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Development of Ultrasound Sensitive eLiposomes Containing Doxorubicin for Drug Delivery

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Authors' contributions

This work was carried out in collaboration between all authors. Author CYL formed the study concept, organized the experiments, interpreted the results, and drafted the manuscript. Author WGP revised and provided comments on the manuscript drafts. Author KCW managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: A novel nanocarrier was formulated by remote loading of doxorubicin (Dox) into a dipalmitoylphosphatidylcholine (DPPC) liposome that also contains various perfluorocarbon (PFC) droplets within its aqueous interior. It was shown that Dox can be loaded to a level of up to 67% into these large unilamellar vesicles composed of DPPC

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and cholesterol by employing a transmembrane pH gradient technique.
Methods: The different encapsulation efficiencies for these eLipoDox constructs of differing PFC composition are 45.5% (PFC5), 31.5% (PFC6) and 66.7% (PFC5/PFC6 mixture, PFCm). At 30 seconds of insonation, the eLipoDox formulation with PFCm droplets appeared to release more Dox than did eLipoDox with pure PFC5 or PFC6 droplets. The thermal stability of these eLipoDox formulations were examined at 37°C at different times; then controlled delivery was demonstrated by applying low-frequency ultrasound (US) at 1 W/cm^2.
Results: The eLipoDox with PFC6 or PFCm showed the best combination of thermal stability and drug release. An immunoblotting analysis indicates that ultrasound-triggered Dox release from eLipoDox could provide a higher quantity of nanodrug into tumor cells and thus may have cytostatic effects in cancer cells.
Conclusion: These eLipoDox constructs with low boiling point PFCs have the potential to provide more effective ultrasonically activated drug therapy to a desired location.

Keywords: Doxorubicin; controlled drug delivery; insonation; emulsion liposome; eLiposome; acoustic droplet vaporization.

ABBREVIATIONS

Dox: Doxorubicin; PFC: Perfluorocarbon; PFC5: Perfluoropentane; PFC6: Pefluorohexane; PFCm: A mixture of equal volumes of PFC5 and PFC6; DPPC: Dipalmitoylphosphatidylcholine; Chol: Cholesterol; DSPE-PEG-2000: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(poly-ethyleneglycol)-2000]; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; US: ultrasound; ADV: Acoustic droplet vaporization; eLiposome: emulsion liposome; eLipoDox: emulsion liposome containing doxorubicin.

1. INTRODUCTION

Acoustic droplet vaporization (ADV) is a phenomenon that can induce the phase change of a liquid droplet into vapor phase by application of cyclic pressure waveforms (acoustic waves) [1]. The proposed mechanism underlying ADV is that during the low pressure phase (rarefaction) of the acoustic wave, the local pressure drops below the vapor pressure of the liquid, at which point boiling may occur. Although many liquids have boiling points near body temperature, perfluorocarbons (PFCs) are very attractive candidates because they are relatively non-toxic, they are easily cleared through the lungs, and they are not very soluble in aqueous systems [2]. Another advantage of liquids that boil near body temperature is that the gas bubble formed will also collapse during each pressure cycle. This phenomenon, called collapse cavitation is extremely energetic; it causes extreme shear stresses that can stress and rupture cell membranes, and can propel fluid jets at velocities sufficient to pierce cell membranes [2]. Such energetic events on the cellular level have many potential biomedical applications, such as delivering molecular imaging agents, activating site-specific drug and gene delivery vesicles, and positioning phase-changed contrast agents for applications in biophysics, biology, and medicine [1-4].

Some PFCs have normal boiling points near body or room temperature [1]. The main advantages of liquid PFCs over conventional ultrasound contrast agents such as microbubbles are that droplets can be made very small, they have a longer biological half-

life, they are chemically and biologically inert, and they appear to be non-toxic [5,6]. In addition, PFCs were chosen for emulsion droplets because of their relatively very low solubility in water [7]. Because of their extreme hydrophobicity, PFC droplets must be stabilized by a shell, typically composed of protein or phospholipid. These surfactants greatly reduce the interfacial energy of the PFC droplets, which reduction decreases the propensity for droplet coalescence and also decreases the Laplace pressure inside the droplet. The Laplace pressure is an additional pressure imposed upon the interior of the droplet due to the highly curved nature of the small droplet surface. Since the Laplace pressure increases the interior pressure, the local pressure must drop even further during the acoustic cycle for boiling to be possible via pressure waves [8,9].

Ultrasound (US) is an ideal source of acoustic waves, and US equipment is prevalent since it is used clinically for diagnostics and therapeutics. ADV of perfluoropentane (PFC5) was used extensively in imaging for pre-clinical trials in the 1990s and has found recent application in acoustic imaging strategies [1]. ADV of very small droplets may have a further clinical application because reduction in droplet size greatly reduces the rate of clearance from the circulatory system, particularly compared to larger conventional contrast agent gas bubbles. PFC droplets provide an ideal combination of ultrasonographic contrast, acoustic cavitation, and non-toxicity that may be ideal for advanced medical diagnosis and therapy.

Chemotherapeutics still play the largest role in non-surgical cancer treatment. As an alternative or even a supplement to traditional chemotherapy, ultrasound-mediated targeted drug delivery is a non-invasive technique for drug administration that has the ability to deliver concentrated doses at target sites with the effect of enhanced cellular uptake. The major mechanisms involved in local drug targeting include heat generation (hyperthermia), acoustic cavitation and acoustic radiation forces [10,11]. Despite its advantages, broader acceptance of ultrasonically activated drug delivery using microbubbles is hindered by controversial issues and concerns related with clinical safety, efficacy, and the half-life of circulating bubbles in vivo. An elegant new approach would be the use of PFC nanoemulsion droplets, transformed to bubbles by ADV, as an alternative to microbubbles for targeting drug delivery [5,12,13].

