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Effect of pH, ultrasound frequency and power density on the release of calcein from stealth liposomes

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Abstract: The use of liposomes as carriers for chemotherapeutic agents in combination with ultrasound as a stimulus to control the time and space of the drug release is a promising approach for cancer treatment, as it can reduce the side effects caused by conventional chemotherapy. This in vitro study investigated the triggered release of calcein from stealth (PEGylated) and non-stealth (non-PEGylated) liposomes, using ultrasound at low (20 kHz) and high (1 and 3 MHz) frequencies, and at different power densities. Release was monitored by the increase in fluorescence due to relieving of calcein's self-quenching upon dilution when the model drug leaks out of the liposomes. The results showed that, independent of the power density, the release was highest at 20 kHz. For the same frequency, release usually increased with increasing power densities. Additionally, for release at 20 kHz, a comparison was done for PEGylated and non-PEGylated liposomes, at two pH values: 5.2 and 7.4. The results were then compared to previously published studies. In all cases, the mechanism of release seems to involve cavitation events that either pierce a hole in or shear open the liposomes, as all the determined power densities are above the transient cavitation threshold.

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Introduction

Liposomes are small spherical nano or microsized carriers composed of a lipid bilayer, similar to the cell membrane, which surrounds an aqueous compartment. The liposomal bilayer is composed of phospholipids, zwitterionic molecules with polar hydrophilic heads corresponding to glycerol and phosphate moieties, attached to long, nonpolar, hydrophobic tails, comprised of fatty acids (Figure 1), and containing other molecules such as cholesterol, carbohydrates and proteins (1). Several groups have reported the delivery of bioactive agents from liposomes, and it has been observed that the encapsulation of agents in these nanocarriers affects their pharmacokinetics and bio-distribution, and thus can reduce the undesirable side effects and improve the therapeutic effectiveness of chemotherapy (2).

Liposomes and other nanoparticles extravasate preferentially and accumulate in tumors, due to several factors including the tumor pH, capillary size, enzymatic concentration, leaky vasculatures and damaged lymphatic drainage systems (3, 4). This phenomenon, known since 1986, is called the enhanced permeability and retention effect (EPR) and has immensely impacted the development of drug delivery systems (DDS). The EPR effect is also referred to as "passive targeting" and is aided by increasing the circulation time of drug delivery vehicles in the body.

Different structures of liposomes can be designed by controlling the phospholipid components and cholesterol content (5), surface charge (6), particle size (7, 8) and steric stabilization (9). Sterically-stabilized liposomes, named stealth liposomes, have been widely researched in vitro and in vivo, as possible chemotherapeutic agents (9–18). These liposomes have their surface decorated with hydrophilic polymers, usually poly(ethylene) glycol

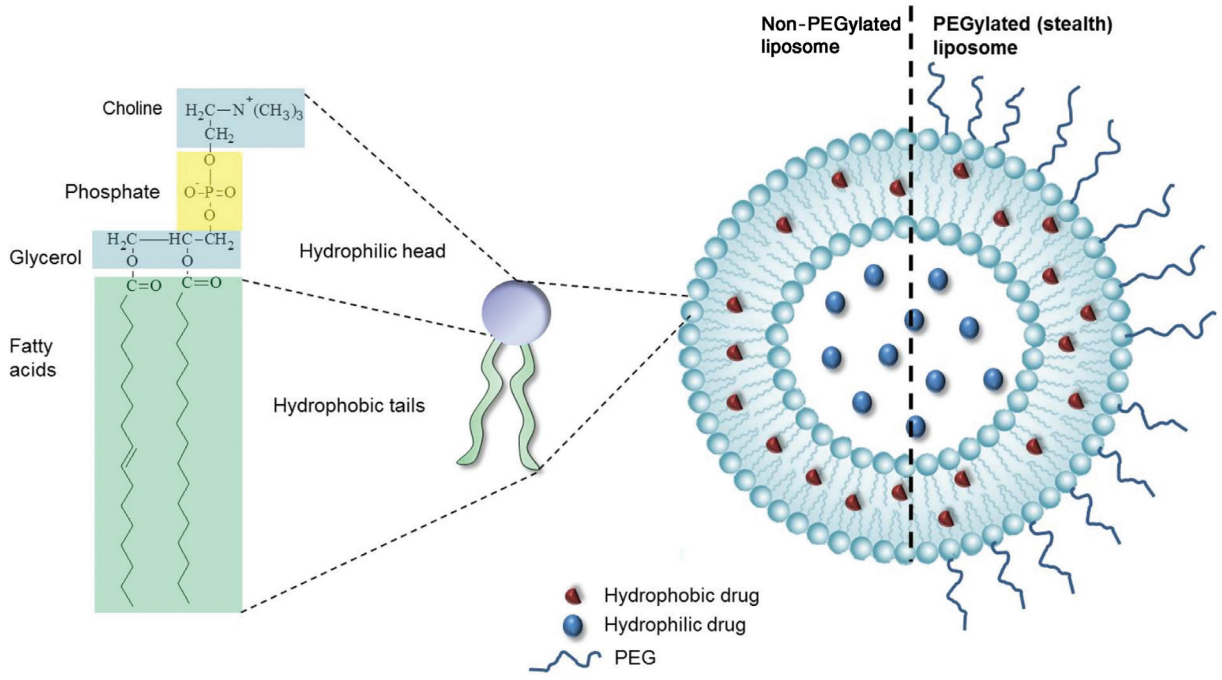

Figure 1: The structure and composition of a liposome.
The liposomal bilayer is composed of phospholipids, molecules with polar hydrophilic heads (glycerol and phosphate moieties) and non-polar hydrophobic tails (fatty acids). Chemotherapeutic drugs can be loaded into the liposome aqueous core, or into the bilayer, depending on their polarity. Furthermore, the liposome surface can be functionalized with polyethylene glycol (PEG) which increases their stability and allows the binding of ligands.

(PEG), which prevents protein adsorption and opsonization, thus increasing their circulation time in the bloodstream (15, 19, 20).

Beside their use in passive targeting, liposomes can be "actively targeted" by conjugating ligands to their surface that will allow their binding to receptors overexpressed on the surface cancer cells (21–23). These ligands can be antibodies – immunoliposomes (24), proteins (25), peptides (26, 27), nucleic acids (28, 29), and small molecules, including the widely researched folic acid (30–32). These multifunctional liposomes can circulate in the body for longer periods of time and, via passive and/or active targeting, reach the desired location (i.e. the tumor site), and release their enclosed cytotoxic agents. Temporal control of release can be achieved by designing liposomes sensitive to a certain stimulus or several stimuli. Several stimuli have been researched and have shown promise in releasing drugs from liposomes: changes in pH and enzyme concentration, light, hyperthermia, magnetic fields, and ultrasound (US) (33–35).

Ultrasound, a potential trigger for drug release from nanocarriers, is gaining increasing attention in creating successful drug delivery systems (DDS). Liposomes modified to respond to acoustic waves are called echogenic liposomes or acoustically activated liposomes (AAL). These liposomes can be designed to respond to an increase in temperature, to mechanical effects, or both (36). The use of US to trigger drug release from liposomes by disrupting their structure mechanically, thus allowing for their contents to spill in or near the diseased area, has been widely studied (10, 37–41). In this case, drug release occurs due to the acoustic and/or thermal energy of the US waves, which, at lower power densities, causes no harm to the healthy tissues. The non-invasive nature of acoustic waves and their ability to penetrate deep into internal tissue are added advantages to the use of this technique. Drug release induced by low-frequency US (LFUS) is mainly associated with mechanical effects (e.g. transient cavitation), whereas high-frequency US (HFUS) is associated with both thermal and mechanical effects (42).

