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# Hydrophobic drug concentration affects the acoustic susceptibility of liposomes

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ABSTRACT

The purpose of this study was to investigate the effect of encapsulated hydrophobic drug concentration on ultrasound-mediated leakage from liposomes. Studies have shown that membrane modifications affect the acoustic susceptibility of liposomes, likely because of changes in membrane packing. An advantage of liposome as drug carrier is its ability to encapsulate drugs of different chemistries. However, incorporation of hydrophobic molecules into the bilayer may cause changes in membrane packing, thereby affecting the release kinetics. Liposomes containing calcein and varying concentrations of papaverine, a hydrophobic drug, were exposed to 20 kHz, 2.2 W $cm^{-2}$ ultrasound. Papaverine concentration was observed to affect calcein leakage although the effects varied widely based on liposome phase. For example, incorporation of 0.5 mg/mL papaverine into $L_d$ liposomes increased the leakage of hydrophilic encapsulants by 3× within the first minute ($p = 0.004$) whereas the same amount of papaverine increased leakage by only 1.5× ($p < 0.0001$). Papaverine was also encapsulated into echogenic liposomes and its concentration did not significantly affect calcein release rates, suggesting that burst release from echogenic liposomes is predictable regardless of encapsulants chemistry and concentration.


## 1. Introduction

Liposomes offer several advantages as drug delivery vehicles. These biocompatible, biodegradable vesicles composed of single or multiple concentric lipid bilayers are able to encapsulate hydrophilic or hydrophobic drugs in the aqueous space or membrane, respectively. Liposomes limit systemic exposure and unintended drug action, decreasing harmful side effects while maintaining chemical integrity of its encapsulants [1]. Recent studies have also shown that antibodies and ligands can be tethered to the liposome surface to enable targeted delivery [2–4]. Various stimuli have been used to control drug release, including the use of pH [5–7], temperature [8–11], light [12], and ultrasound [13–15].

Low-frequency ultrasound (here considered to be on the order of 20 kHz) is a promising external trigger; it does not affect the chemical integrity or potency of the drug, can penetrate without any irreversible damage to the skin, and can be tuned to stimulate a specific release rate at the target location [1]. Schroeder et al. encapsulated three different drugs into identical liposome formulations and showed that 60 s of exposure to low frequency ultrasound, specifically 20 kHz at intensities greater than 1.3 W $cm^{-2}$, released nearly 80% of each drug [16]. Studies have also shown that liposomes can be modified to increase ultrasound-induced leakage, herein referred to as acoustic susceptibility [14,17–22].

Nguyen et al. showed that various membrane modifications such as lipid phase, polyethylene glycol-grafting, and echogenicity affect acoustic susceptibility by systematically modifying liposome membrane properties and monitoring the leakage of calcein, a fluorescent hydrophilic dye [23]. The authors hypothesized that these membrane modifications changed the packing parameter in various regions of the lipid bilayer, creating mismatching interfaces. This is consistent with other studies that demonstrated that liposomes undergoing phase transition are even more permeable than when in their fluid state even without ultrasound exposure, due to large defects in bilayer packing related to the coexistence of phases [24]. Each region with dissimilar packing parameter would behave differently under ultrasonic pressure, causing increased leakage at the mismatched interfaces. Thus, any significant membrane modification could theoretically create bilayer defects and should be examined for potential effects on acoustical susceptibility.

The encapsulation of hydrophobic drugs in the bilayer has been shown to affect liposome stability. In a study examining the influence of drug chemistry and lipid tail length on liposomal stability, Khan et al. encapsulated hydrophilic and hydrophobic fluorescent markers, fluorescein and rhodamine respectively, into liposomes composed of lipids with differing tail lengths [25]. Passive leakage of the fluorescent drug mimics at different temperatures was used to quantify liposomal stability. A markedly different release profile was observed after 4 weeks: ~90% rhodamine leakage and ~50% fluorescein leakage at 37 °C from dipalmitoyl phosphatidylcholine (DPPC) liposomes, a 16-carbon chain with gel–liquid crystalline transition temperature ($T_m$) of 41 °C. On the other hand, leakage of the fluorescent markers

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from liposomes composed of distearoyl phosphatidylcholine (DSPC), an 18-carbon phospholipid with a $T_m$ *of* 55 °C, was both at ~40% after 4 weeks at 37 °C. The authors concluded that the minimal difference in leakage of hydrophilic and hydrophobic dye from DSPC liposomes was due to the minimal phospholipid chain movement in the bilayer relative to the DPPC liposomes. This is consistent with other studies that have shown that lipid bilayers increase in fluidity and phospholipid tail movement as the temperature reaches $T_m$ [26]. Khan et al. also suggested that for liposomes of lower $T_m$, the hydrophobicity of the encapsulated drug can significantly affect the overall stability of the liposome system [25]. Consequently, an aim of this study was to determine the effect of hydrophobic drug content and liposome phase on the liposome system, specifically in the presence of ultrasound.

