Article

Journal of
Nanoscience and Nanotechnology
Vol. 19, 1–8, 2019
www.aspbs.com/jnn

Factors Affecting the Acoustic *In Vitro* Release of Calcein from PEGylated Liposomes

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Typical methods used in cancer treatment, including chemotherapy, are debilitating because of the various adverse side effects experienced by cancer patients. The free drug injected into the patient at given doses affects both healthy and cancerous cells. Therefore, novel methods are being researched to ensure the selectivity of the treatment. The purpose of this study is to test the release of a model fluorescent drug, calcein, from echogenic stealth liposomes, triggered by low-frequency pulsed ultrasound. Several experimental parameters related to the ultrasound (US) and the investigated liposomes were varied in order to examine their effect on the acoustic release. Upon analysis of experimental results, the study concluded that release can be maximized by optimizing the sonication frequency, power density, and US pulse duration. When a non-isothermal chamber is used to conduct the experiments, it is important to have longer *'Off'* than *'On'* US periods in order to avoid overheating the liposomes. Applying such pulsation pattern can also be utilized to achieve slower release rates, which safely meet the desired drug levels at the end of the session. Our study also concluded that optimizing the liposome concentration is vital to delivering desired drug doses. Additionally, the type of lipids used in the synthesis should be carefully selected to produce stable yet acoustically sensitive liposomes capable of releasing at desired rates.

Keywords: Chemotherapy, Drug Concentration, Echogenic Liposomes, Power Densities, Pulse Duration, Saturated and Unsaturated Lipids, Ultrasound.

1. INTRODUCTION

Liposomal drug delivery systems are among the most rapidly evolving technologies in many fields.[1] The variable composition, structure, function, and scalability of a liposome are the underlying key reasons behind its significant contribution to such area of research.[1] Liposomes were discovered and defined about 40 years ago by Bangham and co-workers as nano- or micro-structured concentric spherical vesicles. These vesicles are composed of biocompatible, biodegradable, and non-toxic phospholipids, which are associated with little if any antigenic, pyrogenic or allergic reactions.[1–2]

The phospholipid bilayer that forms a liposome comprises polar hydrophilic heads that are at the inner and outer periphery of the bilayer, and are attached to long, non-polar hydrophobic tails that are trapped between the head groups. Because of its amphiphilic properties, liposomes can be used as carriers for hydrophobic, hydrophilic, lipophilic, or even insoluble matter-sized macromolecules.[2–3] Moreover, the methods of liposomal preparation have advanced in such a way so as to maximize the encapsulation efficiencies of the active agents.[4]

The use of liposomes as drug carriers has been reported by several laboratories, such as in the treatment of leishmaniasis, metabolic disorders, fungal diseases, and various types of cancer.[6–10] Particularly in cancer treatment, liposomes have the advantage of reducing therapeutic toxicity so as to deliver the chemotherapeutic drug directly to the cancer site.[4–11] Several anticancer therapeutics have been commercialized, including Doxil®, which encapsulates the active agent doxorubicin.[13]

Liposomes with polyethylene glycol (PEG) attached to their surface are termed PEGylated liposomes or 'stealth'

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liposomes.[12] The function of PEG is to increase the circulation half-life of liposomes in the body and to reduce the elimination of such liposomes by the mononuclear phagocyte system. Additionally, PEG facilitates the attachment of ligands such as proteins and antibodies to its long chains in order to increase the binding efficiency of targeted liposomes to cancer cell receptors.[12]

Passive liposomal targeting of tumors is a direct consequence of a phenomenon known as the enhanced permeability and retention (EPR) effect.[14–15] When liposomes are introduced intravenously into the body, they make their way through the leaky and imperfect tumor blood vessels. The EPR effect is aided by the malfunction of the tissue's lymphatic drainage system. Once at the site, the liposomes will eventually be taken up by the cancer cells, releasing the drugs inside the cells. On the other hand, *active* targeting uses ligand-targeted liposomes, which bind to specific receptors overexpressed on the surface of cancer cells.[16] This is followed by the receptor-mediated endocytosis of the liposomes into the cells.[12] Targeted liposomes, PEGylated or non-PEGylated, are prepared by conjugating the ligand to their surface. The efficiency of active targeting depends on several factors including tumor penetrability, ligand affinity, binding site barrier, and vascular permeability.[16–18]

Liposomes, under ideal conditions, should be capable of releasing their high concentration chemotherapeutic contents at the tumor site to guarantee high therapeutic efficacies against the disease while reducing its uptake by healthy organs and tissues.[11, 19–20] Moreover, an ideal drug delivery system should be capable of releasing its contents in a controlled manner with respect to time and space.[21] The latter requirement may be achieved using stimuli strategies, such as chemical or biological approaches (e.g., enzymes), or physical approaches (e.g., electric fields, temperature, magnetic fields, visible light, pH, and ultrasound (US)).[22–23]

Ultrasound has garnered considerable interest in the area of drug delivery; due to its non-intrusive nature and its ability to propagate through body tissues and increase the permeability of cell membranes and other body barriers.[19, 24–26] Acoustic waves, being a form of pressure waves, interact with drug carriers, body tissues, and cell membranes through a combination of thermal and mechanical effects (Fig. 1).