High-frequency US is less effective in inducing release from normal and stealth liposomes, hence very high intensities are usually required, which might not be tolerated by the human body (43–45). However, HFUS is preferred when it comes to biological applications, due to the fact that it is more easily focused, which decreases the subjection area. This ensures a DDS with minimal side effects caused by chemotherapeutic agents and/or US waves. Studies reported in the literature have focused on measuring release at either low or high frequencies. In this study,

we report on the effects of a wide range of frequencies (20 kHz–3 MHz).

Several liposomal formulations have been approved for clinical uses. One of the best known is Doxil/Caelyx, stealth liposome-encapsulated doxorubicin (Dox), which was FDA-approved in 1995 for the treatment of Kaposi's sarcoma of AIDS patients, and is currently also used for patients with ovarian cancer and multiple myeloma (2, 10, 46). Other liposomal Dox formulations currently used in clinical settings include DaunoXome and Myocet (47). Several other formulations, combining liposomes and Dox or other drugs, have also been approved or are undergoing clinical trials (47). However, the use of liposomes in conjunction with US as a trigger, has not reached the clinical trial phases yet (https://clinicaltrials.gov).

The advantages of acoustically-triggered liposomes as nanocarrier DDS, and the ongoing research on their use in cancer treatment, make them a very promising DDS. However, several factors still need to be optimized to bring this system into clinical applications (37, 40). In this paper, we present a comprehensive study on the effects of varying frequency (high versus low), and power density (at least four different power densities per frequency examined) on the release kinetics of a model drug from both stealth (PEGylated) liposomes and non-PEGylated liposomes.

On the other hand, the preferred biological route by which nanocarriers, including liposomes, can enter the cancer cells and deliver the chemotherapeutics, is endocytosis, a process which allows for macromolecules present within the extracellular tissue fluid to be internalized by the cancer cell. Endocytosis can be classified under two main categories: pinocytosis and receptor-mediated endocytosis. In pinocytosis, macromolecules are nonspecifically taken up by endocytic vesicles when these molecules are in the proximity of the cell membrane. In contrast, receptor-mediated endocytosis involves the binding of these macromolecules to a specific receptor on the surface of the cell membrane, which initiates a cascade of events that starts via the inward folding of the cell membrane to form a small vesicle. The resultant small vesicle eventually fuses with other endocytic vesicles, and are all delivered to acidic cell compartments known as *endosomes*. These compartments then fuse with *primary lysosomes* to form *secondary lysosomes* where the ingested molecules are broken down into smaller compounds including sugars and peptides, and are consequently eliminated from the cells or tissue. The pH inside an endosome is approximately 5.0 (48), hence, in this paper we also examined the acoustic release at this acidity to test the effect of pH (5.2 vs. 7.4) on drug release from the liposomes. All previously reported acoustic release studies were conducted at physiologically neutral pH.

In all the studies presented in this paper, a careful statistical analysis was performed, with the use of multiple liposome batches and technical replicates. It is worth mentioning that calcein was used instead of Dox, since the use of the latter is not economically feasible when no in vitro experiments with cell cultures are involved.

Materials and methods

Synthesis of pNP-PEG-pNP and DOPE-PEG-pNP

The PEGylated liposomes used in this work were prepared using the amphiphilic PEG derivative DOPE(1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine)-PEG-pNP (*para*-nitro phenol), a group that allows the subsequent attachment of protein ligands to the liposomes. The first step in the procedure was the synthesis of (*para*-nitrophenylcarbonyl-PEG-(*para*-nitrophenylcarbonyl) (pNP-PEG-pNP), by the reaction of PEG with two molar equivalents of 4-nitrophenyl chloroformate (p-NPC) in the presence of dichloromethane and pyridine (49). DOPE-PEG-pNP was prepared by the reaction of the previously synthesized PEG-$(pNP)_2$ with one molar equivalent of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, Inc., Alabaster, AL, USA) in dry chloroform in the presence of triethylamine (TEA), in a modification of Torchilin and co-workers' protocol (50). The pNP-PEG-pNP was dissolved in 32.2 μmol of DOPE in chloroform, in a round bottom flask, followed by the addition of 80 μL of pure TEA (99% concentration), and 5 mL chloroform. The mixture was incubated overnight at room temperature, with stirring, under an argon atmosphere. The chloroform was then removed in a rotary evaporator, and 2 mL of a 0.01 M HCl-0.15 M NaCl were added to hydrate the lipid residue. The solution was sonicated in a 40-kHz sonicating bath (Elma D-78224, Melrose Park, IL, USA), at full power for 10 min and the micelles were separated from the unbound PEG and released pNP, using Sephadex G-25 PD-10 desalting columns (GE Healthcare Life Sciences). The solution was evaporated in a rotary evaporator at high speeds under vacuum for 2 h, and the DOPE-PEG-pNP was extracted 4 times with chloroform. The salt residues were precipitated on ice and removed by centrifugation and the DOPE-PEG-pNP was stored at –20°C as a chloroform solution, with a concentration of 8.4 mM.

Preparation of PEGylated and non-PEGylated liposomes

The PEGylated liposomes were prepared by the reaction of the DOPE-PEG-pNP synthesized as described previously, with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, Inc., Alabaster, AL, USA) and cholesterol (AlfaAesar, Ward Hill, MA, USA), using a molar ratio of 68:30:2 DPPC-Chol-(DOPE-PEG-pNP). The DOPE-PEG-pNP attaches to the liposome via its phospholipid residue and, as mentioned, the water-exposed pNP group can be used to bind a variety of amino group-containing ligands and form stable and non-toxic bonds, making them a convenient tool for protein attachment to the distal ends of liposome-grafted PEG chains (50).

The liposomes were prepared by the lipid film hydration method. Upon evaporation of chloroform, the film was hydrated with a solution of calcein at a self-quenching concentration (~30 mM), with the pH adjusted to 5.2. The resulting solution was sonicated at 40 kHz at full power for 15 min, and extruded three times (10-times each) through 0.2 µm polycarbonate filters using the Avanti® Mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA). Afterwards the liposomes were resuspended in the buffer to be used in the release assays, and cleaned using Sephadex G-25 PD-10 desalting columns (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The liposome solution was stored at 4°C until use.

For the preparation of non-PEGylated liposomes, an identical procedure was followed, but the DOPE-PEG-pNP was substituted by DOPE, keeping the same molar ratio (68:30:2 DPPC:cholesterol:DOPE).

Determination of the liposome size

The size of the liposomes was determined using the DynaPro® NanoStar™ Dynamic Light Scattering (DLS) instrument (Wyatt Technology Corp., Santa Barbara, CA, USA). The samples were appropriately diluted and filtered using 0.45 µm nylon syringe filters (Whatman® Puradisc, Sigma-Aldrich Co. St. Louis, MO, USA) prior to the measurements. Dynamic light scattering autocorrelation data were obtained and analysed using the software Dynamics7 – Static, Dynamic, and Phase Analysis Light Scattering (Wyatt Technology Corp., Santa Barbara, CA, USA).

Release kinetics at LFUS

Release experiments were performed at two different pH values: 5.2 (in 0.1 M sodium citrate buffer) and 7.4 (in PBS buffer). In all cases, release of calcein was monitored by the increase in fluorescence caused by the US-induced disruption of liposomes. The encapsulated calcein is at a self-quenching concentration of 30 mM and once diluted in the buffer upon release, its fluorescence will increase. A schematic of the setting used in these experiments is depicted in Figure 2.