Our previous research has reported acoustically-triggered drug release from a novel liposome containing at least one emulsion droplet (eLiposome), a drug carrier that may provide more safety and reduce the side effects of indiscriminate drug delivery to the whole body [5]. Insonation of these eLiposomes causes vaporization of the PFC nanoemulsion droplets, which expansion breaks the liposomal membrane and releases the drug payload [13]. We have modeled the physics of ADV and found that lower frequencies create greater expansion of the vaporized gas because at a lower frequency there is more time during the rarefaction phase of the pressure cycle to allow for vaporization of liquid to gas [14]. The dynamics of such a system have been observed experimentally and have been modeled for PFC5 and PFC6 droplets in water at different temperatures [14,15]. However, the formulation of PFC5 or PFC6 eLiposomes containing doxorubicin (eLipoDox) for drug targeting delivery in cancer chemotherapeutics has not been thoroughly examined. Moreover, the characteristics of distributing the therapeutic agents through ruptured liposomes also have not yet been examined, particularly in tumors with metastatic potential.

Herein we report on the continued development of eLiposomes containing doxorubicin (eLipoDox) by the remote loading method and demonstrate that drug release is responsive to low frequency ultrasound. The nano-sized emulsion droplets are formed from PFC liquids with high vapor pressures at biological temperatures, making them responsive to ultrasound. We studied the advantages of both ultrasonic sensitive targeting and physical release of Dox

directly inside cells *in vitro*, and hence an improved therapeutic response by eLipoDox employing different types of PFCs. We investigated the effect of insonation upon release of Dox at different power densities and simulated the effect of temperature in vivo upon release of Dox at different power densities using low-frequency ultrasound. We also examined the amount of Dox loaded into the eLiposome using high performance liquid chromatography (HPLC), performed immunoblotting analysis to identify the characteristics of cellular membrane enzyme activity, and studied the effect of this drug delivery technique on cell viability.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Phospholipids dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000](DSPE-PEG-2000) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Doxorubicin (Dox) was purchased from Pharmacia and Upjohn (Kalamazoo, MI, USA). Perfluoropentane (PFC5) was received from SynQuest Labs., Inc. (Alachua, FL). Perfluorohexane (PFC6) was from Sigma-Aldrich (St. Louis, MO). Sucrose and glucose were from Avantor Performance Materials (Phillipsburg, NJ) and United Biochemical Corp. (Cleveland, OH). Anti-glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Millipore Corp., Billerica, MA. Anti-β-actin antibody was received from Cell Signaling Tech., Danvers, MA. Other chemicals were reagent grade from Aldrich-Sigma (St, Louis, MO).

2.2 Perfluorocarbon Emulsion Droplet Formation

Emulsion droplets were made from PFC5, PFC6, and a mixture of equal volumes of PCF5 and PCF6 (PFCm). Briefly, 1.0 mL of DPPC solution (10 mg/mL in chloroform) was dried onto a flask and then hydrated in 1.5 mL of 110mM ammonium sulfate buffer, pH 4.5; then 1.0 g of PFC liquid was added to the flask. The flask was placed in an ice-water bath to reduce evaporation of PFCsduring the subsequent sonication mixing process. Emulsion droplets were formed by using a 20-kHz ultrasonic probe(Vibra-Cell VCX400, Sonics and Materials, Newton, CT), 1.25 W/cm^2 for three 30-second sonications on ice with 1 min of cooling on ice between sonications. The emulsions were then sequentially extruded 10 times through 200-nm polycarbonate filters and 10 times through 100-nm filters using an Avanti Mini Extruder (Alabaster, AL, USA).

2.3 eLiposomes Preparation

Liposomes containing DPPC, cholesterol, DSPE-PEG-2000, and α-tocopherol in a 3:1:1:0.004 molar ratio were made by the hydration method as described previously [5]. The suspension was extruded 10 times through 200-nm and 10 times through 100-nm filters. After cooling to room temperature, 1.0 mL of emulsion droplets was added to 1.0 mL of the liposome suspension and the mixture was sonicated at 20kHz, 1.25 W/cm^2 on ice, 3 times, 30 sec each time with 1 min between sonications. The obtained eLiposomes were extruded 10 times through a 200-nmfilter.

2.4 Pillow Density Separation

A density technique was used to separate un-encapsulated emulsions and empty liposomes from the eLiposomes as previously described [12]. Two different solutions with the same osmolarity and different densities were prepared. Sodium chloride (NaCl) and sucrose were used with an osmolarity of 0.3Os/kg and relative densities of 1.007 kg/L and 1.037 kg/L, respectively [16]. Following extrusion the raw mixture of eLiposomes, free emulsions and empty liposomes was added to the bottom of a 1.5-mL Eppendorf tube. Using a plastic syringe, about 0.5 mL of NaCl solution was gently pipetted to the bottom of the tube, underneath the sample. Similarly, about 0.5 mL of sucrose solution was gently pipetted to the bottom under the NaCl phase. The sample was spun for 10 min at 3000 rpm (50 x*g*) using a fixed rotor centrifuge (Eppendorf 5415 C, Hauppauge, NY). The unencapsulated emulsions have the highest density, so they settled at the bottom, while eLiposomes collected between the sodium chloride and sucrose layers and empty liposomes (having the lowest density) remained in the top layer. The eLiposomes were collected from the sucrose/salt interface by a pipette.

