ULTRASOUND-INDUCED CALCEIN RELEASE FROM eLIPOSOMES

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Abstract—Ultrasound is explored as a method of inducing the release of encapsulated materials from eLiposomes, defined as liposomes containing emulsion droplets. Emulsions were formed using perfluorohexane and perfluoropentane. eLiposomes were formed by folding interdigitated lipid sheets into closed vesicles around the emulsion droplets. Cryogenic transmission electron microscopy was used to verify droplet encapsulation. Self-quenched calcein was also encapsulated inside the vesicles. A fluorometer was used to measure baseline fluorescence, calcein release after ultrasound exposure, and total release from thevesicles. eLiposome samples released 3 to 5 times more of the encapsulated calcein than did controls when exposed to 20-kHz ultrasound. Calcein release increased with exposure time and intensity of ultrasound. eLiposomes with large (400 nm) droplets produced more calcein release than small (100 nm) droplets. These observations suggest that the emulsions are vaporized by ultrasound and that the Laplace pressure in the emulsions has an effect on droplet vaporization. (E-mail: pitt@byu.edu) 2012 World Federation for Ultrasound in Medicine & Biology.

Key Words: Liposome, Emulsion, Perfluorocarbon, Ultrasound, Drug delivery.

INTRODUCTION

Liposomes (bilayered lipid vesicles) have proven to be effective and versatile drug carriers. The ability to change the properties of the lipid membrane by changing its composition adds to their versatility (Gregoriadis and Florence 1993; Lindner and Hossann 2010). Another key characteristic of liposomes is the ability to control their size (Gregoriadis and Florence 1993), which allows them to be used for ''passive'' targeting to cancerous tumors (Seymour 1992). The chaotic and leaky endothelium of many cancerous tissues allows submicron particles to extravasate into the tumor and accumulate in higher concentrations compared to their accumulation in normal tissues. This tendency of submicron particles to extravasate and accumulate in cancerous tissues is known as the enhanced permeability and retention (EPR) effect (Maeda 2001). Data seem to suggest that optimum vesicle sizes for the EPR effect range between 100 nm and 300 nm (Hobbs et al. 1998; Bae and Park 2011). Liposomes can be formed at specific sizes to take advantage of the EPR effect, making them particularly good candidates for delivering therapeutics to tumors. However, although passive targeting does improve selectivity to a target site, substantial amounts of the drug still accumulate in other tissues (Bae and Park 2011).

Active targeting of drug delivery from liposomes could also increase the efficiency of their delivery by more specifically controlling the site of drug delivery after they have accumulated in tissues. Active targeting typically refers to attaching targeting ligands to the surface of the liposomes (Maruyama et al. 1999). These ligands are recognized by a target cell type and aid in preferential attachment and sometimes induce uptake of the therapeutics by targeted cells (Lee and Low 1997; Bareford and Swaan2007;Gabizonetal.2010).Another form of targeting is called actuated targeting and involves the use of external triggers. For example, ultrasound can be used to release drugs from carriers at a specific target site. Ultrasound has the advantages of being noninvasive and allowing control of both the site and the timing of release (Husseini and Pitt 2008a, 2008b) and can be used in conjunction with active and passive targeting. Ultrasound can be focused on targets deep in the body without affecting other tissues, allowing the carriers to sequester their therapeutic content until induced to release the drug load at a specific site (Rahim et al. 2006).Additionally, ultrasound increases vasculature and cell membrane permeability to facilitate drug uptake (Guzman et al. 2001; Prentice et al. 2005; Meijering et al. 2009; Lin et al. 2010). Ultrasound has been used to induce drug carriers such as microbubbles to permeabilize cells or to release their drug load at a specific location or both (Unger et al. 1998; Prentice et al. 2005; Liu et al. 2006). However, it is difficult to form stable gas bubbles at sizes sufficiently small to take advantage of the EPR effect. Micelles have also been investigated as ultrasound-sensitive drug carriers (Rapoport et al. 2004; Husseini and Pitt 2008b). Because of their versatility and other advantages, liposomes are also candidates for ultrasound-induced drug delivery (Schroeder et al. 2007; Klibanov et al. 2010; Evjen et al. 2011; Afadzi et al. 2012). However, liposomes are not inherently responsive to ultrasound. Ultrasonic drug release from conventional liposomes seems to rely on the presence of gas bubbles to cause cavitation and is dependent on lipid composition (Schroeder et al. 2007; Schroeder et al. 2009; Evjen et al. 2011). A relatively new field is that of echogenic liposomes. These liposomal drug carriers are typically designed to contain small amounts of gas in order to increase ultrasound sensitivity (Huang and MacDonald 2004; Huang 2008; Kopechek et al. 2008). Echogenic liposomes have demonstrated ultrasound-induced release of hydrophilic compounds from liposomes (Kopechek et al. 2008) and have shown the potential to be used as diagnostic agents (Kopechek et al. 2011).

The goal of the current work is to develop a submicron-sized liposomal drug carrier that is responsive to low-intensity ultrasound without the requirement of nearby gas bubbles that are too large to extravasate. The proposed drug carrier is called an eLiposome, defined as a liposome containing vaporizable emulsion droplets (Lattin et al. 2012). The nanosized emulsion droplets are formed from perfluorocarbon liquids with high vapor pressures. During the low-pressure phase of an ultrasound wave, the local pressure may drop below this high vapor pressure, allowing the formation and expansion of a vapor phase (Sheeran et al. 2011). As this vapor phase forms and expands, it stretches and disrupts the liposomal membrane, causing local drug release. Emulsion droplets can be formed on a much smaller scale than bubbles, allowing the eLiposome to be small enough to extravasate via the EPR effect while also introducing ultrasound sensitivity to this liposomelike drug carrier. This article presents the release of encapsulated material from eLiposomes when exposed to 20-kHz ultrasound. Calcein, a model drug, was encapsulated inside the eLiposomes, and fluorescence spectroscopy was used to quantify ultrasound-mediated release. Ultrasound intensities and durations of exposure were varied.