Chrzanowski et al. examined the difference in release rates of hydrophilic and hydrophobic drugs from liposomes exposed to ultrasound at a frequency of 6 MHz and pressure amplitude of 2 MPa [22]. They found that 1 s of ultrasound exposure led to the release of a significant amount of calcein (47.5% ± 33.0%) but an insignificant amount of papaverine, a hydrophobic drug (20.1% ± 42.4%). As the study suggests, the difference in the detected amount of drug released in the *in vitro* experimental setup was likely a result of chemical structure and water solubility. Hydrophilic drugs are more likely to be efficiently released because the compounds trapped within the aqueous core would be released into the aqueous external space. On the other hand, hydrophobic drugs are likely to remain associated with the membrane as it reforms in a spontaneous manner. This differed from the work by Khan et al. in that the presence of ultrasound caused the destruction and reformation of liposomes. In an *in vivo* setting, hydrophobic drugs may still be delivered into a cell, as the lipid bilayers of liposomes and cells are known to interact or even fuse [27].

The objective of this study is to examine the effect of hydrophobic drug content and liposome phase on the acoustic susceptibility of liposomes by measuring the leakage of calcein. The leakage of papaverine, the hydrophobic drug of choice, will not be monitored because of its likelihood to remain associated with the lipid bilayer. Calcein fluorescence, on the other hand, will diminish even if it is re-encapsulated, as any exposure to cobalt chloride in the external solution will quench its fluorescence. The goal of this study is not to compare the leakage of hydrophilic and hydrophobic encapsulants; it is to encapsulate both components into liposomes and determine how hydrophobic drug concentration affects ultrasound-mediated leakage of the hydrophilic component. The hydrophobic drug, therefore, is merely treated as a membrane modifier. Since published work showed that release from echogenic liposomes remained nearly the same even with certain modifications, these experiments are also conducted on echogenic liposomes. The results of this study will test the versatility of liposomes and determine whether further studies are needed for each type of drug to be encapsulated.

This paper is organized in the following way: in Section 2, the materials including the drug mimics and lipids used are described along with the experimental procedure. In Section 3, the leakage profiles obtained by monitoring the quenching of calcein fluorescence are displayed and then discussed in Section 4.

## 2. Experimental methods

### 2.1. Materials

Lipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and cholesterol (CHO) dissolved in chloroform were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Calcein, a hydrophilic fluorescent dye (Sigma Aldrich, St. Louis, MO), was encapsulated to monitor leakage from liposomes in conjunction with cobalt chloride (Sigma Aldrich, St. Louis, MO), which quenched external calcein. Papaverine (Sigma Aldrich, St. Louis, MO), a hydrophobic drug, was encapsulated into the membrane. Mannitol (Alfa Aesar, Ward Hill, MA), a cryoprotectant, was used as a key component to form ELIP [17].

### 2.2. Preparation of liposomes

Liposomes were prepared based on an established method [23]. Briefly, lipids, cholesterol, and the hydrophobic drug papaverine were dissolved in chloroform and mixed in a round bottom flask. The organic solvent was removed by vacuum overnight on a rotary evaporator. The resultant lipid film was rehydrated with 0.1 mM calcein, a concentration that would cause fluorescence, in 1× phosphate buffered saline (PBS) and bath sonicated for 5 min to ensure that all lipids were in suspension.

Various concentrations of papaverine (0.1, 0.2, 0.3, and 0.5 mg/mL) were encapsulated and compared to a control without papaverine to determine the effect of hydrophobic drug concentration on acoustic susceptibility. To determine the effect of liposome fluidity on acoustic susceptibility of liposomes with hydrophobic drug, two liposome phases were investigated: a more fluid liquid disordered ($L_d$) phase and a more rigid liquid ordered ($L_o$) phase. The molar ratios of lipids and cholesterol used are shown in Table 1.

Cobalt chloride ($CoCl_2$) was added to quench external calcein. Thus, any fluorescence detected after the addition of $CoCl_2$ was from calcein encapsulated in the aqueous core of liposomes.

For echogenic liposomes, the dry film obtained after the dehydration step was rehydrated with 0.32 M mannitol and bath sonicated for 5 min. Then, the suspension went through five freeze–thaw cycles; in each cycle, the liposome suspension was frozen at −80 °C for 30 min and then thawed to room temperature. The liposomes were lyophilized for 24 h to yield a powder and stored at 4 °C until use. A calcein solution (0.1 mM) in 1× PBS was used to reconstitute the liposomes. The final lipid concentration used was 0.5 mg/mL.