2.5 Loading of Doxorubicin (Dox) into eLiposomes

A doxorubicin solution (20 mg/mL in 0.3 Os/kg of sucrose/HEPES solution, pH 7.4) was added to the pre-formed and separated eLiposomes at 1:1 volume ratio of drug solution and eLiposome suspension; it was incubated at 4℃ for 24 h. Afterward, the free Dox was removed by passing the eLiposome-Dox over a ion exchange column (Dowex 50W-200, Sigma-Aldrich, MO) equilibrated with 110 mM of PBS buffer (pH 7.4). The concentration of Dox in eLiposome-Dox was determined by HPLC system at an excitation wavelength of 488 nm and an emission wavelength of 588 nm, respectively. Encapsulation efficiencies were calculated as the fraction of the original Dox that was incorporated into the eLipoDox vesicles.

2.6 Particle Size

The size distribution of eLipoDox was measured by dynamic light scattering (DLS) on a Brookhaven 90Plus (Brookhaven Instruments Co., Holtsville, New York). The concentration of each sample was adjusted to give between 500 to 1500 kcounts/s. Ten runs of 1 min each were averaged.

2.7 Ultrasound-mediated Dox Release

Ultrasonic release from eLiposomes in vitro was measured using a QuantaMaster spectrofluorometer (Photon Technology International, Birmingham, New Jersey) using the principle that concentrated Dox in the eLipoDox has a very low fluorescence compared to the released Dox. The eLipoDox was diluted 1:100 (v/v) in a cuvette with 110 mM of PBS buffer (pH 7.4) and fluorescence was collected at 4 Hz for 30 seconds to establish a baseline fluorescence with the excitation and emission wavelengths of 488 nm and 588 nm, respectively. The cuvette was removed from the spectrofluorometer and the contents exposed to 20-kHz US by inserting a 3 mm probe tip (VCX400, Sonics and Materials, Newton, CT) at various intensities and durations. The cuvette was then returned to the fluorometer to measure the fluorescence. Finally 60 μL of 2% of Triton X-100 was added and the sample was stirred to lyse any remaining eLipoDox. Fluorescence was measured once again. The percent release of Dox was calculated using the following equation:

$$\% \, Drug \, release = \frac{F_{US} - F_0}{F_{max} - F_0} \times 100\%$$

Where F_0 is initial baseline fluorescence, F_{US} is the fluorescent signal after insonation, and F_{max} is the fluorescence after complete Dox release using Triton X-100. The release experiments were repeated in triplicate.

2.8 Thermal Stability of PFC eLipoDox

After eLipoDox is made, it is stored at 4℃ until use. To examine its thermal stability at higher temperatures prior to insonation, 30 µL samples were placed into cuvettes with 3 mL PBS, and were stirred and incubated at 37℃ for 2, 5 and 10 minutes, respectively. At the designated time point, the baseline fluorescence was measured as described above. The cuvettes were then removed from the fluorometer and insonated for a given time interval at 1 W/cm^2. The fluorescence was again measured after sonication, and then 60 µL of 2% of Triton X-100 was added to lyse any remaining eLipoDox. Percent release of Dox was calculated using the previous equation. These release experiments were repeated in triplicate.

2.9 Cell Viability

HeLa (CCL-2^{TM}) cells were from the American Type Culture Collections (ATCC, MD). The cells (3×10^5 cells/mL) were seeded on 12-well plates and were cultured in folate-free RPMI 1640 medium supplemented with 10% fetal bovine serum in a 37℃, 5% CO_2 incubator for 48 h with complete cell attachment. Cell concentrations were measured using a hemocytometer and the viabilities were assessed using trypan blue exclusion test.

The five experimental groups were 1) control (no drug or US), 2) ultrasound only (no eLipoDox), 3) eLiposomes (no Dox), 4) eLipoDox, 5) eLiposomes with insonation, and 6) eLipoDox with US. The eLiposomes and eLipoDox contained the three different gas bubble compositions listed previously. A 20-kHz ultrasonic probe was inserted into culture wells with the tip approximately 2 cm above the cell monolayer. The power generator was set to an intensity of 1 W/cm^2. Ultrasound was applied for 2 seconds for the groups of the US only, the eLiposomes with US, and the eLipoDox with US. The cells were harvested and a homogeneous cell suspension prepared and counted. The number of viable cells was determined by the dye exclusion method using 0.4% trypan blue and measured by hemocytometer counting at 6 h of cell growth. Cell viability was calculated relative to the HeLa cell control at 6 h.

2.10 Quantitative Determination of Dox Uptake

Dox uptake in cells was quantitated by fluorescence measurements. After exposure to US or sham the cells were incubated at 37℃ for 6 h; then, the culture medium was removed and the cells were rinsed three times with cold PBS. The cells were lysed with 200 µL of a lysing buffer (10% (wt/vol) sucrose, 0.5% (wt/vol) NaCl, and 5% (vol/vol) Triton X-100 in 0.01 M Tris-HCl solution adjusted to pH 7.4). The cells were spun at 12,000 rpm for 10 minutes and the supernatants harvested into fresh tubes.

The supernatants were then filtered through a 0.22 µm Millipore filters before HPLC analysis on a Waters liquid chromatography system employing a C18 column. The mobile phase was

a 75:25 v/v methanol and filtered PBS, pumped at a flow rate of 1.0 mL/min; fluorescence data were measured at an excitation wavelength of 488 nm and emission at 588 nm.