Sonication was performed using a 20 kHz sonicating probe Vibra-Cell (model VC130PB, Sonics & Materials Inc., Newtown, CT, USA). The probe was inserted into a plastic test tube containing the liposomal sample and the transducer was programed to deliver pulsed US (20 s on, 10 s off) at several amplitudes (20%, 25%, 30% and 40% power setting), corresponding to different power densities. Samples were collected at several time points for a total insonation time of 10 min. Release was monitored by the increase in calcein fluorescence due to the reduction of the model drug's self-quenching, using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The fluorescence levels were determined using an excitation wavelength of 494 nm and emission wavelength of 515 nm. These were determined from fluorescence spectra of the calcein-loaded liposome solutions (results not shown). The power densities were estimated using a Brüel & Kjaer Type 8103 Hydrophone (Nærum, Denmark). At the end of each sonication cycle, the detergent Triton X-100 (Tx100) was added to each sample, to a final concentration of 0.48 mM [the critical micellar concentration of Tx100 is 0.24 mM (51)], to lyse any remaining liposomes, allowing the determination of a fluorescence level that represents 100% release (52). The percentage of fluorescence release at each time point was normalized using the equation,

$$\text{Drug release} = \frac{F_t - F_0}{F_{max} - F_0} \times 100\% \quad [1]$$

where, F_t=measured fluorescence intensity in the sample after a given insonation duration (t); F_0=initial fluorescence before insonation (baseline); F_{max}=maximum fluorescence intensity (after addition of Tx100). Controls were measured at both pH, for PEGylated and non-PEGylated liposomes, in the absence of US application.

Release kinetics at HFUS

A similar experimental procedure was followed when using HFUS but all the measurements were made at pH 7.4, using PBS buffer to dilute the calcein-loaded liposomal sample. The fluorescence levels were

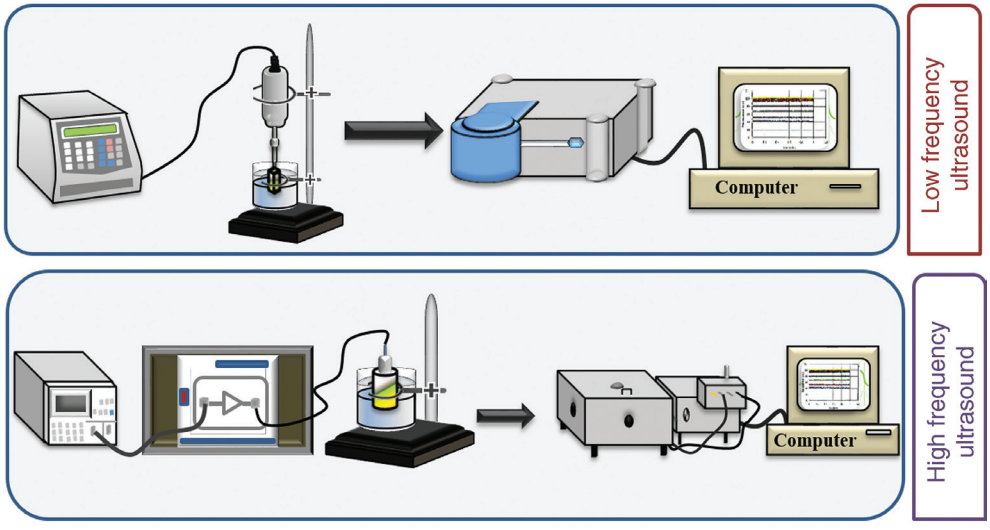

Figure 2: Sonication and fluorescence monitoring settings used in this work.
The settings for LFUS (20 kHz) and HFUS (1 MHz and 3 MHz) experiments are shown.

measured using the QuantaMaster-30 Fluorescence System (Photon Technology Int., Edison NJ, USA). Liposome samples were first diluted in a glass beaker fitted inside a cold water bath to control the temperature of the sample during sonication (Figure 2). The 1-MHz and 3-MHz ultrasonic probes were designed to specifications by H.G. Moussa at the American University of Sharjah (Sharjah, UAE) and manufactured by Precision Acoustics (Dorchester, UK). The probes were connected to an AC High Voltage Amplifier (WMA – 300, Falco Systems, Amsterdam, The Netherlands), which takes its input voltage from a function generator (AFG 310, Tektronix, Beaverton, OR, USA). The probe was fitted just below the surface of the water in the bath and aligned. The sonication was performed in a continuous wave (CW) mode for a total sonication time of 60 min divided into four intervals of 15 min each. After each sonication interval, the fluorescence levels were measured. After 60 min of US exposure, Tx100 was added to a final concentration of 0.48 mM to lyse any remaining liposomes, allowing the calculation of the total release, as described previously. The percentage of release at each time point was calculated using Equation [1].

Statistical analysis

To assess the significance of the differences in release, pairwise comparisons were performed using two-tailed t-tests with the assumption of equal variances of the two samples, in Excel 2010. Two values were considered significantly different when $p<0.05$ (unless otherwise stated).

Results and discussion

In this work, we compared the US-induced release of the model drug calcein from PEGylated and non-PEGylated liposomes, at low frequencies-20 kHz (4 power densities), and high frequencies-1 MHz (5 power densities) and 3 MHz (6 power densities). The power densities at each frequency were determined using a hydrophone. Additionally, at 20 kHz, the release was compared at two different pH values (5.2 and 7.4).

Liposome size

The size of both, the PEGylated and non-PEGylated liposomes, was determined by DLS measurements. The average radii of the PEGylated liposomes was