MATERIALS AND METHODS

Materials

Perfluorohexane (PFC6) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Perfluoropentane (PFC5) was purchased from SynQuest Labs (Alachua, FL, USA). Dipalmitoyl phosphatidyl choline (DPPC) dissolved in chloroform was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Calcein was purchased from MP Biomedicals (Solon, Ohio, USA). All water used was double distilled.

Emulsion preparation

Emulsions were made using PFC5 and PFC6. A thin film of DPPC was deposited on a round-bottomed flask by evaporation of the chloroform. The lipid film was then hydrated at 30 mg/mL by adding water and stirring at 50C. Then 167 mL of this hydrated DPPC suspension (5 mg DPPC) was added to a vial along with 0.2 g of PFC and 1.5 mL of water. The water was degassed prior to emulsion formation. Degassing could not be performed after emulsion preparation without destroying the emulsion. The flask was placed in an ice bath and allowed to cool. The emulsion was formed by sonicating the contents with a 20-kHz ultrasound probe 3 mm in diameter (Vibra-Cell VCX400, Sonics and Materials, Newton, CT). Ultrasound intensity was previously calibrated in degassed and deionized water using a low-frequency hydrophone (Model 8103, Bruel & Kjaer, Naerum, Denmark). The hydrophone was positioned 3 mm from the transducer and connected to an oscilloscope. This calibration was performed in a 100-L water tank lined with corrugated rho C rubber to minimize potential interference at the hydrophone tip resulting from standing waves. The hydrophone voltage signal was converted into intensity (W/cm^2) values using the factory calibration parameters. Large emulsion droplets were prepared by sonicating for 1 min at 0.5 W/cm^2. Small emulsion droplets were formed by sonicating for 1 min at 1 W/cm^2. The droplet size was further reduced and controlled by extrusion through a 100-nm polycarbonate membrane; the PFC emulsion was heated to 50C and passed through the membrane 15 times using an Avanti Mini Extruder (Alabaster, AL, USA). Droplet size was verified by dynamic light scattering on a Brookhaven 90Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY, USA). Large emulsion droplets were considered suitable to form eLiposomes if the median diameter ranged between 400 and 500 nm. Small emulsion droplets were used if the median diameter was between 90 and 120 nm.

Ultrasonic vaporization of emulsion droplets was verified by applying ultrasound to the emulsion sandwiched between a microscope slide and a cover slip at room temperature (24C). Then 100 mL of emulsion was diluted in 1 mL of water. The diluted emulsion was viewed with an Olympus IX70 microscope using a 340 objective. The slide was then inverted and exposed to 1 W/cm^2 ultrasound for 5 s by applying a small amount of ultrasound gel to the slide and positioning the 20 kHz transducer directly into the gel. The slide was again imaged after ultrasound exposure. Control experiments were performed using plain water and a DPPC lipid solution to verify that bubbles were not formed in the absence of PFC emulsion.

eLiposome Preparation

eLiposomes were formed using a lipid sheet refolding technique (Kisak et al. 2004; Boyer and Zasadzinski 2007). Small (30 to 70 nm) DPPC liposomes were prepared by thin film hydration followed by 20-kHz sonication for 15 min at 1 W/cm^2. Size was verified by dynamic light scattering and, if necessary, the sample was sonicated for an additional 15 min. The resulting solution of small DPPC vesicles was translucent. Interdigitated DPPC sheets were formed from these small vesicles by adding ethanol dropwise to a total concentration of 3 M. Formation of lipid sheets was manifested by a large increase in viscosity and the loss of translucence. After sheet formation, excess ethanol was removed from the sheet suspension by centrifugation and washing. The sheets were suspended and diluted in 50 mL of water at room temperature and centrifuged at 18003 g for 3 min, resulting in a large pellet of DPPC sheets. The alcohol-rich supernatant was removed, and the pellet was resuspended in water and centrifuged again. The pellet was again suspended in a small volume of water in order to transfer the sheets to microcentrifuge tubes, with 10 mg of DPPC allotted to each tube. The extra water was removed through 1 more cycle of centrifugation, and 0.2 mL of emulsion was added to 10 mg of interdigitated sheets along with 0.2 mL of water (for imaging by transmission electron microscopy [TEM]) or a 30-mM calcein solution (for release experiments). The sheet solution was repipetted and briefly vortexed to ensure complete mixing. The solution was then heated to 50C and stirred with a magnetic stir bar for 30 min, allowing the sheets to fold back into vesicles and trapping some nanoemulsion droplets inside (Lattin et al. 2012). The size distribution of the resulting eLiposomes was controlled by extrusion through an 800-nm polycarbonate filter at 50C. Empty eLiposomes, defined as liposomes refolded from interdigitated sheets in the absence of emulsion, were prepared as negative controls.

Transmission electron microscopy imaging

The eLiposomes were imaged using cryogenic TEM (cryoTEM). eLiposome samples were pipetted onto a holey-carbon-coated copper grid. A Vitrobot (FEI, Hillsboro, OR, USA) was used to blot the grid with filter paper and plunge it into liquid ethane for freezing. Frozen grids were stored in liquid nitrogen. A 626 cryoholder (Gatan, Pleasanton, CA, USA) maintained the samples at approximately 2180C in the microscope. The microscope stage was rotated and the sample was viewed at 245, 0, and 145 to verify encapsulation of the emulsion droplets (Lattin et al. 2012). Images were recorded at 300 kVon a Tecnai F30 transmission electron microscope (FEI) using a Gatan 1024 3 1024 CCD camera. The objective lens was under focused by several micrometers to improve contrast.