2.11 Protein Assay

The treated cells were lysed with lysis buffer. After harvesting supernatants as previously described, these fresh lysates were immediately measured for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. Western blot analysis was performed by the method of Gillespie [17]. All blots were normalized to a β-actin standard.

2.12 Statistics

Statistical analysis was performed on a personal computer using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) statistical software. Statistical differences were assessed using the Mann-Whitney U test. The statistical significance was defined as $P < 0.05$.

3. RESULTS

3.1 Characteristics of eLipoDox

eLipoDox constructs containing one of three types of PFC droplets were developed as novel chemotherapeutics for cancer treatment. Dox was loaded into eLiposomes by first forming eLiposomes containing ammonium sulfate (pH 4.5) and one of 3 types of emulsion droplets, and then incubating in a Dox solution at pH 7.4 to load Dox by the pH gradient technique [18] as shown in Fig. 1. After loading the eLiposomes and removing the unincorporated Dox by ion exchange chromatography, the size distribution of eLipoDox with emulsions of PFC5, PFC6, and PFCm had average diameters from 146 nm to 460 nm, 160 nm to 370 nm, and 170 nm to 460 nm, respectively (Fig. 2A). Thus the sizes for each construct are comparable.

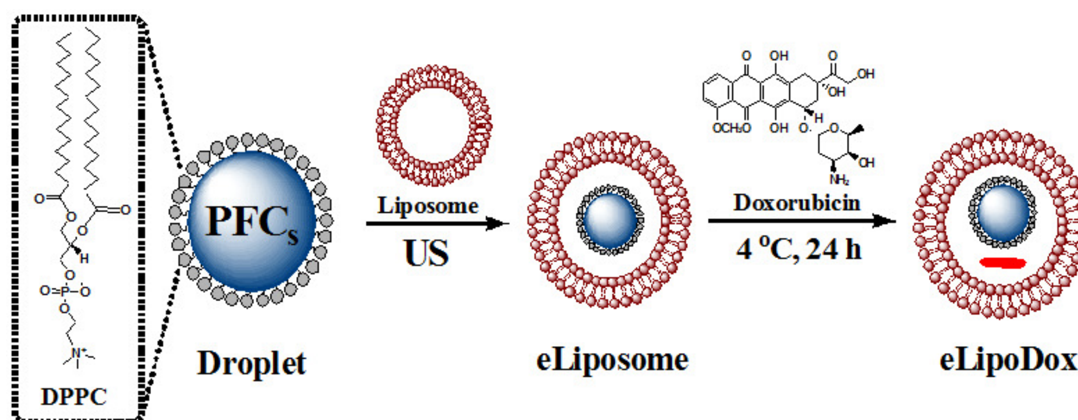

Fig. 1. Schematic representation of the process of remote loading of doxorubicin (Dox) into eLiposome

Different PFCs emulsion droplets were added to DPPC liposomes, and the mixture was sonicated using low-intensity ultrasound to encapsulate the droplets. Dox molecules were incorporated into the resulting eLiposomes by pH gradient loading at 4℃ for 24 h. The red bar in eLipoDox represent a doxorubicin sulfate crystal. PFCs, perfluorocarbons; US, ultrasound; DPPC, dipalmitoylphosphatidylcholine; Dox, doxorubicin. Sizes are not to scale

As measured by HPLC, the eLipoDox has a Dox entrapment efficiency of 45.5%, 31.5%, and 66.7% for constructs containing PFC5, PFC6, and PFCm droplets, respectively. It is noteworthy that the eLipoDox with the mixed PFCs had the highest loading efficiency. The HPLC retention times for Dox in the PFC5, PFC6, and PFCm constructs are 2.71, 2.71, and 2.76 min, respectively (Fig. 2B). The slightly longer retention time for the eLipoDox with PFCm may be due to the larger amount of Dox in the sample.

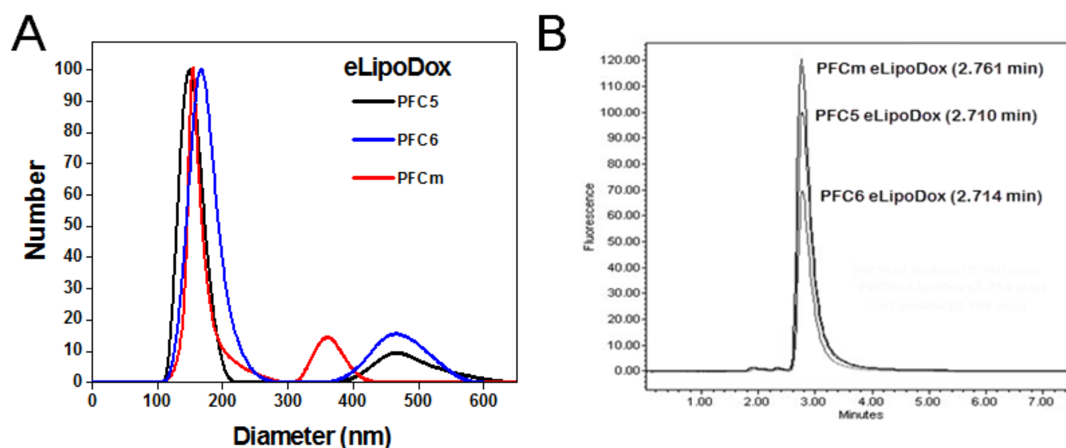

Fig. 2(A). Particle size distribution of eLipoDox with various PFCs measured by dynamic light scattering. (B) Dox encapsulated efficacy determined by HPLC