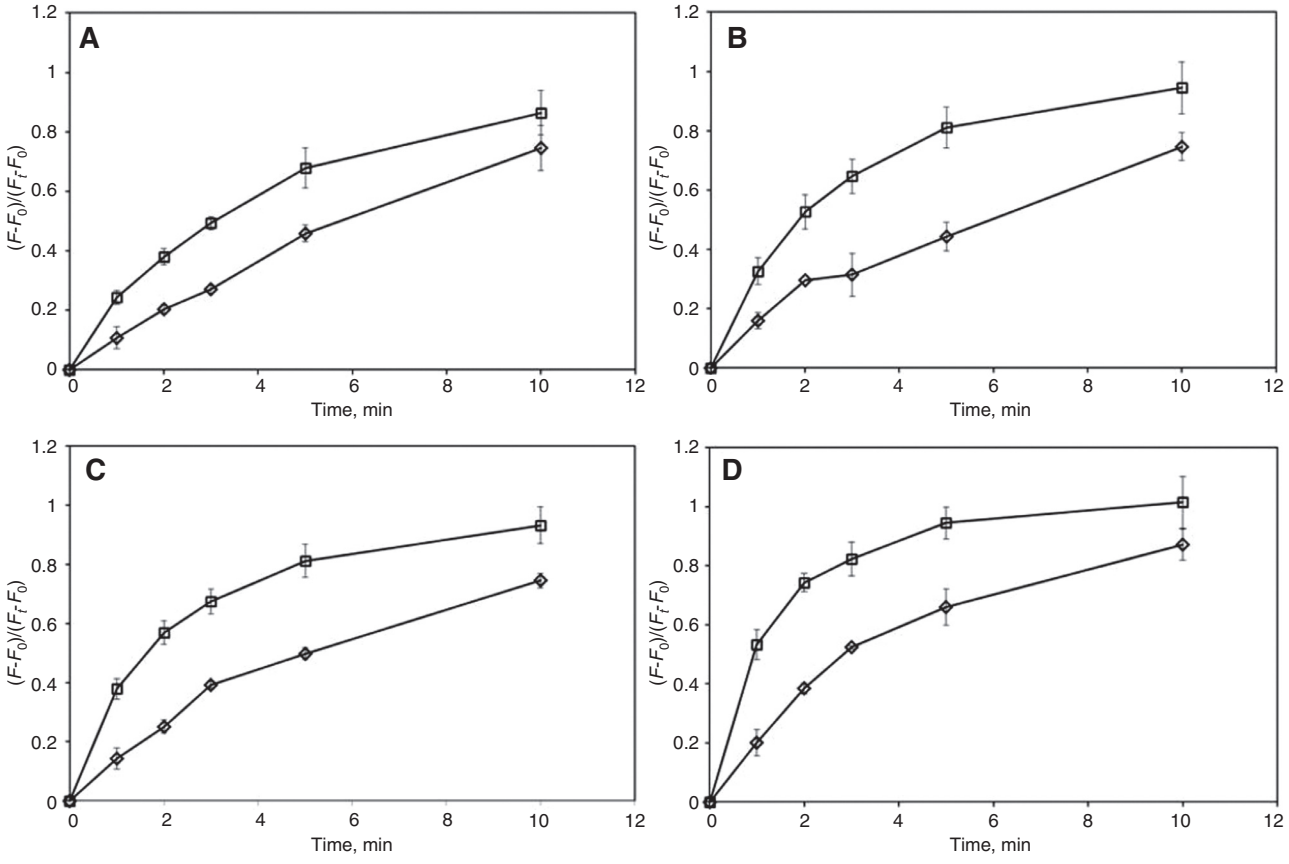

Figure 3: Comparison of release curves of calcein from non-PEGylated liposomes at pH 5.2 (◊) and pH 7.4 (□).
Samples were sonicated at 20 kHz at different power densities: (A) 6.08 W/cm², (B) 6.97 W/cm², (C) 11.83 W/cm² and (D) 17.14 W/cm². Results are average±standard deviation of three measurements (one liposome batch) for release at pH 5.2 (0.1 M sodium citrate pH 5.2), and nine measurements (three liposome batches) for release at pH 7.4 (PBS). The graphs were built from the raw data provided as supplementary material.

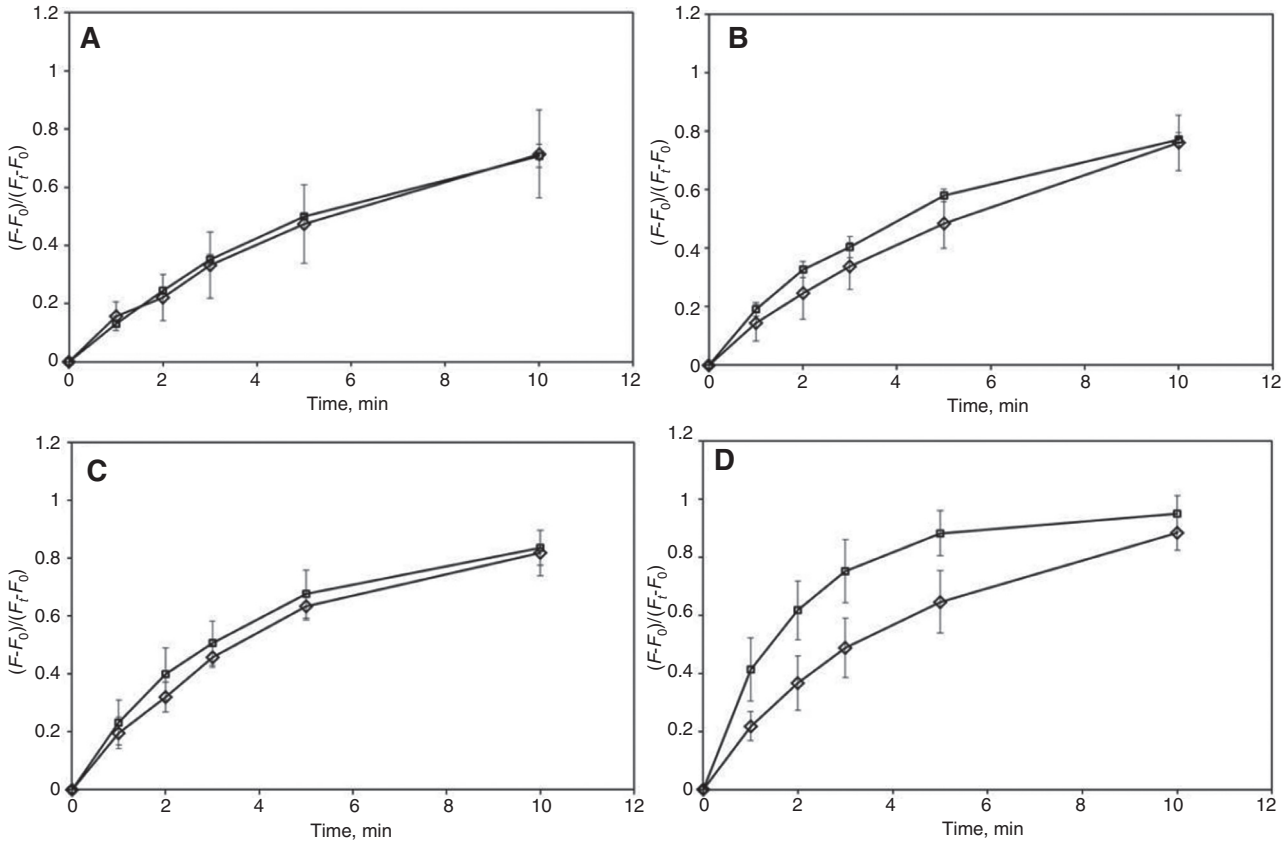

Figure 4: Comparison of calcein release curves (20-kHz US) from PEGylated liposomes at pH 5.2 (◊) and pH 7.4 (□). Samples were sonicated at 20 kHz at different power densities: (A) 6.08 W/cm², (B) 6.97 W/cm², (C) 11.83 W/cm² and (D) 17.14 W/cm². Results are average±standard deviation of four measurements (three liposome batches) for release at pH 5.2 (0.1 M sodium citrate pH 5.2), and 18 measurements (six liposome batches) for release at pH 7.4 (PBS).

130.12.±4.28 nm (n=10 batches of liposomes, at least two technical replicates each), whereas that of the non-PEGylated ones was 131.46±1.16 nm (n=3 batches of liposomes, at least two technical replicates each). These results indicate that both types of liposomes are large unilamellar vesicles (LUV), with average diameters of 260.24 and 262.91 nm, respectively. A statistical two-tailed t-test with the assumption of equal variances of the two samples was conducted to compare the sizes of both liposomes and it was found that the differences in size were not statistically significant ($p>0.3$).

LFUS-induced release

The release at 20-kHz US was performed using a sonicator probe. Results were obtained at pH 5.2 and pH 7.4 and were compared for PEGylated and non-PEGylated liposomes. First, we compared release for the same type of liposomes at different pH (Figures 3 and 4) and afterwards, for each pH, we compared release from PEGylated and non-PEGylated liposomes (Figures 5 and 6). The average final release percentages that were obtained at the different frequencies and power densities are summarized in the Supplementary Material (Table S.1). No release was observed from non-PEGylated or PEGylated liposomes, at any pH, when no US was applied. The detected fluorescence was stable up to 60 min of monitoring. These negative controls were performed to make sure that all the observed release was due to the US application.

Non-PEGylated liposomes

The release profiles obtained from non-PEGylated liposomes at pH 5.2 and 7.4 are shown in Figure 3, for each power density at 20-kHz US.