Liposomes that respond to ultrasound are known as echogenic or acoustically-activated liposomes (AAL). The ability of these liposomes to interact with US increases as the composing lipids become more saturated.[19, 27] There are two broad categories of US: low-frequency (LFUS) (up to 100 kHz) and high-frequency (HFUS) (>100 kHz).[28] For the purpose of this *in vitro* study, a fluorescent dye known as calcein was used as a model drug to test its release using LFUS.[28] After preparing and characterizing our calcein-encapsulated liposomes, several factors and their individual impact are thoroughly investigated and reported in this paper.

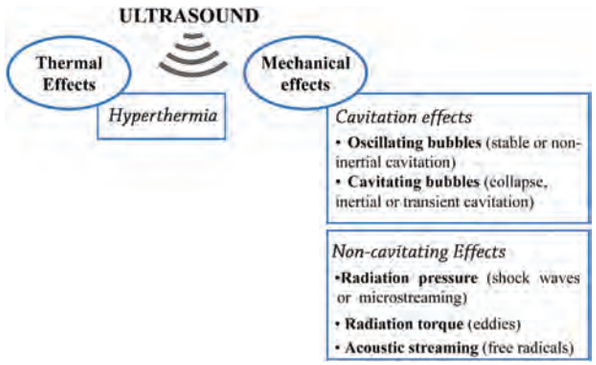

Figure 1. The physical effects of ultrasound.

2. MATERIALS AND METHODS

Two types of PEGylated, echogenic liposomes are reported in this study. Saturated 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is the main contributing phospholipid in both carriers, as it gives them their echogenic properties. The other lipids are either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), or its saturated analogue; 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE). These lipids have reactive groups at their ends, thus can be utilized to bio-conjugate targeting ligands for future applications.[3]

The two types of liposomes studied here are referred to as DOPE- and DSPE-liposomes. The synthesis procedure is illustrated in the following sections.

2.1. Synthesis of DOPE-PEG-pNP

The DOPE-PEG-pNP (para-nitrophenol) lipids were synthesized from DOPE lipids and pNP-PEG-pNP (para-nitrophenylcarbonyl-PEG-para-nitrophenylcarbonyl), as described in our previous paper.[28] In short, 4-nitrophenyl chloro-formate (p-NPC) was reacted with half its molar equivalent of PEG to produce pNP-PEG-pNP. Dichloromethane and pyridine were added to the reaction to act as an organic solvent and a nucleophilic catalyst, respectively. 32.2 μmol of DOPE was dissolved in dry chloroform (~5 mL) before being reacted with the previously synthesized pNP-PEG-pNP, in the presence of an excess amount of trimethylamine (TEA) (80 μL), added to remove the produced hydrochloride (HCL). This reaction was left w/continuous stirring at room temperature overnight under an argon atmosphere. The mixture was then transferred to a rotary-evaporator to evaporate the solvent. The formed lipids were then hydrated with 2 mL of a 0.01 M HCl–0.15 M NaCl solution, before being sonicated to allow the DOPE-PEG-pNP micelles to form. The latter were then purified using a Sephadex G-25 PD-10 desalting column. The hydrating solution was

then removed using a rotary evaporator, running under vacuum at high speed for two hours. Finally, micelles were extracted with chloroform while salt residues were centrifuged after being allowed to precipitate on ice. The produced 8.4 mM DOPE-PEG-pNP/chloroform solution was stored at 20 °C.

2.2. Preparation of DOPE- and DSPE-Liposomes

The previously synthesized DOPE-PEG-pNP micelles were reacted with DPPC (Avanti Polar Lipids, Inc., Alabaster, AL, USA), and cholesterol (AlfaAesar, Ward Hill, MA, USA) in a molar ratio of 68:30:2 in chloroform. DOPE-liposomes were prepared using the lipid film hydration method, as described in Ref. [28], where, after dissolving the lipids in chloroform, the latter was removed in a rotary evaporator, before the lipids being hydrated with a calcein/buffer solution (pH = 5.2), prepared at a self-quenching concentration (~30 mM). Following a 15-min full-power sonication at 40 kHz, the formed liposomes were extruded three times before being filtered and re-suspended in an adequate buffer, using a Sephadex G-25 PD-10 desalting column. DOPE-liposomes were finally stored at 4 °C until use.

