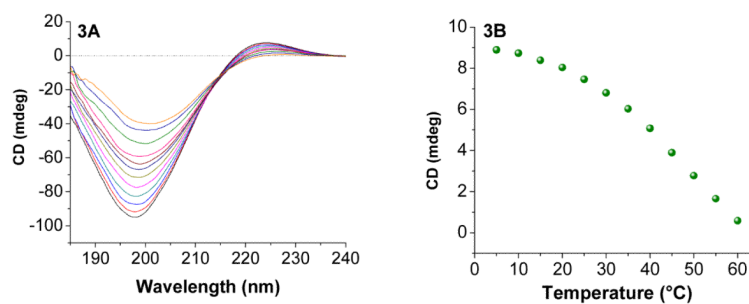
Figure 3.
(A) Temperature dependent CD spectra (5-60 °C) of LP4 (0.5 mg/mL) in 4 mM phosphate buffer (pH = 4). The presence of positive peak at 225 nm and negative peak at 198 nm indicates the triple helical nature of LP4. (B) The melting curve for LP4 monitored at 225 nm is shown.
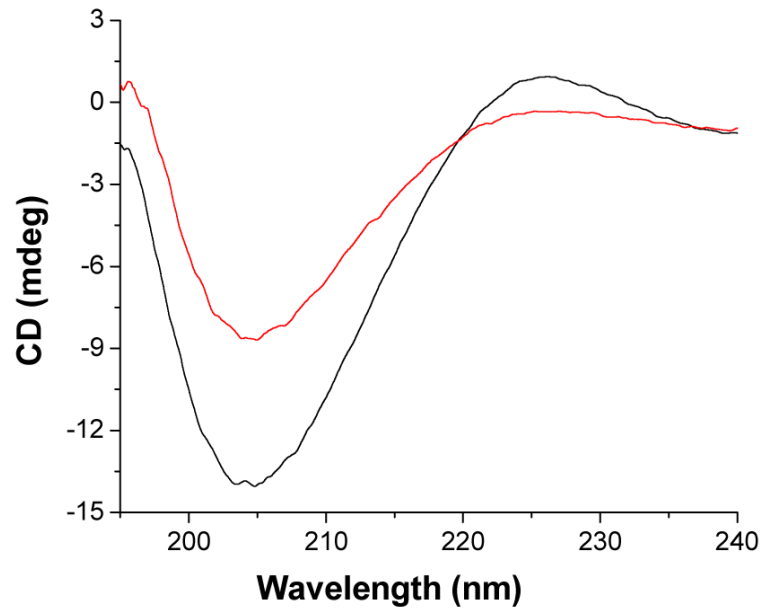
Figure 4.
CD spectra of liposome incorporated LP4 is shown before (black trace) and after (red trace) incubation with 2 QM MMP-9 for an hour. These experiments were conducted in 25 mM phosphate buffer, pH = 8.0.
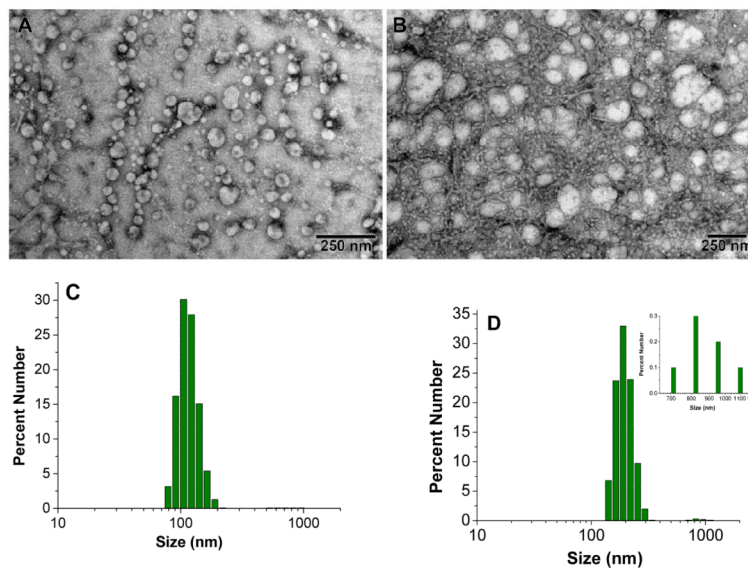
Figure 5.
TEM images (A: before freeze drying B: after freeze drying) and DLS size distributions (by number) of carboxyfluorescein encapsulated ELIPs (C: before freeze drying; D: after freeze drying). The inset plot of panel D shows the presence of liposomes in the size range 700 – 1100 nm. The TEM images of the samples were obtained using a LaB_6 emitter at low magnifications and with the beam spread to reduce the amount of electron beam interaction per unit area and hence beam damage to sample.
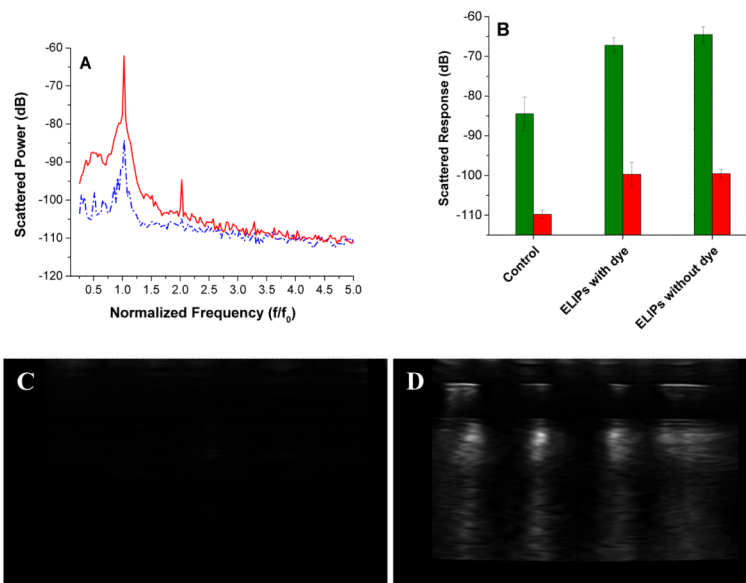
Figure 6.
(A) FFT of the scattered signal from a suspension with (red trace) and without (blue trace) liposomes at an acoustic pressure of 500 kPa. (B) Fundamental (green columns) and second harmonic (red columns) of ultrasound scattered response from ELIPs prepared with or without dye loading and reconstituted in PBS and PBS-BSA solutions. (C, D): Ultrasound images (5 – 10 MHz transducer) of 4 wells of a 96 well plate containing buffer (C) and echogenic liposomes (0.2 mg/mL in each well) incorporating LP4 peptide and encapsulating the dye carboxyfluorescein (D).