Calcein release

Calcein-containing samples were prepared for quantification of release. During vesicle formation ,calcein was encapsulated inside of the eLiposomes at a concentration of approximately 15 mM. At this concentration, the calcein was self-quenched. The external calcein concentration was reduced by allowing the sample to settle at the bottom of the microcentrifuge tube for a few hours. Because of their relatively high density, the vesicles settled to the bottom of the tube in a thick gel-like phase. The top vesicle-free layer was removed, and the samples were resuspended in an NaCl solution with the osmolarity matched to the calcein solution inside the eLiposomes. This created samples with concentrated calcein inside the eLiposomes and dilute calcein on the outside. A correlation of calcein fluorescence versus concentration was prepared using a QuantaMaster fluorometer (Photon Technology International, Birmingham, NJ, USA) with excitation and emission wavelengths of 488 nm and 525 nm,respectively.Atconcentrationsbelow7.5mMcalcein, the plot of fluorescence intensity versus calcein concentration is linear. The eLiposome samples were diluted by adding 20 mL of sample to a disposable UV/VISrange cuvettewith a 10-mm path length (Fisher Scientific, Pittsburgh, PA, USA). Then 2 mL of NaCl solution was added to the cuvette to achieve a target external calcein concentration of 1 to 5 mM in order to operate in the linear region of the concentration curve for calcein. Standards were prepared at 1 mM and 5 mM to test the fluorescence emission (counts per second) expected for the target concentration range in order to verify that experiments were being run within these concentrations.

Baseline fluorescence data were collected for 10 s at 4datapointspers.Thecuvettewasthenremovedfromthe fluorometer and 20-kHz ultrasound was applied using a 3mm transducer (Sonics and Materials, Newton, CT, USA) inserted directly into the cuvette. As concentrated (selfquenched) calcein was released from the interior of the eLiposomes into the surrounding solution, it was diluted below its self-quenching concentration. Fluorescence was again measured after sonication. Finally, 25 mL of

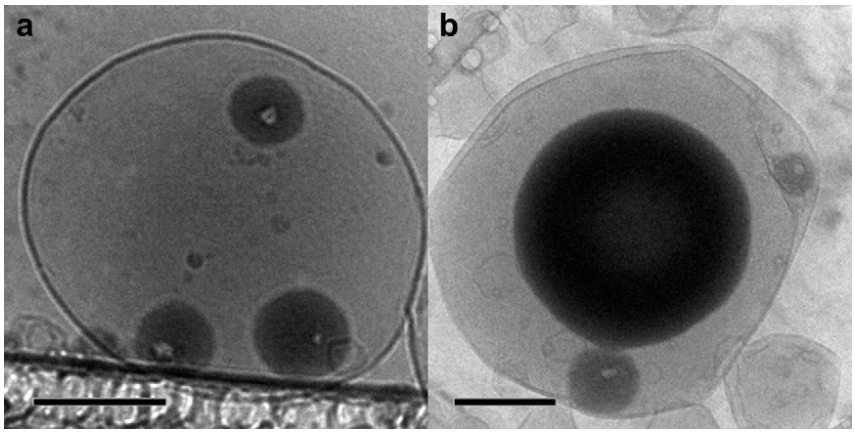

Fig. 1. CryoTEM images of 800 nm eLiposomes containing (a) 3 emulsion droplets 100 to 150 nm PFC6; (b) 1 emulsion droplet 470 nm PFC6 and 2 smaller droplets. Scale bars represent 200 nm. The straight (b, top left) and mottled (a, bottom) structures are supporting entities of the holey carbon support film.

5% Triton X-100 was added to lyse any remaining liposomes, and a final fluorescence was measured. All experiments were conducted at 24C. Percent release of calcein was determined using the following equation:

$$\%Release = \frac{f_{us} - f_i}{f_{full} - f_i} * 100$$

where f_i is initial fluorescence, f_{US} is the fluorescent signal after sonication, and f_{full} is the fluorescence after complete calcein release using Triton X-100. Release experiments were performed on eLiposome samples and on empty control liposome samples. Experiments were also performed on empty control samples mixed with emulsion droplets after the formation of the control vesicles, such that the emulsion droplets were external to the liposomes instead of internal.

RESULTS

CryoTEM images of eLiposomes verify that PFC6 emulsion droplets were encapsulated inside the lipid vesicles using the sheet refolding technique. Figure 1 shows examples of eLiposomes containing emulsion droplets. The interdigitated sheet refolding technique was used to encapsulate both small and large emulsion droplets. The dark circular structures are the perfluorocarbon droplets. These droplets are observed within a DPPC membrane layer. The microscope stage was rotated to 245, 0, and 145 to verify encapsulation of the emulsion droplets within the lipid vesicle. The emulsion droplets were observed within the membrane boundary as the microscope stage was rotated, demonstrating that they were encapsulated inside of the eLiposome (data not shown) (Lattin et al. 2012). Figure 1 demonstrates that both small and large droplets can be encapsulated inside the newly formed DPPC vesicles. Figure 1a shows an example of an800nmeLiposomewith3distinct100to150-nmemulsion droplets. Figure 1b shows an example of an 800-nm eLiposome with a single 470-nm emulsion droplet as well as 2 smaller droplets.