At pH 5.2, for each power density at 20 kHz, the release increases with insonation time, as expected, since liposomes do not re-form between US pulses as is the case with micelles (46, 53). Statistical analysis revealed that the release values obtained using 6.08 and 6.97 W/cm²

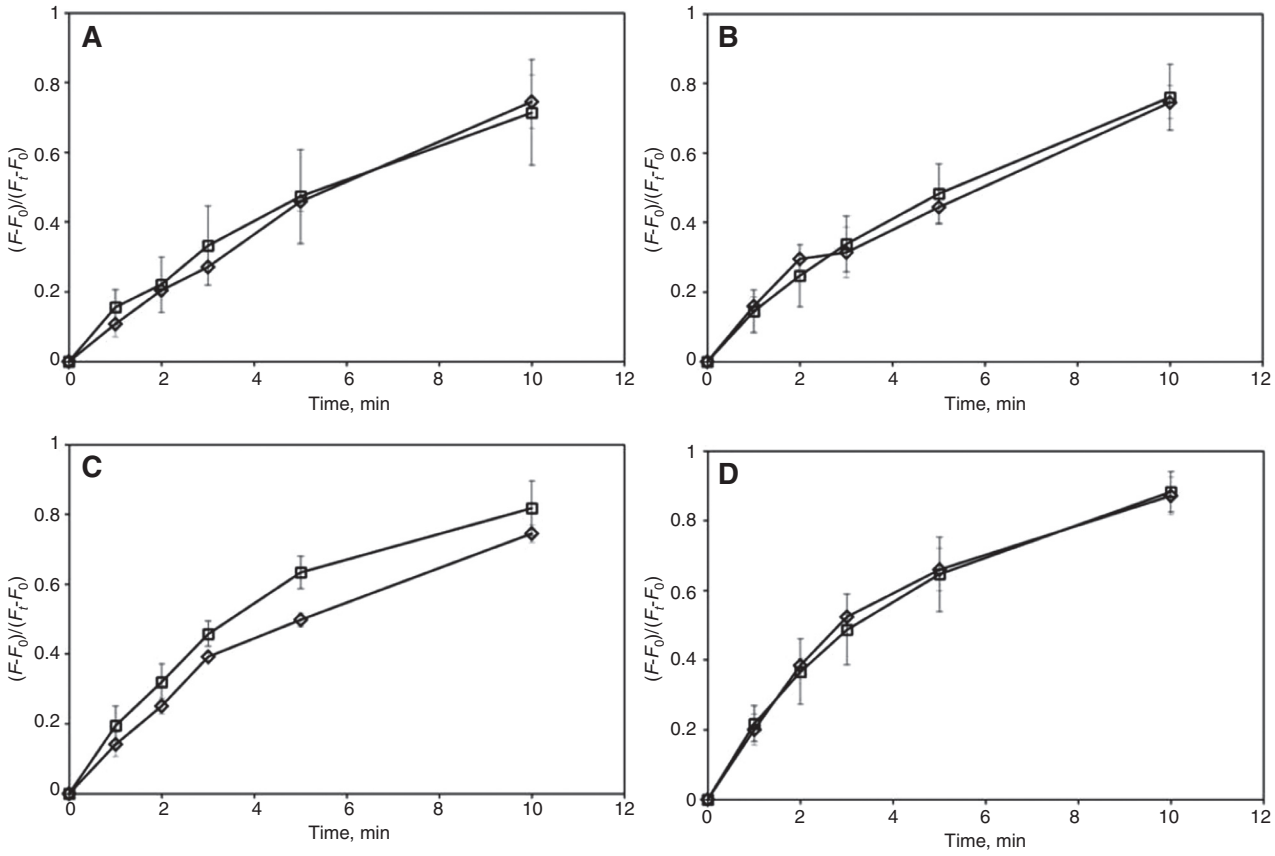

Figure 5: Comparison of calcein release curves at pH 5.2 (0.1 M sodium citrate buffer) from non-PEGylated (◊) and PEGylated (□) liposomes. Samples were sonicated at 20 kHz at: (A) 6.08 W/cm², (B) 6.97 W/cm², (C) 11.83 W/cm² and (D) 17.14 W/cm². Results are average±standard deviation of three measurements (one batch) for the nonPEGylated liposomes, and six measurements (three batches) for the PEGylated liposomes.

power densities are not significantly different ($p>0.05$) for any time point. The same situation was observed when comparing release at 6.97 and 11.83 W/cm². In contrast, the release obtained at the highest power intensity used (17.14 W/cm²) was significantly higher ($p<0.05$) than for any other power density. The maximum release after 10 min of insonation was obtained at a power density of 17.14 W/cm², whereas the maximum releases at the other 3 power densities are very similar (Supplementary Material Table S.1).

The US-induced release from non-PEGylated liposomes was also studied at pH 7.4 (also shown in Figure 3). All the curves show an increase in fluorescence as the total insonation time increases, as was also observed at pH 5.2. However, the differences between release at different power densities are more pronounced at pH 7.4. A statistical analysis of the results revealed that the release obtained at each power density is significantly different ($p<0.01$) than at any other power at the same time point, except for the similarity obtained in the case of sonicating at 6.97 and 11.83 W/cm².

The comparison between the release obtained for non-PEGylated liposomes at each pH studied, showed that release at pH 5.2 is significantly lower ($p<0.05$) than at pH 7.4 for all the power densities, and for all the time points investigated (Figure 3A–D).

PEGylated liposomes

A similar study was conducted with PEGylated liposomes and the comparison for each power density at 20-kHz US is shown in Figure 4.

At pH 5.2, the release from PEGylated liposomes increases with insonation time, for each US condition used. No statistically significant difference ($p>0.05$) was observed for release percentages obtained at 6.08 and 6.97 W/cm², and 11.83 and 17.14 W/cm². The most significant differences ($p<0.03$) are between the intermediate power densities (6.97 and 11.83 W/cm²), after 3 and 5 min of sonication. The values for final release, obtained after 10 min of total insonation time, are shown in