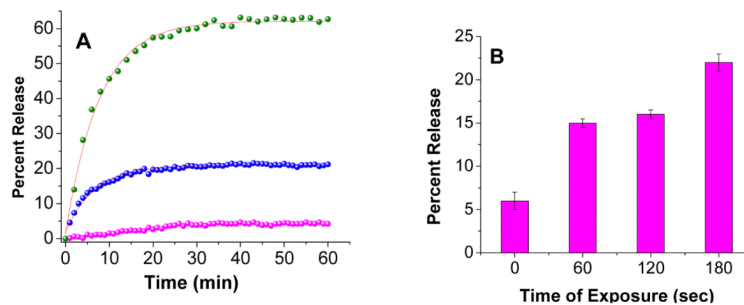
Figure 7.
(A) MMP-9 triggered release profile for encapsulated carboxyfluorescein from ELIPs after incubation for an hour with 1 QM (blue spheres) and 2 QM (green spheres) recombinant MMP-9. The magenta spheres indicate the release from the liposomes in the absence of any added enzyme. The red lines indicate the fitted curves through these data points using a single exponential rate equation. (B) High frequency ultrasound (3 MHz, continuous wave, 1 MPa) triggered release from ELIPs with varying exposure time.
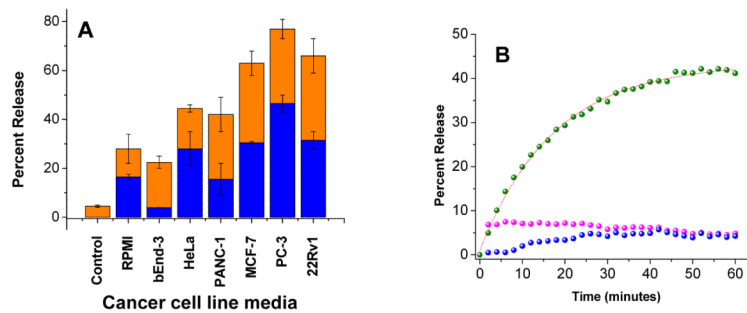
Figure 8.
(A) Release of liposomal contents in presence of conditioned cell culture media from metastatic cancer cells (blue bars) and upon subsequent application of 3 minutes of 3 MHz ultrasound pulse (orange bars) are shown. Control represent release in absence of media as well as ultrasound (B) The kinetic profiles of the contents release in the presence of conditioned media of PC-3 (Green spheres) and bEnd-3 (Magenta spheres) cells and in the absence of any media ie. Control (Blue spheres) are shown. The red line shows the fitted curve using a single exponential rate equation.

Table 1

Ultrasound (US) enhanced recombinant MMP-9 triggered contents release from ELIPs.

Conditions	Release (% ± SD) with MMP-9	Release (% ± SD) with US	Total Release (% ± SD)
MMP-9 followed by US	62 ± 10	9 ± 1	71 ± 5
US followed by MMP-9	50 ± 12	15 ± 4	65 ± 10
Simultaneous			30 ± 2
Control (no US)			4 ± 4