The DSPE-liposomes were also prepared using the lipid film hydration method, but the DOPE-PEG-pNP was substituted by DSPE-PEG-NH_2 (Avanti Polar Lipids, Inc., Alabaster, AL, USA).

It should be mentioned that, when loaded into a Sephadex column, liposomes filtered first from the desalting columns, while the majority of the (much smaller) dye was trapped within the beads. The two phases (lipidic phase vs. free dye) can easily be spotted by the bare eye due to the distinct yellow colour of calcein. The extracted liposomes were then used in the release experiments.

2.3. Determination of Liposome Sizes

Liposome samples were diluted in buffer, then filtered using 0.45 μm nylon syringe filters (Whatman® Puradisc, Sigma-Aldrich Co. St. Louis, MO, USA). The size of the liposomes was measured using the DynaPro® NanoStar™ DLS instrument (Wyatt Technology Corp., Santa Barbara, CA, USA). DLS autocorrelation data were reported and analyzed using the Dynamics7—Static, Dynamic, and Phase Analysis Light Scattering (Wyatt Technology Corp., Santa Barbara, CA, USA) software.

2.4. Determination of Phospholipids

The amount of phospholipids in the synthesized liposomes was determined using a Stewart Assay.[29] This simple assay applies a conversion factor to translate the absorbance values (measured by a spectrophotometer) of complexes formed from liposomal phospholipids and ammonium ferrothiocyanate, into milligrams of phospholipids. The ferrothiocyanate reagent was prepared by dissolving 2.7 g of ferric chloride hexahydrate and 3 g of ammonium thiocyanate into deionized water. The final volume of the reagent was adjusted to 100 ml by adding deionized water and stored at room temperature. The measurement protocol started by preparing a solution of 0.1 mg DPPC/ml chloroform. Duplicate volumes (0.1–1 ml) of this solution were then prepared, with the final volume of each aliquot adjusted to 2 ml by the addition of chloroform. Two ml of the ferrothiocyanate reagent were then added to each aliquot, before vortexing and centrifuging the samples at high velocity. The lower phase of each aliquot was then removed using a Pasteur pipette. The optical density of chloroform was finally read at λ 485 nm. Similarly, the buffer solution was evaporated in a rotary evaporator from a liposomal sample (50 ul sample). One ml chloroform was added to dissolve the lipids, with 40-kHz US applied at full power. Liposomal/chloroform solution was then divided into triplicates of varying volumes, having the final volume of each adjusted to 2 ml by the addition of chloroform. Two ml of the ferrothiocyanate reagent were then added to the aliquots. Each aliquot was then vortexed and centrifuged at a high velocity for 10 min. The lower phase was removed using a Pasteur pipette. The optical density of chloroform was finally read at λ 485 nm. Average optical densities were plotted against the values obtained earlier from DPPC aliquots. Milligrams of DPPC in these aliquots were calculated using the resulting calibration curve.

2.5. Release Dynamics Using LFUS

LFUS release experiments were carried out at a pH value of 7.4 (in PBS buffer). The objective of these experiments was to track the release of calcein from the liposomes subjected to LFUS. The release was continuously monitored by recording the fluorescence level of the liposome solution throughout the ongoing sonication process. A non-isothermal QuantaMaster QM 30 Phosphorescence/Fluorescence Spectrofluorometer (Photon Technology International, Edison NJ, USA) was used. An excitation wavelength of 494 nm and an emission wavelength of 515 nm were used as fluorescence emitted by the calcein is visible in this range. The sample was directly diluted in a plastic disposable fluorescence cuvette, which was placed in the sample chamber. The 20-kHz ultrasonic probe (Vibra-Cell model VC130PB, Sonics and Materials Inc., Newtown, CT, USA) was introduced through the opening in the top cover of the fluorometer and slightly immersed inside the sample in the cuvette, in such a way that it did not block the path of the emitted light. The initial fluorescence (prior to US) was recorded (i.e., the baseline), and the acoustic exposure was initiated 60 s later. Sonication was done at different power settings using 3 different pulse patterns (10 s *On*/10 s *Off*, 20 s *On*/10 s *Off* and 20 s *On*/30 s *Off*) until the fluorescence reached a plateau. During the *On* period of each sonication cycle, the ultrasound was applied continuously. After

the fluorescence level plateaued, the fluorometer measurements were paused, and a 2% (w/v) Triton X-100 solution was added to a final concentration of 0.48 mM to lyse any undisrupted/intact liposomes, and then the final fluorescence level representing 100% release was measured.[29–30] Fluorescence release was normalized using Eq. (1):

$$\text{Drug Release} = \frac{F_T - F_o}{F_{\max} - F_o} \times 100\% \quad (1)$$

where F_t is the measured fluorescence intensity in the sample after a given sonication duration t, F_0 is the initial fluorescence before sonication, and $F_{\max}$ is the maximum fluorescence intensity after addition of $T \times 100$. A Brüel and Kjaer Type 8103 Hydrophone (Nærum, Denmark) was used to approximate the power density delivered to the samples. Equivalent densities to the 20%, 25% and 30% power settings on the sonicator display map to 6.09, 7.8, and 11.7 W/cm^2 respectively.