3.2 Ultrasonically Triggered Release

The ultrasonically triggered release of Dox from eLipoDox was evaluated at 20-kHz and 1 W/cm^2. Fig. 3 shows that the release of Dox from all eLipoDox formulations increased with increasing insonation time from 2 seconds to 30 seconds. After 30 seconds of exposure, the eLiposomes with PCF5, PFC6, and PFCm droplets had released approximately 50%, 55%, and 70% of the encapsulated Dox compared to only 20%, 12%, and 39% release at 2 seconds. The results suggested that the release of Dox by insonation was influenced by the composition of the PFC droplets. eLipoDox with PFCm had the highest encapsulation efficiency and also had the highest percentage of Dox release upon insonation. The same trend of increasing amounts of Dox release with increasing time was consistently observed with all samples.

The ultrasonically triggered release of Dox from eLipoDox with PFC5, PFC6, and PFCm droplets was also examined at different power densities at 20-kHz. Figs. 4A-C show the *in vitro* release profiles of Dox from these constructs insonated at 1, 2, 3, 4, and 5 W/cm^2 for 2, 5, and 10 seconds, respectively. It appears that as sonication time increases, even eLipoDox with the higher-boiling-points PFC6 droplets can significantly release Dox. Release from PFCm eLipoDox was larger after 2, 5, and 10 seconds of insonation compared to release from either PFC5 and PFC6 eLipoDox, nearly 15% and 20% higher than PFC5 or PFC6 eLipoDoxat the same insonation conditions. In contrast, PFC6 eLipoDox showed a slower Dox release upon insonation in the range from 1 W/cm^2 to 5 W/cm^2. These results with respect to the duration and intensity of ultrasound application are consistent with the results above at low-intensity frequency in that drug release increases with time and power.

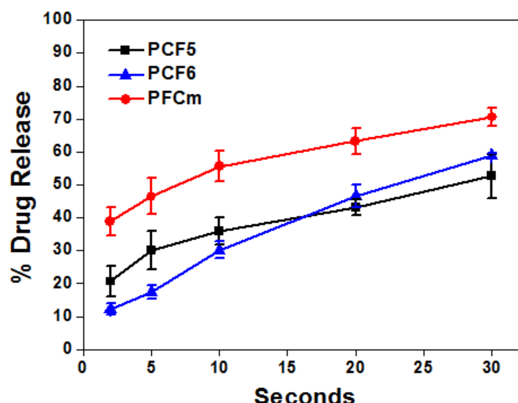

Fig. 3. Doxorubicin release from the three eLipoDox formulations when exposed to 20-kHz ultrasound at 1 W/cm^2 for different sonication times

Error bars represent the standard deviation of repeat experiments (n ≥ 3).

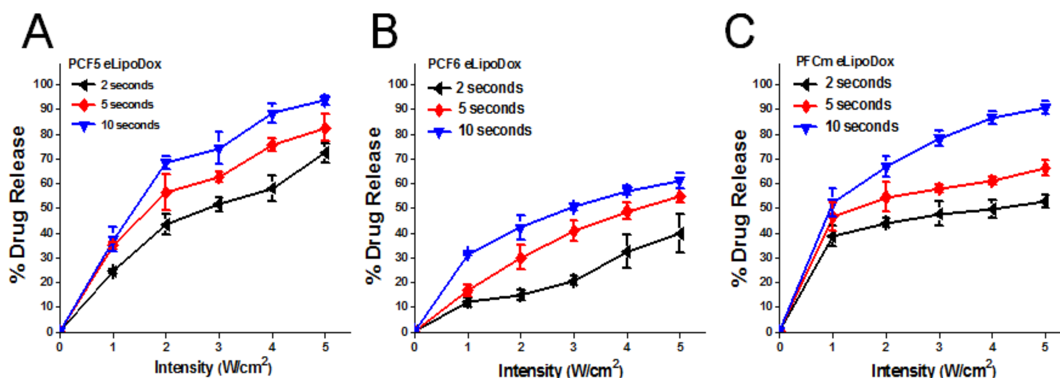

Fig. 4. Percentage of Dox released from eLipoDox as a function of ultrasound intensity and sonication with 20-kHz ultrasound

Error bars represent the standard deviation of repeat experiments (n ≥ 3).

3.3 Thermal Stability

Figs. 5A-C show that the release of Dox from eLipoDox formulations increased with increasing time of insonation from 2 seconds to 30 seconds after up to 10 minutes of incubation (without insonation) at 37℃. Fig. 5A shows that compared to 2 minutes of incubation, the release after 5 and 10 minutes of incubation was 10% to 15% less, perhaps due to slow Dox leakage from low boiling point of PFC5 at 37℃. Pure PFC5 has a normal boiling point of 29℃, but the small emulsion droplets do not boil because the pressure inside the droplet is greater than the surrounding hydrostatic pressure because of the Laplace pressure of the highly curved droplets [19]. In contrast, PFC6 has a normal boiling point of 57℃, and the PFCm has a calculated normal boiling point of 38℃. Figs. 5B and 5C show that the same amount of release occurred for PFC6 and PFCm eLipoDox at 2, 5, and 10 minutes of pre-incubation.