In order to verify acoustically produced vaporization of emulsion droplets, large PFC5 emulsions were placed on a microscope slide, were covered with a thin glass coverslip, and were exposed to 1 W/cm^2 ultrasound for 5 s. Prior to ultrasound exposure only the largest droplets (approximately3to4 ⅿⅿ)are visible(Fig. 2a).After ultrasound exposure, many larger structures can be observed that have the appearance of gas bubbles (Fig. 2b). These newly formed bubbles vary in size and can be as large as 20 ⅿⅿ. The study included 2 control experiments involving a DPPC solution and plain water. No bubbles were observed before or after ultrasound exposure in either of the control samples (data not shown).

When exposed to ultrasound, eLiposome samples demonstrated an increase in calcein release compared to controls. Figure 3 shows the percent released after 100 ms of exposure to 20 kHz ultrasound (n5 3, and error bars represent 1 standard deviation). Ultrasound intensity was varied between 0.5 W/cm^2 and 5 W/cm^2. eLiposomes were prepared with large (400 nm) and small (100 nm) emulsion droplets. The amount of release increased as ultrasound intensity increased. After 100ms of ultrasound exposure, PFC5 eLiposomes released approximately 3 to 4 times more calcein than controls at each intensity (Fig. 3a). For example at 5 W/cm^2, eLiposomes with large PFC5 emulsion droplets released 39% and eLiposomes containing small PFC5 emulsion droplets released 31%, while control samples released only 10% of the encapsulated calcein. PFC6 eLiposomes also released more of the encapsulated calcein than control samples. Figure 3b shows calcein release from PFC6 eLiposomes after 100 ms of insonation. Although this release was slightly less than that observed in PFC5 eLiposomes, PFC6 eLiposomes also released up to 3 times more calcein than control samples. After 100 ms at 5 W/cm^2, eLiposomes with 100-nm PFC6 emulsions released 22% of the available calcein. The difference in calcein release between controls and eLiposomes was statistically significant (Student t-test, p , 0.05) for both PFC5 and PFC6 eLiposomes with both large and small emulsions. Negative controls included both empty vesicles and empty vesicles with emulsion droplets added to the exterior. All of these negative controls released less of their encapsulated calcein than did the eLiposome samples. At 100 ms of ultrasound exposure there was no significant difference (p . 0.05) between empty control liposomes and empty liposomes with emulsion droplets added to the outside solution, regardless of emulsion size or material (Fig. 3).

There was, however, more variability in samples with emulsion droplets added to the outside of the vesicles; empty vesicles had an average standard deviation of 3% compared to a standard deviation of 5% when emulsion droplets were added to the exterior solution(n53). There was no significant difference (p . 0.05) between release from empty vesicles with small exterior droplets and release from empty vesicles with large exterior droplets.

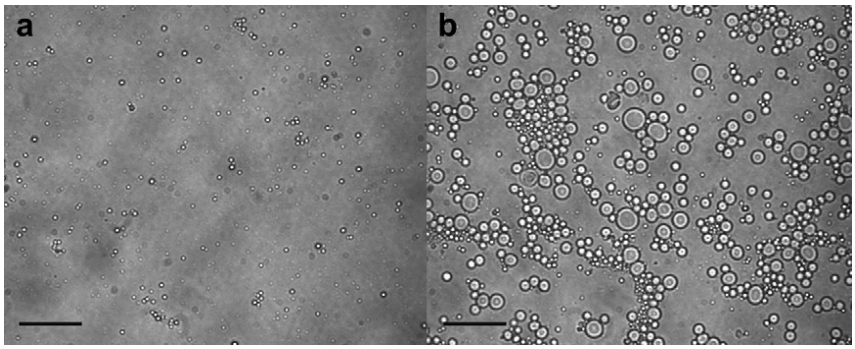

Fig. 2. PFC5 emulsion before ultrasound exposure (a) and after ultrasound exposure (b). Large emulsion droplets were placed on a microscope slide under a coverslip and imaged prior to ultrasound exposure. Then 20-kHz ultrasound was applied for 5 s at 1 W/cm^2 and the slide was imaged again. Before ultrasound exposure, only the largest droplets in the emulsion can be observed (a). After ultrasound exposure, droplets had vaporized and perhaps coalesced into much larger bubbles (b). Scale bars represent 50 mm.

The amount of calcein release also increased with increasing time of ultrasound exposure, as shown in Figure 4, which plots the release as the time of ultrasound exposure was varied from 100 ms to 10 s at 1 W/cm^2. Each point is the mean of 3 measurements. After 10 s, the PFC5 eLiposomes with 400-nm emulsion droplets had released 94% of the encapsulated calcein compared to only a 20% release from control samples. The same