In addition to the above-mentioned power values, a 23% power setting, which is equivalent to a power density of 6.84 W/cm^2 was applied to test release from aliquots prepared at different liposomal (thus calcein) concentrations, namely at concentrations of (75 μl liposomes/3ml PBS), (100 μl liposomes/3 ml PBS) and (125 μl liposomes/3 ml PBS), using a pulse cycle of 10 s *On*/10 s *Off*.

3. RESULTS AND DISCUSSION

In this work, we studied the effect of 20-kHz LFUS on the release of the model drug calcein from two different types of PEGylated carriers: DOPE- and DSPE-liposomes. In this section, unless otherwise stated, the term "liposomes" refers to the first type (DOPE-liposomes).

The effect of US power intensity on drug release from liposomes has been widely studied, and our previous study is an example.[28] Besides varying the power density, in this work, we also varied the pulse pattern, using equal time *On*/*Off* (10 s *On*/10 s *Off*), an *On* pulse longer than an *Off* pulse (20 s *On*/10 s *Off*) and an *On* pulse shorter than an *Off* pulse (20 s *On*/30 s *Off*). This resulted in different total insonation times, different release curves, different initial release rates, and different maximum releases.

3.1. Liposome Size

The size of the DOPE-liposomes was determined as 130.12 ± 4.28 nm ($n = 10$ batches of liposomes, at least two technical replicates each), while that of the DSPE-liposomes was 96.75 ± 3.83 nm ($n = 3$ batches of liposomes, at least two technical replicates each). Hence, both types of liposomes are classified as large unilamellar vesicles (LUVs). While both vesicles were synthesized following the same procedure, the difference in size might have been due to the micelles used in preparation. For instance, the DOPE-PEG-pNP micelles (used to prepare the unsaturated liposomes) were synthesized in our lab in two lengthy steps totalling a preparation time of over 48 hours; hence, the presence of more impurities and larger molecules were expected. Whereas the DSPE-PEG-NH_2 micelles (used to prepare the saturated liposomes) were synthesized using more sophisticated techniques and equipment at Avanti Polar Lipids, Inc.

3.2. Effect of Changing Amplitude and Pulse Duration

Figure 2 shows the initial and maximum release levels, determined by the increase in fluorescence due to the relief of calcein's self-quenching when ultrasound is applied, at different amplitudes and pulse durations. The figure also indicates that, when applying different pulses, the only significant differences in initial and final release percentages and rates are obtained when the *'On'* period is less or equal to the *'Off'* period. Experimental results also show that the minimum initial release was obtained when the pulse of (10 s *On*/10 s *Off*) was applied. Table I lists the p-values for the statistical comparisons between different sonication pulses, while Table II lists the p-values for the statistical comparisons between different frequencies.

Figure 3, on the other hand, shows release patterns of calcein from DOPE-liposomes, during our 440 s experiments (referred to as *total experimental time*, and it includes the whole *'On'* and *'Off'* periods), at different amplitudes and pulse durations. It should be mentioned that the part preceding the *total experimental time*, which shows the coinciding baselines of the runs (prior to applying US), was deducted from this figure, as the main focus of this illustration is the patterns of release after sonication. Nevertheless, the average value of each baseline was integrated in Eq. (1), so as to obtain the normalized data.

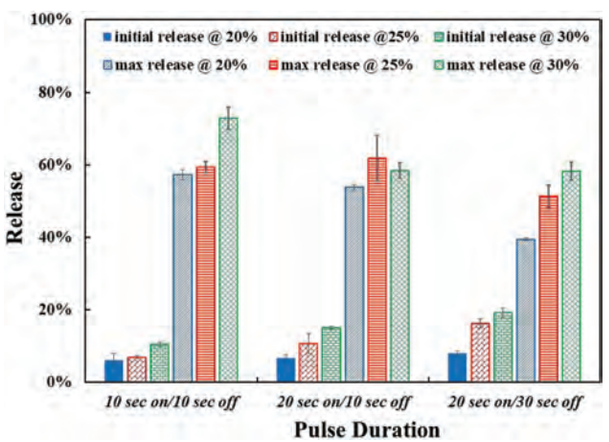

Figure 2. Average initial and final releases at different amplitudes and pulse durations. Results are average of 10 measurements (3 liposome batches) for release at 20%, 25% and 30% power settings, at 10 s *On*/10 s *Off* pulse cycle, 7 measurements (one liposome batch) for release at 20%, 25% and 30% power settings, at 20 s *On*/10 s *Off* pulse cycle, and 6 measurements (one liposome batch) for release at 20%, 25% and 30% power settings, at 20 s *On*/30 s *Off* pulse cycle

Table I. Statistical analysis for release data while comparing different pulses for a given frequency.