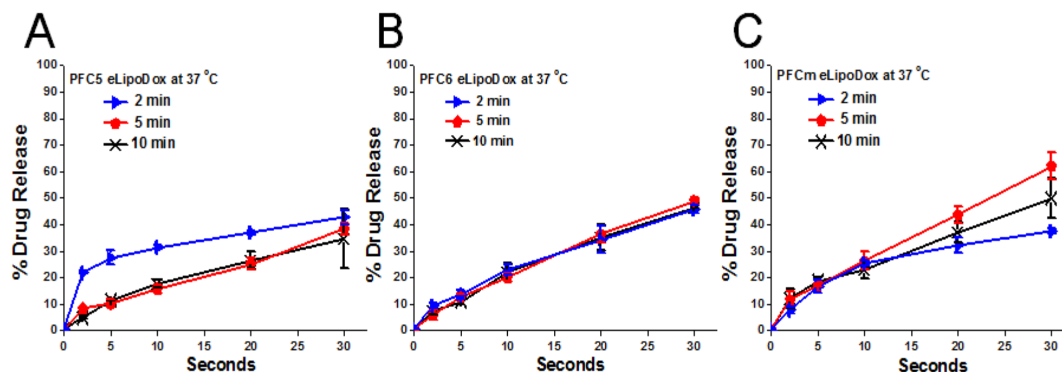

Fig. 5. Percentage of Dox released as a function of insonation time and the pre-insonation incubation time at 37℃ before insonation commenced. Insonation was at 20-kHz and 1 W/cm^2 after heating individually at 37℃ for 2, 5 and 10 minutes

Error bars represent the standard deviation of repeat experiments (n≥3)

3.4 Cellular Effects for eLipoDox with US *In vitro*

To evaluate the biocompatibility and toxicity of eLipoDox for its anticipated therapeutic applications, HeLa tumor cells were exposed to the PBS (control without Dox), the eLiposomes, and the various eLipoDox formulations, with insonation or with sham exposure (no insonation). The control had no eLipoDox and no insonation. Ultrasound at 20-kHz and 1 W/cm^2 was applied for 2 seconds for the insonated groups. The cell viability was measured for all experimental groups 6 h after applying real or sham insonation [5]. Fig. 6A shows that the cytotoxicity was effectively enhanced by insonation. The results indicate that insonation might increase the effectiveness of drug release even though there is no statistically significant difference between groups ($P > 0.05$).

Fig. 6B shows the amount of Dox quantified from the cells for various treatmentgroups. These results show that the concentration of Dox in the sonicated cells is always higher than for the unsonicated group at6 h, and is statistically higher for PFC6 and PFCm eLipoDox formulations. The amount of Dox delivered from PFCm eLipoDox with insonation was much greater than that for insonation of eLipoDox with of PFC5 or PFC6, and the difference is statistically significant. This suggests that insonation in the presence of eLipoDox has the ability to increase the transport of Dox into the sonicated cells.

The glyceraldehyde-3-phosphate dehydrogenase protein (GAPDH) served as a marker for cellular stress associated with the Dox delivery and distribution in the tumor cells after insonation of eLipoDox. GAPDH expression on endothelial cells indicates Dox-induced hydrogen peroxide production, which inhibited the key thiol-enzyme, GAPDH [20]. Cellular membrane disruption is useful for studies regarding whether or not any insonation in the presence of emulsion droplet induces the Dox released from eLipoDox and disruption of cell membrane, thereby increasing Dox uptake into cells. The results shown in Fig. 6C indicate that the GAPDH expressions following ultrasonic treatment with PFC6 and PFCm eLipoDox are slightly lower than for the other conditions. The difference of GAPDH expression between the cases without insonation indicates that the interaction of ultrasound with PFC emulsion droplets may produce gas bubbles that undergo stable or inertial cavitation, which result in the release of Dox and disruption of cellular membrane and the subsequent inhibition of GAPDH production.