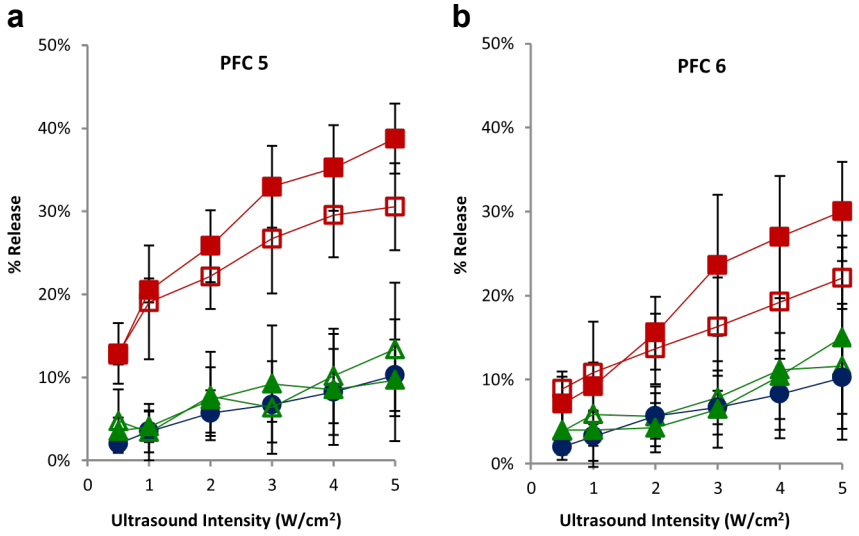

Fig. 3. Calcein release from PFC5 eLiposomes (a) and PFC6 eLiposomes (b) when exposed to 100 ms of 20 kHz ultrasound at varying intensities. Data are presented for eLiposomes with large (■) and small (□) emulsion droplets, empty control vesicles (●), and empty vesicles with large (▲) or small (△) emulsion droplets added to the exterior solution. Error bars represent 61 standard deviation.

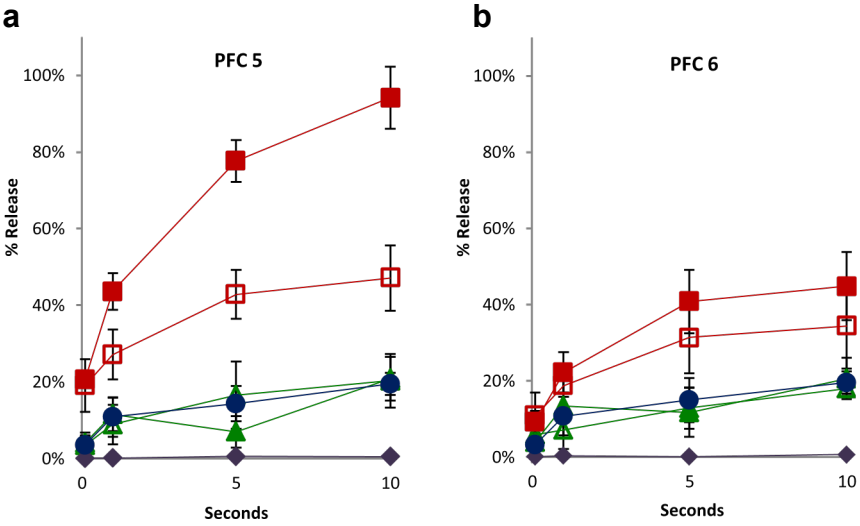

Fig. 4. Calcein release from PFC5 eLiposomes (a) and PFC6 eLiposomes (b) when exposed to 20 kHz ultrasound at 1 W/cm for varying times. Data are presented for eLiposomes with large (■) and small (□) emulsion droplets, empty control vesicles (●), and empty vesicles with large (▲) or small (△) emulsion droplets added to the exterior solution. Sham experiments (no ultrasound) were also performed using eLiposomes with large droplets (◆). Error bars represent ±1 standard deviation.

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trend of increased calcein release with increased time was observed in all samples. PFC6 eLiposomes with 400-nm droplets released 47% of encapsulated calcein after 10 s. This was much less than PFC5 eLiposomes with large droplets, but more than twice as much release as the controls.

The size of the emulsion droplets had only a small effect at 100 ms (Fig. 3), but this effect became more pronounced with PFC5 eLiposomes at longer ultrasound exposures. PFC5 eLiposomes with 400-nm emulsion droplets released approximately 94% of encapsulated calcein after 10 s of 1 W/cm^2 ultrasound, whereas PFC5 eLiposomes with 100-nm droplets released only 47% after 10 s. The difference in calcein release with droplet size was less pronounced for PFC6 eLiposomes; eLiposomes containing 400-nm droplets released 45% of the encapsulated calcein, and eLiposomes with 100-nm PFC6 droplets released 34%. All eLiposome samples released more calcein than did negative controls, which released 20% of the encapsulated calcein after 10 s at 1 W/cm^2.Thedifferenceincalceinreleasebetweencontrols and eLiposomes was statistically significant for PFC5 and

PFC6 eLiposomes at each time point for both large and small emulsion droplets (p , 0.05). Similar to the results shown in Figure 3 (various intensities after 100 ms of ultrasound exposure) ,control samples with external emulsion droplets released the same average amount as empty negative controls but showed more variability. Sham experiments were also performed in which eLiposomes with large emulsion droplets were submitted to the entire release procedure, including inserting the probe into the cuvette for the same amount of time as in active experiments. These sham experiments showed no evidence of leaking or eLiposome damage resulting from the experimental procedure (Fig. 4).

Ultrasound intensity was also varied at 10 s of exposure (Table 1). In most cases, there was significantly more release from eLiposomes exposed to 1 W/cm^2 than from those exposed to 0.5 W/cm^2 for 10 s. For example, PFC5 eLiposomes with 400-nm droplets released 75% of encapsulated calcein at 0.5 W/cm^2 and 94% at 1 W/cm^2.

Table 1. Percent release of calcein from eLiposomes exposed to 20 kHz ultrasound for 10 s

	eLiposomes with internal emulsion				Liposomes with external emulsion *				
	PFC5		PFC6		PFC5		PFC6		Empty
Intensity	Large	Small	Large	Small	Large	Small	Large	Small	Control[v]
0.5 W/cm^2	75%	33%	33%	31%	20%	15%	23%	21%	19%
1 W/cm^2	94%	47%	45%	34%	21%	20%	21%	18%	20%
2 W/cm^2	94%	47%	47%	36%	26%	35%	34%	33%	30%

PFC5 5 perfluoropentane; PFC6 5 perfluorohexane.
* External emulsion refers to calcein-filled liposomes with emulsion droplets added externally to the liposome. [v]
Control refers to calcein-filled vesicles formed from DPPC sheets with no emulsions.