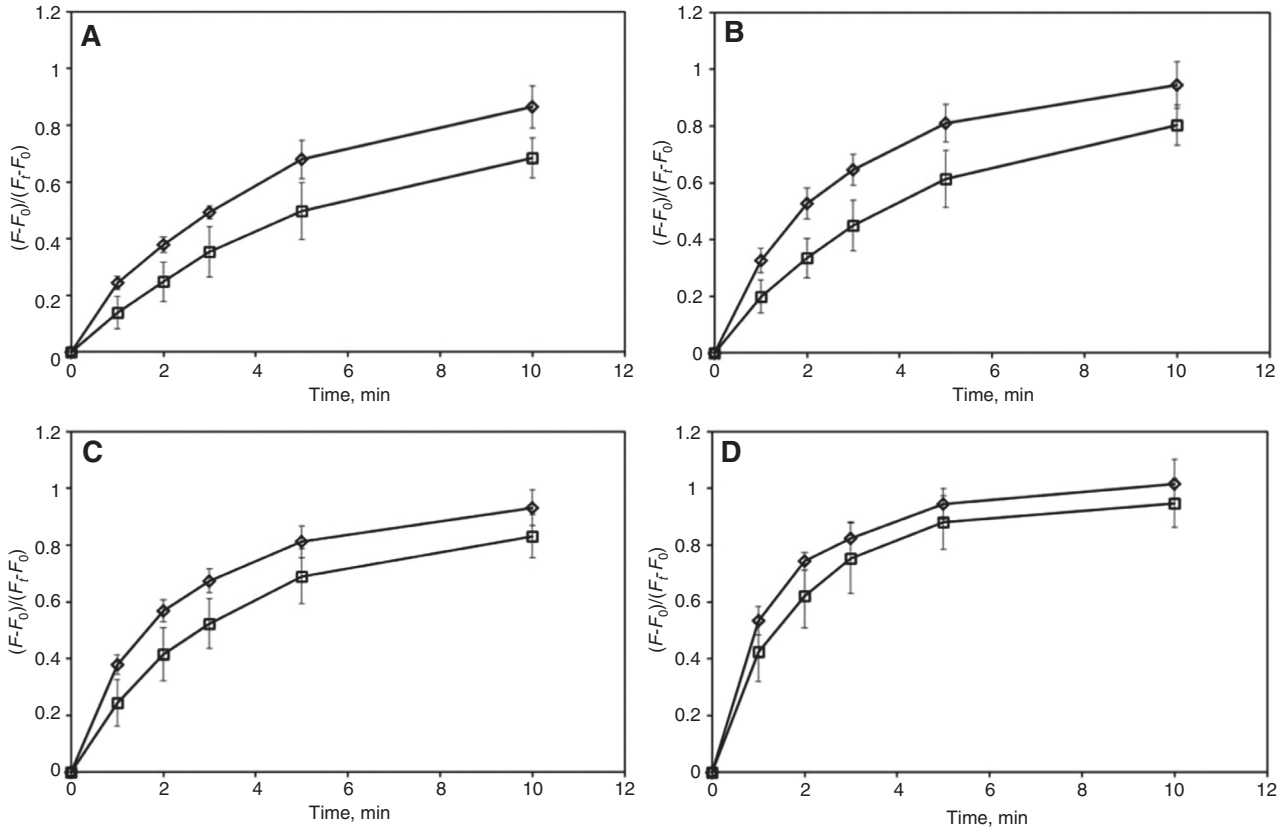

Figure 6: Comparison of calcein release curves at pH 7.4 (PBS buffer) from nonPEGylated and PEGylated liposomes.
Samples were sonicated at 20 kHz at: (A) 6.08 W/cm², (B) 6.97 W/cm², (C) 11.83 W/cm² and (D) 17.14 W/cm². Results are average±standard deviation of: (i) nine measurements (three batches) for the non-PEGylated liposomes, (ii) 18 measurements (six batches) for the PEGylated liposomes.

Supplementary Material Table S.1. The release at 20 kHz and 6.08 W/cm² is significantly lower ($p<0.05$) than the release at 17.14 W/cm² at the same frequency.

At pH 7.4, the highest release from PEGylated liposomes was observed at the highest power density of 17.14 W/cm². Under these conditions, the releases observed at each time point are all significantly different ($p<0.05$), (except for the points of initial and final release obtained at 20 kHz, 6.97 and 11.83 W/cm²). The total release percentages observed after 10 min of insonation are shown in the Supplementary Material Table S.1, and are not statistically different from the ones measured at pH 5.2.

The comparison of the release curves for each power density at 20 kHz, at the two pH buffers investigated, showed that there are no significant differences ($p>0.05$) (Figure 4A, B and C), except for the highest power density, when the release at pH 7.4 was significantly higher ($p<0.001$) than at pH 5.2 (Figure 4D). This result is different than the one obtained for non-PEGylated liposomes (Figure 3), where the differences at the two different pH were significant at several time points.

Comparison non-PEGylated versus PEGylated

Finally, we compared the release from non-PEGylated and PEGylated liposomes, upon insonation with pulsed 20-kHz US, at different power settings, and at both pH. A comparison between the temporal release curves at pH 5.2 is shown in Figure 5, and at pH 7.4 is shown in Figure 6. The levels of calcein released from non-PEGylated and PEGylated liposomes at pH 5.2 are not significantly different ($p>0.05$), as can be observed from the almost superimposed curves shown in Figure 5. The only statistically significant difference ($p<0.05$) was observed for a power density of 11.83 W/cm² (Figure 5C), showing that the release from PEGylated liposomes is slightly higher than from non-PEGylated ones at some data points. After 10 min of insonation, the release percentages for each power density are not statistically significantly different ($p>0.05$) for both types of liposomes (Supplementary Material Table S.1). However, the results obtained at pH 7.4 are different. It was observed that the release from the non-PEGylated liposomes is significantly higher

($p<0.03$) than when sonicating PEGylated liposomes (Figure 6).

Lin and Thomas reported that the release from PEGylated liposomes is higher than from the non-PEGylated ones at 20-kHz US, in a HEPES buffer, pH 7.6 (54). However, their study is different from the one presented here. The highest power density they used was 3.8 W/cm^2, which is lower than any of the power densities used in this work. Also, they continuously monitored the increase in fluorescence, which allowed them to determine initial release rates. Here, we performed a discrete kinetic study, following the release from 1 to 10 min insonation. Additionally, they used different methods for the synthesis of the liposomes, with different composition and type of lipids used, as well as different PEG-lipid to phospholipid ratios. Also, the liposomes used in this study are larger in diameter (~260 nm) than the one used by Lin and Thomas, with a diameter of 100 nm. Another factor that differs between the two studies is the use of batch replicates in this study, whereas the previous study used only technical replicates of the same liposome batch. Hence, with such different experimental conditions, it cannot be said that the study presented here contradicts the one reported by Lin and Thomas (54).

HFUS-induced release

Two probes were specifically designed for the HFUS experiments, with two different frequencies: 1 and 3 MHz. Release experiments from PEGylated liposomes at HFUS were performed at pH 7.4, using these frequencies, each at different power densities (indicated in Figure 7), for a total insonation time of 60 min.

Figure 7A shows the release curves obtained with the 1-MHz US probe. Similar to what was previously observed at the lower frequencies, there is an increase in release as the insonation time increases. Results obtained at this frequency also show that as the power density increases, from 10.5 to 50.2 W/cm^2, the release also increases. The release values obtained at the different power densities are significantly different ($p<0.05$) for each time point. The maximum release achieved after 60 min of sonication at 1 MHz was approximately 57%, when the highest power density was used (50.2 W/cm^2).

For 3-MHz US, the release curves are not as well defined, as can be seen in Figure 7B. Also in this case, there is an increase in release over time for all power densities. However, there is an overlap between the 158.5 and 173.3 W/cm^2 power densities. The highest release percentage achieved at 3 MHz was approximately 68%, obtained at 183.0 W/cm^2.

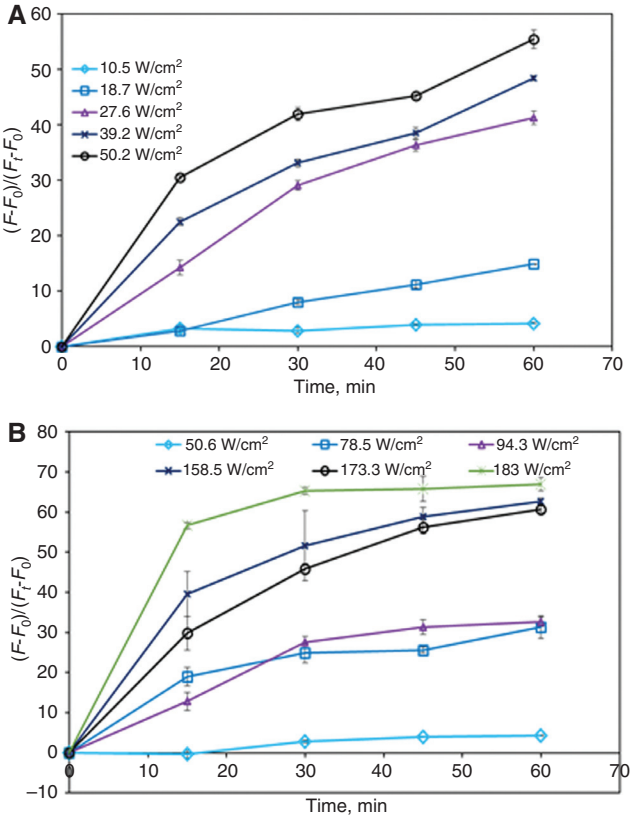

Figure 7: Calcein release kinetics from PEGylated liposomes at pH 7.4 using (A) 1-MHz US and (B) 3-MHz US.
Calcein release curves at different power densities: samples were sonicated at 1 MHz (A) or 3 MHz (B) for a total time of 60 min, using the power densities indicated in the legend. Results are average±standard deviation of three different liposomes batches.