Frequency	Pulse comparison	*p*-value (average initial release)	*p*-value (maximum release)
20%	10/10 versus 20/10	0.7856	0.0353
	10/10 versus 20/30	0.2428	8.29E–5
	20/10 versus 20/30	0.2353	0.0218
25%	10/10 versus 20/10	0.1668	0.6305
	10/10 versus 20/30	0.0101	0.0087
	20/10 versus 20/30	0.0858	0.0186
30%	10/10 versus 20/10	0.0008	0.0042
	10/10 versus 20/30	0.0003	0.0033
	20/10 versus 20/30	0.0519	0.3894

Notes: $p \geq 0.05$, $0.04 \leq p < 0.05$, $0.03 \leq p < 0.04$, $0.02 \leq p < 0.03$, $0.01 \leq p < 0.02$, $p < 0.01$.

Both figures (Figs. 2 and 3) confirm previously published results[26] that liposomes release their contents regularly and slowly as the sonication proceeds, and that as the amplitude increases, the release increases. They also show that the release increases as the US *'On'* time increases, until a plateau is reached, which corresponds to the maximum amount (equilibrium amount) of the drug that the acoustic waves are capable of releasing (at the frequency and power density investigated). Once the fluorescence level plateaus, the release levels cease to increase, unless the process was non-isothermal. In such a case, the sample might overheat, and the spectrofluorometer starts to read faulty fluorescence output levels. This is clearly illustrated in Figure 4, where the release levels were increasing initially when applying the 10 s *On*/10 s *Off*-cycle US (has a total of 220 s *'On'* out of the *total experimental time*), achieving the highest final acoustic release, even before reaching equilibrium.

On the other hand, the curves of the 20 s *On*/30 s *Off*-pulses have the shortest *'On'* sonication period (equivalent to 176 s *'On'* out of the *total experimental time*) and took the longest time to achieve maximum release. In this case, the release levels were initially "slightly" higher

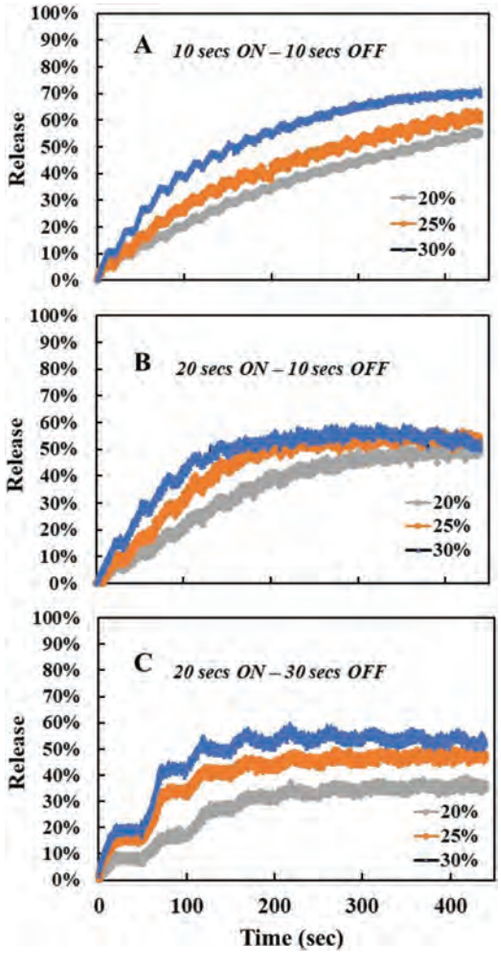

Figure 3. Release curves at different amplitudes and pulses. (A) 10 s *On*/10 s *Off*, results are average of 10 measurements (3 liposome batches); (B) 20 s *On*/10 s *Off*, results were obtained with 1 liposome batch and are average ± standard deviation of 2 technical replicates (blue line), 3 technical replicates (red line) and 2 technical replicates (red line); (C) 20 s *On*/30 s *Off*, results were obtained with 1 liposome batch and are average ± standard deviation of 2 technical replicates for each line. The blue lines correspond to 20% power setting, the red lines are 25% and the green lines are 30%. The gray shades are the error bars.

Table II. Statistical analysis of release data while comparing different frequencies for a given pulse.