Similarly, PFC6 eLiposomes with 400-nm droplets released 33% of encapsulated calcein at 0.5 W/cm^2 and 45% at 1 W/cm^2. These differences were statistically significant in all eLiposome samples except PFC6 eLiposomes with small droplets(p,0.05). However, when ultrasound intensity was increased from 1 W/cm^2 to 2 W/cm^2, there was no significant additional change in the amount of calcein released from any of the eLiposome samples. In contrast, the difference in calcein release by controls was generally not significant when ultrasound was increasedfrom0.5W/cm^{2}to1W/cm^2.However,allcontrol samples (both empty and with external emulsion) showed a statistically significant increase in calcein release after 10 s of ultrasound when the intensity was increased from 1 W/cm^2 to 2 W/cm^2. The temperature of the solution was measured after sonication. The temperatures of samples exposed to 2 W/cm^2 for 10 s increased by an averageofapproximately4Candthoseofsamplesexposed to 2 W/cm^2 for 5 s increased by approximately 2C. Increased temperature was not measurable in other samples at lower intensities and times. Because the resulting temperatures were still well below the transition temperature for DPPC and below the temperature at which the nanoemulsions were extruded (50C), it is unlikely that the increase in temperature was responsible for the increase in calcein release from eLiposomes or from control samples.

DISCUSSION

eLiposomes were successfully formed by adding emulsion droplets to interdigitated DPPC sheets and refolding the lipid sheets into vesicles in the presence of emulsion. This method was able to encapsulate both large (400 nm) and small (100 nm) emulsion droplets, as demonstrated by CryoTEM. Rotating the microscope stage verified that the droplets were inside the vesicles. PFC5 and PFC6 were chosen for emulsion droplets because of their relatively high vapor pressures. Additionally, both of these perfluorocarbons have very low solubility in water, enabling nanodroplets to persist for an extended time in an aqueous environment. Finally, PFC5 and PFC6 have low toxicity (Correas et al. 2001; Nieuwoudt et al. 2009), making them good candidates for use in a drug delivery system. The ability of ultrasound to vaporize PFC emulsion droplets was verified by visualizing emulsion droplets before and after ultrasound exposure (Fig. 2). Images taken after ultrasound exposure revealed that large bubbles had formed. These resulting bubbles were larger than expected and persisted at room temperature. The larger size could be due to bubbles' colliding and combining or due to dissolved nitrogen and oxygen accumulating in the PFC bubble during and perhaps after insonation.

Although most medical ultrasound is of higher frequency, ultrasound was applied at 20 kHz in order to employ an acoustic system that could be applied within an optical cuvette. The work presented in this article is

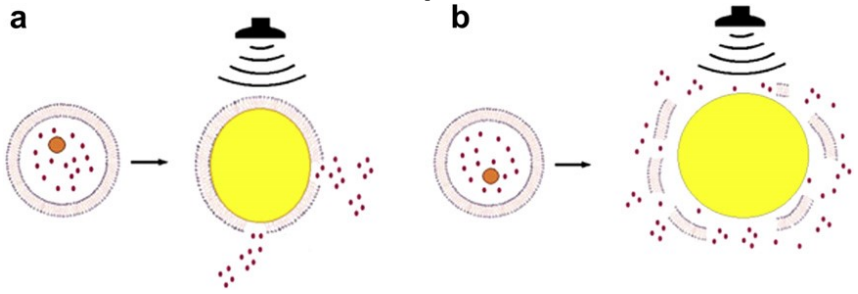

Fig. 5. Proposed mechanisms of ultrasound-induced release from eLiposomes. The small ovals represent calcein or encapsulated drugs. In each panel the medium-sized circle inside the liposome represents a liquid perfluorocarbon emulsion droplet. When exposed to the low pressure phase of an ultrasound wave, the droplet vaporizes and expands, disrupting the bilayered membrane and releasing the contents from the eLiposome. This expansion may stretch and rip the membrane (a), or it may more completely destroy the liposome by fragmenting the bilayered membrane into small sections (b).

intended to demonstrate the greater sensitivity of eLiposomes to ultrasound, as compared to conventional liposomes, in order to explore the potential of liposomes to be disrupted by acoustic emulsion droplet vaporization at short exposure times and relatively low ultrasound intensity. Although ultrasound from a probe sonicator would not be used in a clinical setting (because of focus and potential safety limitations),investigation of the ultrasound parameters provides insight into the ability to use eLiposomes at higher frequencies in a clinical setting or at the same frequency in a biological laboratory. The short exposure times and lower intensities chosen provide ultrasound exposures that would be comparable to higher frequency ultrasound administered in short pulses over a longer period of time. Acoustic droplet vaporization of perfluorocarbons has been reported by others at higher frequencies and similar mechanical indexes (Fabiilli et al. 2010; Rapoport et al. 2011; Sheeran et al. 2011). The mechanical indexes at the lowest 3 intensities used (0.5, 1, and 2 W/cm^2) were 0.63, 1.22, and 1.73, respectively, which may be clinically acceptable for short pulses to tumors. Furthermore, 20-kHz ultrasound at similar mechanical indexes has been used for drug delivery in cancer therapy in animal models (Larina et al. 2005; Staples et al. 2009). The eLiposome samples demonstrated increased calcein release compared to conventional liposomes (containing only calcein) and compared to conventional liposomes with external emulsions, suggesting that the encapsulated emulsion droplets add an ultrasound-sensitive element to the liposomes. The proposed mechanism for this increased sensitivity to ultrasound is the ability of ultrasound to vaporize the emulsion droplets (Kripfgans et al. 2000; Giesecke and Hynynen 2003; Rapoport et al. 2011; Singh et al. 2012). When the local pressure inside of the emulsion droplet falls below the vapor pressure of the emulsion there is a thermodynamic potential to vaporize. The expanding vapor phase stretches and disrupts the surrounding membrane and releases the encapsulated material because lipid bilayers can sustain only about a 3% expansion (Evans et al. 1976; Netz and Schick 1996). Two possible representations of this are illustrated in Figure 5. As the vapor phase expands, it may stretch the membrane, creating small rips and expelling the encapsulated material (Fig. 5a). This scenario is perhaps most likely with small droplets relative to eLiposome size and with a liquid having a lower vapor pressure. Perfluorocarbons with lower vapor pressures may be more difficult to vaporize and will collapse back into liquid droplets when no longer exposed to the low-pressure phase of an ultrasound wave. In this scenario, the gaps formed in the stretching membrane could potentially close during each cycle of ultrasound and following ultrasound exposure. Alternatively, if the expansion is more violent and/or if the volume of perfluorocarbon is more substantial, the membrane could be fragmented by the expanding vapor phase and associated cavitation events (Fig. 5b).