Comparison between release using LFUS and HFUS

Here, we compare the release results from PEGylated liposomes at pH 7.4, using three different US frequencies –20 kHz, 1 MHz and 3 MHz – at different power densities.

As shown in Figure 8, the release levels reached when using 20-kHz US are much higher than when using HFUS, for much lower power densities and shorter insonation times (10 min versus 60 min). Additionally, it can also be observed that the level of release obtained at 1 MHz for a certain power density, can only be obtained at 3 MHz when using a higher power intensity. As an example, the lowest release percentage obtained at 1 MHz was 4.18%±0.15%, very similar to the one obtained at 3 MHz, 4.32%±0.16%. However, the power density used at 1 MHz

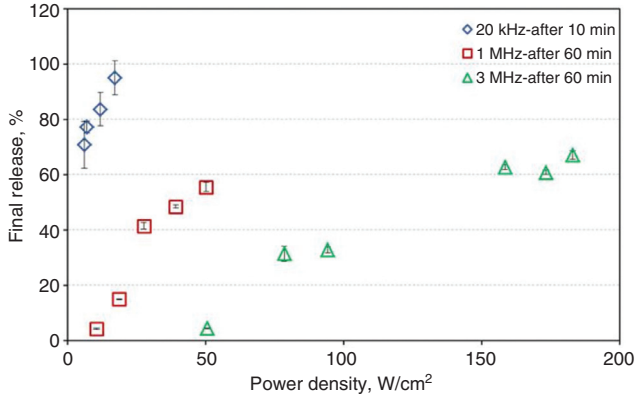

Figure 8: Comparison between the final release percentages of calcein from PEGylated liposomes at 20 kHz, 1 MHz and 3 MHz, as a function of power density.
Experiments were carried out at pH 7.4 in PBS buffer. Results are average±standard deviation of 18 measurements (six liposome batches) for 20-kHz US, and three measurements (three liposomes batches) for HFUS.

was about five times lower than that at 3 MHz, 10.5 versus 50.6 W/cm^2. Similarly, a comparison of similar power densities at the two different frequencies (e.g. 50.2 W/cm^2 at 1 MHz and 50.6 W/cm^2 at 3 MHz), shows that the release at 1 MHz is much higher than at 3 MHz: 55.43%±1.66% versus 4.34%±0.16%, respectively. Hence, the advantage of using lower LFUS is that higher release can be achieved at lower power densities. This result is in agreement with the fact that the release decreases as the US frequency increases (taking the intensity into consideration) (55, 56). As the frequency decreases, so does the collapse cavitation threshold, and thus, the mechanical index (MI; the peak negative pressure of the US wave divided by the square root of the center frequency of the wave) increases, which leads to an increase in the release rate (taking the intensity in consideration) (51).

Release mechanism

As mentioned in the Methods section, a hydrophone was used to measure the power densities delivered to the liposomes, at each frequency. Schroeder et al. previously reported that the collapse cavitation threshold when LFUS is used is ~1.2 W/cm^2 (56), whereas Lin and Thomas refer to a value of 2 W/cm^2 (54). The lowest power density used at 20 kHz frequency was 6.08 W/cm^2, which indicates that the release obtained using this US frequency is mainly due to cavitation events and not due to US thermal effects. The power density values measured for HFUS, 1 MHz and 3 MHz, also indicate that the release observed is mainly due to collapse cavitation events, since they are above the collapse cavitation thresholds. It was previously described that the onset of collapse cavitation occurs at a mechanical index of 0.3–0.4 (57). This corresponds to power densities of 3–5.3 W/cm^2 for 1-MHz US, and 9–16 W/cm^2 for 3-MHz US. All the power densities determined in this work, for both HFUS frequencies used, are above the referred thresholds.

We have previously concluded that micelles release their contents acoustically due to cavitation effects whereby cavitating bubbles reach the collapse cavitation power densities (58). Upon the collapse of these microbubbles, microjets and shock waves shear the micellar structure open leading to the release of its anti-neoplastic contents. We believe that a similar mechanism is at play here, with the liposomes being pierced by a microjet or sheared by a shockwave, with the subsequent release of their contents.

Conclusion

Several drug delivery systems have been investigated to reduce the side effects of chemotherapy by encapsulating the therapeutic agent in a synthetic nanosized carrier until it reaches the tumor site. Many of these particles are designed to be responsive to the mechanical and thermal perturbations delivered by US.

In this study, we used non-PEGylated and PEGylated liposomes, and performed a careful statistical analysis, to compare the US-induced release of calcein, at low and high frequencies. The release was successfully triggered from normal and stealth liposomes, at the three frequencies used. Several experimental problems were addressed, including the solubility of calcein, a fluorescence model drug widely used in in vitro studies, at different pHs, and the determination of the variance between similar liposome batches, which significantly affects the comparison of the results. It was observed that the release at lower frequencies is higher than that at high frequencies. At the same frequency, as the power density increased, so did the release of the encapsulated molecule.

Our results also showed that, for 20-kHz US at pH 7.4, the release from non-PEGylated liposomes is significantly higher than from PEGylated liposomes. It was previously described that the initial US-induced (20 kHz, 3.8 W/cm^2) release rate from PEGylated liposomes is higher than when using non-PEGylated liposomes (54). The differences between the two studies were discussed. More work is currently being conducted in our group, to synthesize liposomes that are both ligand-targeted and echogenic.

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References

1. Mozafari MR, Khosravi-Darani K. An overview of liposome-derived nanocarrier technologies. In: Mozafari MR, editor. Nanomaterials and nanosystems for biomedical applications. Dordrecht: Springer, 2007:113–23.
2. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther 2008;83:761–9.
3. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv Enzyme Regul 2001;41:189–207.
4. Maeda H, Nakamura H, Fang J. The EPR effect for macromolecular drug delivery to solid tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo. Adv Drug Deliv Rev 2013;65:71–9.
5. Kirby C, Clarke J, Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem J 1980;186:591–8.
6. Lee KD, Hong K, Papahadjopoulos D. Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density. Biochim Biophys Acta 1992;1103:185–97.
7. Gabizon A, Price DC, Huberty J, Bresalier RS, Papahadjopoulos D. Effect of liposome composition and other factors on the targeting of liposomes to experimental tumors: biodistribution and imaging studies. Cancer Res 1990;50:6371–8.
8. Willis M, Forssen E. Ligand-targeted liposomes. Adv Drug Deliv Rev 1998;29:249–71.
9. Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today 2005;10:1451–8.
10. Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. Adv Drug Deliv Rev 2013;65:36–48.
11. Gabizon A, Martin F. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Drugs 1997;54:15–21.
12. Gabizon AA, Barenholz Y, Bialer M. Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmacokinetic studies in rodents and dogs. Pharm Res 1993;10:703–8.
13. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomedicine 2006;1:297–315.
14. Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, et al. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 1991;88:11460–4.
15. Sharpe M, Easthope SE, Keating GM, Lamb HM. Polyethylene glycol-liposomal doxorubicin: a review of its use in the management of solid and haematological malignancies and AIDS-related Kaposi's sarcoma. Drugs 2002;62:2089–126.
16. Slepushkin VA, Simoes S, Dazin P, Newman MS, Guo LS, Pedroso de Lima MC, et al. Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation in vivo. J Biol Chem 1997;272:2382–8.
17. Woodle MC. Sterically stabilized liposome therapeutics. Adv Drug Deliver Rev 1995;16:249–65.
18. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171–99.
19. Andresen TL, Jensen SS, Jorgensen K. Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release. Prog Lipid Res 2005;44:68–97.
20. Hasirci N. Micro and nano systems in biomedicine and drug delivery. In: Mozafari MR, editor. Nanomaterials and nanosystems for biomedical applications. Dordrecht: Springer, 2007:1–26.
21. Basile L, Pignatello R, Passirani C. Active targeting strategies for anticancer drug nanocarriers. Curr Drug Deliv 2012;9:255–68.
22. Bertrand N, Wu J, Xu X, Kamaly N, Farokhzad OC. Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. Adv Drug Deliv Rev 2014;66:2–25.
23. Wang M, Thanou M. Targeting nanoparticles to cancer. Pharmacol Res 2010;62:90–9.
24. Fay F, Scott CJ. Antibody-targeted nanoparticles for cancer therapy. Immunotherapy 2011;3:381–94.
25. Li X, Ding L, Xu Y, Wang Y, Ping Q. Targeted delivery of doxorubicin using stealth liposomes modified with transferrin. Int J Pharm 2009;373:116–23.
26. Dai W, Yang T, Wang Y, Wang X, Wang J, Zhang X, et al. Peptide PHSCNK as an integrin alpha5beta1 antagonist targets stealth liposomes to integrin-overexpressing melanoma. Nanomedicine 2012;8:1152–61.
27. Torchilin VP. Tat peptide-mediated intracellular delivery of pharmaceutical nanocarriers. Adv Drug Deliv Rev 2008;60:548–58.
28. Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, et al. Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy in vivo. Proc Natl Acad Sci 2006;103:6315–20.
29. Mi J, Liu Y, Rabbani ZN, Yang Z, Urban JH, Sullenger BA, et al. In vivo selection of tumor-targeting RNA motifs. Nat Chem Biol 2010;6:22–4.
30. Leamon CP, Reddy JA. Folate-targeted chemotherapy. Adv Drug Deliv Rev 2004;56:1127–41.
31. Sudimack J, Lee RJ. Targeted drug delivery via the folate receptor. Adv Drug Deliv Rev 2000;41:147–62.
32. Zhao X, Li H, Lee RJ. Targeted drug delivery via folate receptors. Expert Opin Drug Deliv 2008;5:309–19.
33. Bibi S, Lattmann E, Mohammed AR, Perrie Y. Trigger release liposome systems: local and remote controlled delivery? J Microencapsul 2012;29:262–76.