Pulse	Frequency comparison	*p*-value (average initial release)	*p*-value (maximum release)
10 sec *On*	20% versus 25%	0.5636	0.1791
10 sec *Off*	20% versus 30%	0.0037	0.0001
	25% versus 30%	0.1282	0.1361
20 sec *On*	20% versus 25%	0.1425	0.1802
10 sec *Off*	20% versus 30%	0.0083	0.0918
	25% versus 30%	0.1153	0.9604
20 sec *On*	20% versus 25%	0.0150	0.0320
30 sec *Off*	20% versus 30%	0.0088	0.0088
	25% versus 30%	0.1282	0.1361

Notes: $p \geq 0.05$, $0.04 \leq p < 0.05$, $0.03 \leq p < 0.04$, $0.02 \leq p < 0.03$, $0.01 \leq p < 0.02$, $p < 0.01$.

than the previous ones as the *'On'* cycle was initially longer (20 s compared to 10 s). However, as the experiment progressed, the increase in release slowed down as the cumulative *'On'* time became less, eventually reaching equilibrium (and maximum release) at the slowest rate recorded. Alternatively, the 20 s *On*/10 s *Off*-cycle curves, which had the longest *'On'* sonication periods investigated (293.3 s *'On'* out of the *total experimental time*), though they started to increase initially with similar rates to the curves of the previous cycle (both cycles had 20 s *'On'*), the short *'Off'* periods allowed them to plateau faster than the two other sets. However, at the highest US power density investigated (30% power setting), the curves of this set (20 s *On*/10 s *Off*) started to decline, possibly driven

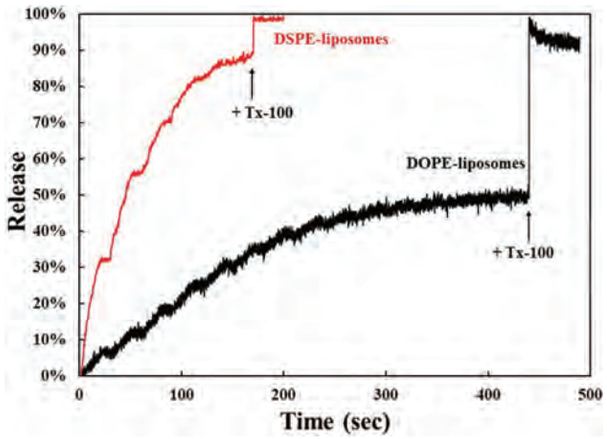

Figure 4. Effect of changing lipids used. Results are average ± standard deviation of 2 measurements (one liposome batches) for release from DOPE-liposomes and of 2 measurements (one liposome batches) for release from DSPE-liposomes, at 20% power setting and 20 s *On*/10 s *Off* pulse cycle.

by the excess thermal heat generated by the US probe, which might be the reason for the lower fluorescence level observed, and hence the lower final calculated release percent, compared to values obtained at other pulse durations or lower power densities. Our experiments showed that it is essential to choose the optimum *'On'* and *'Off'* sonication periods in future *in vitro* studies, so that the *'On'* period would be long enough to induce drug release at concentrations that meet the therapeutic levels, and the *'Off'* period is long enough to allow the surrounding tissues to cool down, without disturbing the drug concentration or its signal.

It should be stressed that our work does not necessarily suggest that one pattern of release is better than the other, as the one to be chosen would primarily be determined by an oncologist depending on various factors, including the patient's condition, the type and expected toxicity of the applied drug, and release mechanisms and conditions.[31] Our work mainly points out that tuning specific factors and conditions related to drug synthesis and release can be used to optimize and directly affect the outcomes of drug release. That is to say, we only present a way to make such tuning possible—by understanding which factor/condition needs to be manipulated, in order to achieve a desired therapeutic effect.

3.3. Effect of Changing Lipids of Liposome

Different lipids may be used to synthesize liposomes. These lipids differ in their fatty acids constituents, lengths, net charge and their transition temperature.[32]