Remarkably, in the absence of ultrasound the eLiposome membranes appear to be sufficiently robust to completely sequester their cargo of calcein through various laboratory manipulations. The sham experiments showed no leakage (Fig. 4).

The eLiposomes were most sensitive to ultrasound when large emulsion droplets were encapsulated. This is most likely due to the greater Laplace pressure imposed on the droplets as their sizes decrease. The pressure on the nanodroplet interior is the sum of the environmental pressure and the Laplace pressure, or:

$$P_{inside} = P_{\infty} + \frac{2.\gamma}{r}$$

where P_{inside} is the pressure inside of the droplet, P_N is the local pressure of the continuous water phase, γ is the interfacial tension of the PFC/water interface, and r is the radius of the droplet. The increased pressure on the droplet adds to the ultrasound intensity required to vaporize the droplet. Because Laplace pressure is inversely proportional to radius, the amount of pressure on the

droplet increases as droplet diameter decreases. The Laplace pressure across the interface of the nanoemulsions can be estimated by using the interfacial tension reported for other perfluorocarbon emulsions stabilized by phosphocholines (Kabalnov et al. 1995). Interfacial tension for a perfluorocarbon droplet in water stabilized with DPPC can be estimated to be about 3.5 mN/m. This predicts a Laplace pressure of 140 kPa for 100-nm droplets and 35 kPa for 400-nm droplets. It may be noted that PFC5 has a normal boiling point of 29C, suggesting that it would boil at body temperature (37C). However, the additional Laplace pressure imposed on PFC5 emulsion droplets effectively raises the boiling point of small droplets above their normal boiling point, allowing them to remain in the liquid phase well above biological temperatures (Giesecke and Hynynen 2003; Sheeran et al. 2011), creating a PFC5 emulsion droplet that is meta-stable until disrupted by ultrasound. For example, when heated to 50C for extrusion in our lab, emulsion droplets persisted despite being heated above the normal boiling point of PFC5. A slight foam layer sometimes formed after extrusion. The presence of emulsion droplets after heating and extrusion was verified by adding an emulsion to the top layer of a ''sucrose cushion.'' The 1 M sucrose layer had a density much greater than that of perfluorocarbon bubbles, slightly greater than that of an aqueous suspension of liposomes, but less than that of liquid perfluorocarbons. The collection of a pellet at the bottom of the sucrose layer verified the preservation of emulsion droplets. Persistence of nanosized emulsion droplets was further confirmed by TEM. In the case of a meta-stable emulsion formed from a perfluorocarbon with a boiling point that is lower than biologic temperatures, droplet vaporization may be irreversible at biologic temperatures due to the decrease of the Laplace pressure as the bubble radius increases upon gas expansion. This irreversible phase change would create a persistent oscillating bubble that would continue to disrupt and shear the lipid membrane (Rapoport et al. 2011).

The increase in Laplace pressure with decreased droplet size results in a higher required ultrasound intensity to vaporize smaller droplets. As mentioned previously, both PFC5 and PFC6 eLiposomes with large emulsion droplets released more calcein than those with small droplets. In this study, the effect of droplet size can be best observed with PFC5 eLiposomes: eLiposomes with large emulsion droplets released approximately twice as much alceinasthosewithsmalldropletsafter10sofultrasound exposure at all 3 intensities tested (Table 1). After 100 ms of ultrasound exposure, the difference in calcein release from eLiposomes with large PFC5 emulsion droplets was statistically significant (p , 0.05) compared to that of eLiposomes with small droplets at intensities of 2 W/ cm^2 or greater. At 1 W/cm^2, these differences were significantforsamplesexposedtoultrasoundfor1sorlonger.I n PFC6 samples exposed to 100ms of ultrasound, the difference in calcein release from eLiposomes with large emulsion droplets was statistically significant compared to that of eLiposomes with small droplets at intensities of 3 W/cm^2 or greater. At 1 W/cm^2, these differences were statistically significant for samples exposed to ultrasound for 5 s or longer.