34. Esser-Kahn AP, Odom SA, Sottos NR, White SR, Moore JS. Triggered release from polymer capsules. Macromolecules 2011;44:5539–53.
35. Timko BP, Dvir T, Kohane DS. Remotely triggerable drug delivery systems. Adv Mater 2010;22:4925–43.
36. Schroeder A, Honen R, Turjeman K, Gabizon A, Kost J, Barenholz Y. Ultrasound triggered release of cisplatin from liposomes in murine tumors. J Control Release 2009;137:63–8.
37. Ahmed SE, Martins AM, Husseini GA. The use of ultrasound to release chemotherapeutic drugs from micelles and liposomes. J Drug Target 2015;23:16–42.
38. Bohmer MR, Klibanov AL, Tiemann K, Hall CS, Gruell H, Steinbach OC. Ultrasound triggered image-guided drug delivery. Eur J Radiol 2009;70:242–53.
39. Deckers R, Moonen CT. Ultrasound triggered, image guided, local drug delivery. J Control Release 2010;148:25–33.
40. Husseini GA, Pitt WG, Martins AM. Ultrasonically triggered drug delivery: breaking the barrier. Colloid Surf B Biointerfaces 2014;123C:364–86.
41. Rapoport NY, Christensen DA, Fain HD, Barrows L, Gao Z. Ultrasound-triggered drug targeting of tumors in vitro and in vivo. Ultrasonics 2004;42:943–50.
42. Schroeder A, Avnir Y, Weisman S, Najajreh Y, Gabizon A, Talmon Y, et al. Controlling liposomal drug release with low frequency ultrasound: mechanism and feasibility. Langmuir 2007;23:4019–25.
43. Ernsting MJ, Worthington A, May JP, Tagami T, Kolios MC, Li S-D. Ultrasound drug targeting to tumors with thermosensitive liposomes. In: 2011 IEEE Int Ultrasonics Symp 2011:1–4.
44. Gasselhuber A, Dreher MR, Partanen A, Yarmolenko PS, Woods D, Wood BJ, et al. Targeted drug delivery by high intensity focused ultrasound mediated hyperthermia combined with temperature-sensitive liposomes: computational modelling and preliminary in vivovalidation. Int J Hyperthermia 2012;28:337–48.
45. Karshafian R, Bevan PD, Williams R, Samac S, Burns PN. Sonoporation by ultrasound-activated microbubble contrast agents: effect of acoustic exposure parameters on cell membrane permeability and cell viability. Ultrasound Med Biol 2009;35:847–60.
46. Huang SL. Liposomes in ultrasonic drug and gene delivery. Adv Drug Deliv Rev 2008;60:1167–76.
47. Pillai G. Nanomedicines for cancer therapy: an update of FDA approved and those under various stages development. SOJ Pharm Pharm Sci 2014;1:1–13.
48. Zhang X, Lin Y, Gillies RJ. Tumor pH and its measurement. J Nucl Med 2010;51:1167–70.
49. Sun H, Guo B, Cheng R, Meng F, Liu H, Zhong Z. Biodegradable micelles with sheddable poly(ethylene glycol) shells for triggered intracellular release of doxorubicin. Biomaterials 2009;30:6358–66.
50. Torchilin VP, Levchenko TS, Lukyanov AN, Khaw BA, Klibanov AL, Rammohan R, et al. p-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via p-nitrophenylcarbonyl groups. Biochim Biophys Acta 2001;1511:397–411.
51. Lin HY, Thomas JL. Factors affecting responsivity of unilamellar liposomes to 20 kHz ultrasound. Langmuir 2004;20:6100–6.
52. Huang SL, MacDonald RC. Acoustically active liposomes for drug encapsulation and ultrasound-triggered release. Biochim Biophys Acta 2004;1665:134–41.
53. Husseini GA, Pitt WG. Micelles and nanoparticles for ultrasonic drug and gene delivery. Adv Drug Deliv Rev 2008;60:1137–52.
54. Lin HY, Thomas JL. PEG-Lipids and oligo(ethylene glycol) surfactants enhance the ultrasonic permeabilizability of liposomes. Langmuir 2003;19:1098–105.
55. Cohen-Levi D. Ultrasound for targeted delivery of cytotoxic drugs from liposomes. M.Sc. Thesis. Beer Sheva, Israel: Ben Gurion University, 2000.
56. Schroeder A, Kost J, Barenholz Y. Ultrasound, liposomes, and drug delivery: principles for using ultrasound to control the release of drugs from liposomes. Chem Phys Lipids 2009;162:1–16.
57. Barnett S. Ultrasound. Threshold for nonthermal bioeffects: theoretical and experimental basis for a threshold index. Ultrasound Med Biol 1998;24 (Suppl 1):S41–9.
58. Husseini GA, Diaz de la Rosa MA, Richardson ES, Christensen DA, Pitt WG. The role of cavitation in acoustically activated drug delivery. J Control Release 2005;107:253–61.

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Graphical abstract

Salma E. Ahmed, Hesham G. Moussa, Ana M. Martins, Mohammad H. Al-Sayah and Ghaleb A. Husseini

Effect of pH, ultrasound frequency and power density on the release of calcein from stealth liposomes

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Original Article: This work provides a comprehensive study on the effects of ultrasound frequency (20 kHz, 1 MHz and 3 MHz), power density and pH (5.2 versus 7.4) on the release of calcein from non-PEGylated and stealth-PEGylated liposomes.

Keywords: drug delivery; liposomes; PEGylated; power density; triggered release; ultrasound.