Figure 4 shows two release curves, representing the performance of two types of liposomes; the first contains the unsaturated DOPE lipids (DOPE-liposomes), while in the second, all the lipids are saturated (DSPE-liposomes). Both were sonicated for 2 minutes at a power setting of 20% and a pulse duration of 20 s *On*/10 s *Off*. The same (DPPC: Cholesterol: PEG-derivatives) ratios were used in both sets, with the same concentration of the entrapped calcein. The almost identical fluorescence levels upon adding TX-100 at the end of the experiments suggests that both types of liposomes encapsulated (approximately) equivalent amounts of calcein. This was also supported by Stewart assay measurements. That is to say, the Stewart assay showed equivalent phospholipid content in both types of liposomes. This might suggest that equivalent amounts of liposomes were formed using both types. Also, as exact amounts of calcein were used to prepare all liposomal samples, and as fluorescence levels before applying US (baselines) and at the end of the release experiments (after TX-100) were equivalent, it can be safely hypothesized that the concentrations of encapsulated calcein in both types were equivalent. Nevertheless, DSPE-liposomes were considerably more responsive to US than the DOPE-liposomes at the conditions investigated, and thus released their contents at faster rates (see also Fig. 5). This faster release from DSPE-liposomes (and from saturated liposomes in general) might be due to the non-bilayer forming characteristics of the saturated lipids, which allows ultrasound to perturb the liposomal membrane in a more rigorous way than when interacting with unsaturated layers; consequently, the carriers would release significantly higher drug amounts from DSPE-liposomes compared to DOPE-liposomes at the same time point.[28, 33–34] It is important to stress that these findings do not necessarily imply that using saturated lipids for the synthesis of liposomes is always recommended. In fact, this merely depends on the application in which liposomes are used, and whether fast or slow rates of release are desired.

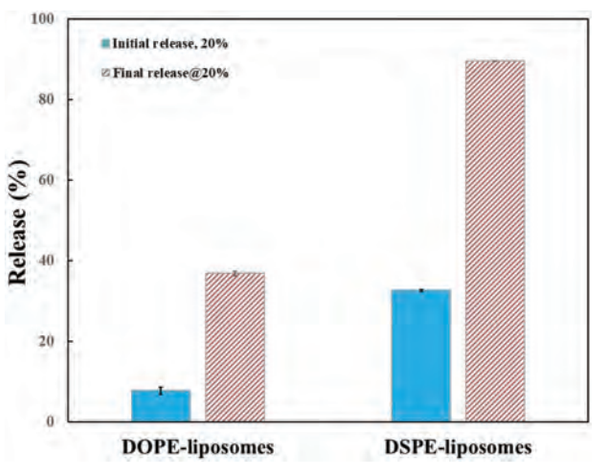

Figure 5. Average initial and final releases from DOPE- and DSPE-liposomes. Results are average of 2 measurements (one liposome batches) for release from DOPE-liposomes and of 2 measurements (one liposome batches) for release from DSPE-liposomes, at 20% power setting and 20 s *On*/10 s *Off* pulse cycle.

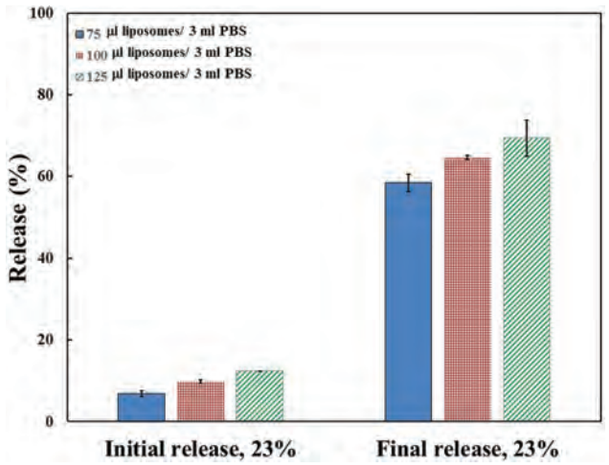

Figure 6. Average initial and final releases when changing liposomes concentration in buffer solution. Results are average ± standard deviation of 6 measurements (2 liposome batches) for release at concentrations of 75 μl liposomes/3 ml PBS, 100 μl liposomes/3 ml PBS and 125 μl liposomes/3 ml PBS, at 23% power setting and 10 s *On*/10 s *Off* pulse cycle.

3.4. Effect of Changing Concentration of Encapsulated Drug

Choosing the optimal concentration of the therapeutic agent to be encapsulated then released, is essential in preventing the development of multi-drug resistance in tumors if the drug concentration falls below the therapeutic level.

In our sets of experiments, the differences in calcein concentrations were obtained by changing liposomal concentrations in the aliquots instead of adjusting the amounts of calcein initially encapsulated. This was done in an attempt to avoid any changes that might occur during the synthesis and purification steps. Accordingly, and as hypothesized all liposomes encapsulated similar amounts of calcein; by increasing the amount of liposomes diluted in the same amount of PBS, we, therefore, increase the amount of calcein that can be released, depending on the power of US.