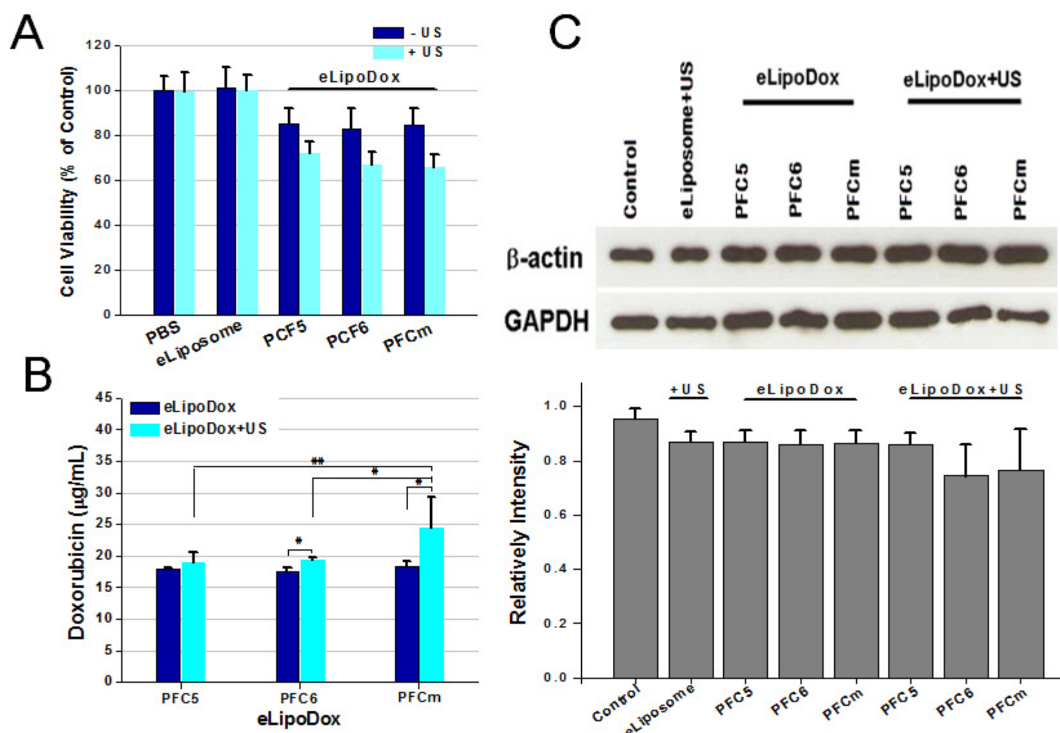

Fig. 6(A). Cell viability of the HeLa cells after different treatment conditions: PBS control, PBS and US only, eLiposome and PFCs eLipoDox without and with 2 seconds of US exposure at 6 h post exposure. Columns are the mean; bars are the SD (each condition, n=6). (B) Quantification of Dox amount in the cellular uptake after insonation without or with eLipoDox at 6 h after treatment (*P < 0.05; **P < 0.01). (C) Western blotting analysis and its GAPDH expression from tumor cellular lysates. The band for the eLipoDox with insonation exhibited a slightly weak expression of GAPDH. β-actin functioned as a control for protein loading (for each condition, n=3; mean plus SD)

4. DISCUSSION

In this study, we successfully loaded Dox into eLiposomes formulated with 3 different types of perfluorocarbon emulsion droplets using the pH gradient method [5]. We showed that Dox release could be manipulated by ultrasonic exposure at different intensities and times in order to cause the phase-change ultrasound contrast agents to expand to gas. A mixture of equal volumes of PFC5 and PFC6 (PFCm) inside eLiposomes exhibited a higher Dox encapsulation efficiency (approximately 68% of encapsulation; eLipoDox contains 1.8 mg/mL Dox) than eLipoDox formulated with PFC5 or PFC6 droplets. Not only was the loading greater, but the percentage of release upon insonation was also greater with the PFCm droplets. For example, 30 seconds of low-intensity ultrasound produced a release of up to 70% when compared to release from eLipoDox with PFC5 or PFC6 droplets. These PFCm droplets have a normal boiling point of 38ºC and should remain liquid at 1 atm pressure. The Laplace pressure further stabilizes this mixture. The *in vivo* enhancement of cytotoxicity toward HeLa cells is consistent with the *in vitro* results that show Dox can be loaded to high concentrations into eLipoDox by the remote loading method, with most

cytotoxicity from the PFCm eLipoDox formulation. Finally the PFCm and PFC6 versions of remained thermally stable when incubated at 37℃. The eLipoDox with PFCm apparently produced sufficient Dox release to inhibit GAPDH production.

The conjugation of folate to liposomal drug carriers increases their potency and specificity to cancer cells in a ligand-targeted therapeutics by a folate-mediated endocytosis, leading to highly selective cell killing [12,21]. We hypothesize that during insonation of endocytosed folated eLipoDox, the ultrasound pressure waves would interact with the PFC emulsion droplets within the eLipoDox and cause the liquid to expand to gas, possibly followed by bubble creation, growth, oscillation/deformation, and perhaps even violent cavitation [15]. These latter cavitation phenomena may shear and disrupt endosomal vesicle membranes or produce disruption of cell membranes for enhancing drugs transport within or into tumor cells [13-15]. Using folate-tethered targeting, eLipoDox would further enhance the efficiency of chemotherapy and reduce side effects by constructing a vesicle that would tightly sequester drug until the vesicle is endocytosed, and then release it to the cytosol only in desired tissues that are targeted by focused ultrasound. PCF5 and PFC6 were chosen as emulsion droplets because of their relatively high vapor pressure, low solubility in water and boiling point near body temperature. The Laplace pressure created by the curved interface of small emulsions enable PCF5 droplets to remain as a liquid even when above the normal boiling point of PFC5 [14,15]. The PFC6, which has a normal boiling point of 57℃, will remain liquid before and after insonation at body temperature, but still can be activated to a gas phase transiently during insonation [13,14]. Likewise the 1:1 volume mixture of PFC5 and PFC6 has a normal boiling point slightly above body temperature, so it will revert to a liquid form after a pulse of ultrasound, unless it was in a tumor or other situation of elevated temperature.

The Dox was remotely loaded into PFC eLiposomes using an ammonium sulfate transmembrane gradient [18,22]. Extra liposomal Dox at pH 7.4, being slightly hydrophobic and not charged, can easily diffuse through the DPPC/Chol/DSPE-PEG-2000 membrane. Once inside the acidified intraliposomal compartment at pH 4.5, it becomes protonated, which precludes membrane repermeation and results in an accumulation inside the eLipoDox vesicle, eventually forming doxorubicin sulfate [23]. Compared with previous publications showing loading efficiency, eLipoDox differs in regard to loading procedure and incubation condition [24,25]. We loaded Dox by forming eLiposomes containing ammonium sulfate buffer and emulsion droplets at 4℃ for 24 h in order to reduce evaporation of the PFC droplets.