There was also a noticeable difference between eLiposomes formed with PFC5 and PFC6. This difference is most likely due to the difference in vapor pressures. Experiments were conducted at 24C. At this temperature, the vapor pressure of PFC6 is 28 kPa and the vapor pressure of PFC5 is 84 kPa. Because of the higher vapor pressure of PFC5, less acoustic amplitude is required to drop the local pressure below the PFC5 vapor pressure. At only 0.5 W/cm^2, the ultrasound imposes a negative pressure of 122 kPa, sufficient to overcome the difference in the atmospheric pressure and the vapor pressure of PFC5 or PFC6. This amplitude is also sufficient to overcome the Laplace pressure on 400-nm droplets. For 100-nm PFC5 droplets, 1 W/cm^2 is required to overcome the Laplace pressure, with ultrasound at that intensity imposing 173 kPa of negative pressure. Higher intensity ultrasound (2 W/cm^2) is required to provide 245 kPaof negativepressure in order to overcome the Laplace pressure and the lower vapor pressure for 100-nm PFC6 droplets. The expected pattern of PFC5 eLiposomes releasing more calcein than PFC6 eLiposomes was consistent for all analogous samples. These differences were statistically significant in a Student t-test (p , 0.05) for all intensities and times reported except for eLiposomes with small droplets after 10 s of ultrasound exposure at 0.5 W/cm^2 (Table 1).

Our eLiposomes released more calcein than the negative controls of liposomes without any emulsions or liposomes with external emulsions. This is consistent with the hypothesis that increased release was due to internal emulsion droplets' expanding and disrupting the membrane. Short exposure times (100 ms) led to increased release from eLiposomes compared to controls, which is also consistent with this hypothesis. In order to vaporize a liquid emulsion droplet with ultrasound, the applied negative pressure must lower the environmental pressure below the liquid vapor pressure and overcome the Laplace pressure imposed on the droplet. When these conditions are not met, the liquid droplet will remain in the liquid phase. After imposing the necessary pressure, a vapor phase should nucleate and expand during phases of low pressure and rupture the eLiposome membrane. The required ultrasound intensity for vaporization and time for nucleation should result in some kind of intensity threshold

for calcein release from eLiposomes. However, obvious thresholds were not observed with respect to time or intensity, despite operating at ultrasound parameters above and below those calculated to overcome both the Laplace pressure on the droplets and the difference between the local pressure and the droplet vapor pressure. This lack of obvious thresholds is most likely due to the distribution of sizes in both emulsion droplets and eLiposomes. The distribution of emulsion sizes results in differing Laplace pressures being imposed from droplet to droplet. Some emulsion droplets will therefore require higher or lower negative pressure in order to vaporize, and this distribution may be responsible for the lack of an intensity threshold for calcein release. Furthermore, nucleation probably does not occur instantly but may be a stochastic process that is related to the degree of sub pressurization of the emulsion. The experimental data indicate that nucleation occurs, but there may be a distribution in nucleation times that may smear out a theoretically sharp threshold in experimental data.

At longer exposures and higher intensities, control samples began to release more calcein, and the gap in percent of calcein released between eLiposomes and negative controls became narrower. However, these higher intensities of ultrasound are less desirable for clinical use. eLiposomes have the advantage of releasing encapsulated loads at low ultrasound intensities and relatively short exposure times. Low-intensity exposure provides leverage to increase the amount of drug that can be released while limiting unwanted heating and cavitation in adjacent tissues. Furthermore, eLiposomes with large PFC5 droplets approached 100% release at reasonable ultrasound parameters. This release was much higher than that observed in control samples, even at longer exposure times and with higher ultrasound intensities. However, in order to take best advantage of the EPR, eLiposomes have to be reduced to sizes closer to 250 nm. This will require the use of smaller (100 to 125 nm) emulsion droplets. At these sizes, the additional Laplace pressure may limit the ability to release 100% of encapsulated drug from PFC5 or PFC6 eLiposomes at reasonable ultrasound intensities. Ideally, a 250-nm eLiposome with small emulsion droplets could accumulate in cancer tumors or even in cells followed by an ultrasound-induced release of the entire encapsulated drug load. In order to approach this goal with eLiposomes, more efficient methods of inducing emulsion vaporization or other materials may be required, such as using lower-boiling-point perfluorocarbons or mixtures thereof with PFC5.

Finally, we caution readers that it is probable that applying 20-kHz ultrasound (which has a relatively long wavelength of 7.5 cm) within a small plastic chamber created standing waves. Constructive and destructive interference caused by these standing waves may have resulted in local regions of higher and lower pressures than the reported calibrated values. Lower pressure caused by destructive interference could have reduced the amount of calcein release expected at any specific intensity, while constructive interference may have caused local regions of higher intensity. Because of mixing in the cuvette, it is likely that eLiposomes in regions of lower intensity would have been quickly convected to regions of higher intensity. Thus the results presented herein represent the average release over the entire volume of a cuvette, and there may have been local regions of more intense insonation than the nominal intensity values listed in this report. Because the amount of release from the eLiposomes is not a linear function of intensity or time (Figs. 2, 3), extrapolation of these data to other experimental or clinical systems should be done with caution.

CONCLUSION

Liposomes formed from DPPC were made more responsive to ultrasound by encapsulating emulsion droplets inside of them. This was accomplished by forming lipid sheets, adding a suspension of emulsion droplets, and refolding the sheets into vesicles, encapsulating emulsion droplets in the process. The resulting eLiposomes demonstrated excellent sequestration of payload in the absence of ultrasound and yet were sensitive to short pulses of low-intensity ultrasound. Upon insonation, severalfold more calcein was released from eLiposomes than from controls. As expected by thermodynamic considerations, the small emulsion droplets were not as efficient as the large droplets, and the PFC6 was not as efficient as the PFC5 in activating calcein release. These data point to the use of large PFC5 emulsions in eLiposomes, but the need to construct eLiposomes small enough to be extravasated suggests that an optimized intermediate size would be most clinically relevant for drug delivery applications to tumors exhibiting the EPR effect.

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