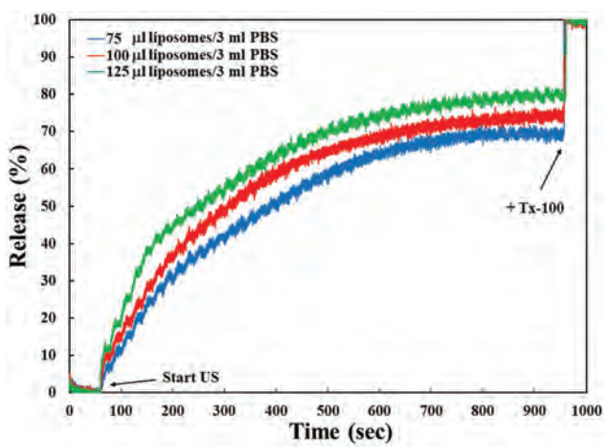

Figure 7. Release curves showing effect of using different liposomes concentration in aliquots solutions. Results are average of 6 measurements (2 liposome batches) for release at concentrations of 75 μl liposomes/3 ml PBS, 100 μl liposomes/3 ml PBS and 125 μl liposomes/3 ml PBS, at 23% power setting and 10 s *On*/10 s *Off* pulse cycle.

Table III. Statistical analysis of release data while comparing different concentrations for a given pulse and frequency.

Pulse	Concentration comparison	p-value (average initial release)	p-value (maximum release)
10 sec *On*	75 versus 100	0.0364	0.0587
10 sec *Off*	75 versus 125	0.0083	0.0874
@23%	100 versus 125	0.0125	0.2667

Notes: $p \geq 0.05$, $0.04 \leq p < 0.05$, $0.03 \leq p < 0.04$, $0.02 \leq p < 0.03$, $0.01 \leq p < 0.02$, $p < 0.01$.

Figure 6 shows the initial and final release percentages for a set of release experiments, conducted at 25% power settings, using a 10 s *On*/10 s *Off* cycles and a 890 s-*total experimental time*, at different liposomal concentrations (75 μl liposomes/3 ml PBS, 100 μl liposomes/3 ml PBS and 125 μl liposomes/3 ml PBS). Figure 7 shows the actual release profiles under these conditions. The figure reveals that as the concentration of the encapsulated calcein increases, more drug is released under ultrasound.

Table III displays the p-values for the statistical comparisons between the different concentrations. Results show that only the average initial releases were significantly different (p-value <0.05). However, the final release values were, to a great extent, equivalent at the end of the experiments. On these sets, the lower concentration used is more preferable, as it is able to achieve the same final release levels of the higher concentrations while using less drug/carriers during the synthesis steps.

By examining the above results, we hypothesize that US is not able to completely break down the liposomal membrane, it rather disrupts the surface, allowing the drug to passively diffuse out of these nanocarriers. However, at a certain point, the drug will no longer diffuse, as its concentration inside and outside of these nanocarriers reach equilibrium. This is evident by the declining release rate as the sonication ensued (followed by the plateauing behavior of the release after 5 minutes of US). Clearly, all curves eventually reach a plateau, but the final release level slightly increases as the concentration increases.

4. CONCLUSIONS

Triggered-stealth liposomal combinations have been widely investigated as prominent delivery systems, which can be utilized in a wide range of applications. The synergistic effects of LFUS, when used as a trigger for echogenic-PEGylated liposomes, have rendered these carriers even more useful in the fields of gene and drug delivery. The present study indicates that several factors should

be considered when designing liposomal carriers and using ultrasound as a trigger.

All release experiments reported in this study were conducted in a non-isothermal chamber, using two types of PEGylated liposomes. A 20-kHz LFUS was applied at three power densities, three *On*/*Off* pulse durations and varying insonation times, using three aliquot concentrations and two types of lipids. The behavior of liposomes at these conditions was studied quantitatively, and the results were summarized graphically, and statistically.

The use of ultrasound to release chemotherapeutic and other drugs from nanocarriers has shown promise. The fact that ultrasound is non-invasive and can be focused on the diseased tissue with minimum effects on other healthy cells renders this technique even more useful. The enormous amount of data presented and analyzed in this work were merely collected to study possible factors that affect synthesis and release conditions of a fluorescent chemical from liposomes, and further, determine possible effects and/or tuning techniques that can be used to reach certain desired outcomes of each set of experiments. That is to say; we suggest that it is possible to reach the desired outcome from a release experiment when enough knowledge of the effects caused by each factor and condition are investigated. Such knowledge would sharply minimize the number of experiments needed in the lab.

One of the main objectives of acoustic cancer research is the optimization of ultrasound parameters that may be employed clinically in the future. This paper is our humble attempt to help achieve this goal.

Acknowledgments: The authors would like to acknowledge the funding from the American University of Sharjah Faculty Research Grant (FRG1-2012), Patient's Friends Committee-Sharjah, AlJalila Foundation, Al Qasimi Foundation, and the Dana Gas Endowed Chair of Chemical Engineering.

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Received: 21 September 2018. Accepted: 12 October 2018.