We are intrigued and encouraged by the good performance of the eLipoDox with PFCm. The 1/1 volume mixture was selected because it has a vapor pressure of 94 kPa, assuming ideal thermodynamic mixing properties. This mixture has a bubble point around 38℃, meaning that in the absence of any Laplace pressure, it would start to form a gas (boil) at this temperature. The highly curved surface of the small emulsion droplet produces additional Laplace pressure that keeps the droplet from vaporizing until higher temperatures or lower local pressures are encountered. Because of the low surface tension for these mixed perfluorocarbons (PFCm) surrounded by phosphatidylcholines, the increase in Laplace pressure is relatively low such that the rarefaction pressure of ultrasound can produce gas formation [26].

Our experiments showed that eLipoDox with PFCm could be loaded with 1.8 mg Dox per mL eLipoDox suspension, a fairly high encapsulated efficiency, which was about 1.5-2 fold higher than we observed for loading eLipoDox containing pure PFC5 or PFC6 as shown in

Fig. 2. While the physical chemistry behind this higher loading efficiency is still under investigation, it is encouraging to note that an eLipoDox construct that whose PFC droplets have a normal boiling point slightly above body temperature can be efficiently loaded with Dox and be easily triggered by ultrasound to release its cargo.

In Fig. 3, the eLipoDox with PFCm displayed more release resulting in approximately 70% Dox release after 30 seconds of insonation. This represents a statistically significant increase in release at 30 seconds compared to delivery from eLipoDox with pure PFC5 or PFC6 droplets. The results show that the formulation of PFC emulsion nanodroplets apparently plays a key role to liposomal disruption.

The eLipoDox formulations were sonicated by using 20-kHz ultrasound transducer at power densities of 1 to 5 W/cm^2 from 2 to 10 seconds. Under these conditions, the eLipoDox with PFC5 and PFCm droplets released more Dox than did the eLipoDox with PFC6 droplets as seen in Figs. 4A-C. This suggested that the phase transition of PFCs emulsion droplets might be easier to produce at the low intensities of US used in these experiments. As we previously reported, the minimum intensity required for inertial cavitation for PFC emulsion droplets at 20-kHz is on the order of 0.4 mW/cm^2 [15]. The acoustic intensity used in this study was 1 W/cm^2 or above, and hence it would have enough energy to cause the PFC emulsions located within the eLiposome to produce a gas and even to cavitate, which gas production expands and breaks the liposomal membrane, enhances the permeability of local cell membranes, and releases the drug to promote drug transport into cells [5,12-15].

Fig. 5, shows that the relationship between the eLipoDox temperature stability, the change in response to heating, and the results of possible leakage of Dox from the eLipoDox after insonation. The eLipoDox formulations with PFCm and PFC6 were incubated below the normal boiling point of their droplets, while the PFC5 eLipoDox was incubated above the normal boiling point of PFC5. The PFC5 appears to have suffered the largest decrease in initial release rate with prolonged pre-insonation incubation time. One possible explanation may be that the temperature induced phase changes are not sufficient to evaporate the PFC6 and the PFCm droplets, but the PFC5 droplets were affected. However, experiments in our lab and others show that PFC5 droplets of sub-micron size are stable, even up to 20℃ or more beyond their normal boiling point [12,13,27]. We speculate that the acoustically triggered responsiveness of eLipoDox might be further optimized after further research to better understand the physical chemistry of the liquid PFC formulations within the eLiposomes.

Figs. 6A-C shows the cell viabilities, Dox quantification, and protein expression for HeLa tumor cells 6 h after insonation (or sham). This data is consistent with previous results showing that this type of mild insonation at 20-kHz does significantly decrease the cell viability. It also shows that the eLipoDox without insonation does not hinder the cell proliferation within 6 h after exposure [5]. In this study, we also investigated the cytotoxicity of eLipoDox combined with insonation at 6 h post-exposure. The cell viabilities after exposure to any of the eLipoDox formulations with insonation decreased to between 70% and 60% of the viability of the non-insonated control group. Also, the amount of Dox accumulated in cells with eLipoDox and insonation was enhanced, particularly for eLipoDox with PFCm droplets. These results are consistent with our hypothesis that insonation of these eLiposomes causes acoustic droplet vaporization (ADV) of the PFCs, which expansion breaks the liposomal membrane and possibly the nearby cell membranes. It is also interesting that our observations show that Dox treatment seems reduce cellular proliferation, without significantly altering cell viability.

The Dox used in this study has the potential to reduce oxidative metabolism purely as a function of limited available substrate [20,28]. The role of GAPDH in oxidative metabolism might be affected by this or other therapeutic drugs [29]. In the cells of this study, the GAPDH protein appeared as a major band at approximately 37 kDa and its intensity value following ultrasonic delivery of Dox from PFC6 and PFCme LipoDox was slightly smaller than for the other conditions (control, eLiposome, and eLipoDox without insonation). These findings suggest that insonation-mediated Dox released from eLipoDox may inhibit GAPDH enzyme expression on the cellular level [20,30], but the effect was not significant at 6 h. Although the precise mechanism as to how Dox alters metabolism and chemotherapy sensitivity remains unknown, our data supports the hypothesis that alterations in enzymatic regulation may result from this method of Dox delivery.

5. CONCLUSION

To summarize, this report shows that development of eLipoDox as a targeted drug carrier provides more efficient delivery, most probably resulting in a higher intracellular concentration of Dox than when free Dox is administered to the extracellular environment. The results also demonstrate that these eLipoDox carriers may provide an efficient carrier for chemotherapy for delivering significant quantities of Dox to a specific area, thus enhancing the local concentration and reducing the necessary whole-body doses that may damage other organs.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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