The resultant multilamellar liposomes were approximately 1 μm in diameter, as measured by dynamic light scattering using a Zetasizer Nano S90 (Malvern, UK). Liposome echogenicity was confirmed using a Sonosite 180 standard diagnostic ultrasound device (Bothell, WA) as described by the authors previously [23]. Briefly, a 5–10 MHz linear array transducer was used to image the samples in 10 mm × 10 mm × 10 mm silicone containers. The relative amount of reflections per sample was visualized by brightness level and compared to control samples. The mean gray scale value of the regions of interest of the images ($n = 3$) was measured with ImageJ software (National Institutes of Health, Bethesda, MD) and shown to be statistically significant ($p < 0.05$).

### 2.3. Methodology of ultrasound-induced release

Ten milliliters of each liposome formulation (varying lipid phase and papaverine concentration) was placed into 20 mL glass vials and the tip of a Misonix XL2020 probe sonicator (Misonix Inc., NY) was submerged approximately 3 mm from the bottom of the vial. The setup was placed in ice to maintain a constant temperature for the duration of the experiment because liposome fluidity and phase have been found to be dependent on temperature [26]. Each liposome suspension was exposed to 20 kHz continuous wave ultrasound for a total of 10 min, with samples gathered at 1, 3, 6, and 10 min for comparison to the initial sample ($n = 3$).

The acoustic energy delivered by the probe sonicator was determined by a calibrated Reson 4038 hydrophone probe (−227 dB re 1 V/uPa at 20 kHz). The pressure amplitude was measured from

**Table 1**
Lipid formulations for liquid ordered and liquid disordered liposomes.

| Liposome phase | DOPC (mol.%) | DPPC (mol.%) | CHO (mol.%) |
|---|---|---|---|
| Liquid disordered ($L_d$) | 75 | 15 | 10 |
| Liquid ordered ($L_o$) | 5 | 57 | 38 |

10 mm to 50 mm from the sonicator tip to minimize the potential damage of the probe due to inertial cavitation. The data was then plotted against the distance and extrapolated to the tip surface, so that the intensity of interest could be calculated. Intensity was calculated from the pressure amplitude at 3 mm to be equal to 2.2 W $cm^{-2}$ spatial peak, temporal peak. Conversion of the pressure amplitude data to the intensity was performed to facilitate the comparison with the reported literature data.

The mechanical index (MI) has been used to estimate the potential for cavitation. The MI is defined by

$$\mathrm{MI} = \frac{P}{\sqrt{f}} \tag{1}$$

where $P$ is the peak rarefactional pressure in MPa and $f$ is the ultrasound frequency in MHz. It has been assumed that below a MI of 0.7, cavitation does not occur [28]. Thus, at higher frequencies, higher intensities are required for cavitation whereas at lower frequencies, lower intensities are able to initiate cavitation. The Food and Drug Administration (FDA) allows a maximum MI of 1.9 [29]. According to these standards, the acoustic pressure may not exceed 300 kPa or 2.4 W/$cm^2$ at 20 kHz. The ultrasound parameters used in this study induce cavitation but also comply with FDA standards.

The potential of introducing undesirable artifacts such as reflections and standing waves during insonification was acknowledged and given thoughtful consideration. Due to the relatively long (75 mm) wavelengths of 20 kHz ultrasound, the pressure gradient across the sample (3–10 mm, from the sonicator tip to the sides of the container) at any time was negligible. With this, the experimental setup was not subject to nodes or anti-node formation within the medium and therefore was not a concern.

### 2.4. Spectrofluorometric techniques

A spectrophotometric technique was used to quantify leakage using a cobalt quenching assay. Calcein fluorescence of an initial sample before ultrasound exposure and samples from each time point were analyzed with a spectrometer (Tecan, Crailsheim, Germany) at an excitation and emission wavelength of 488 and 527 nm, respectively. Leakage was defined as

$$\text{Experimental Percent Leakeage } (\%) = \frac{F_i - F(t)}{F_i - F_f} \times 100 \tag{2}$$

where $F_i$ is the maximal fluorescence of the initial liposome suspension, $F(t)$ is the fluorescence of the remaining calcein inside the liposomes at any time in minutes, and $F_f$ is the fluorescence after lysing the liposomes with an equal volume of 1% Triton, or the background fluorescence once all of the calcein has been quenched by $CoCl_2$.

Kopechek et al. found that fluorescence measurements used as a means of quantifying leakage from echogenic liposomes may give rise to misleading results because of the scattering caused by the bubbles [15]. The authors have discussed this issue in detail in a previous investigation and shown that the results are unaffected by the presence of bubbles [30].

For another means of comparison, the rate of leakage was assumed to be a first order process, modeled by

$$\text{Expected Percent Leakage } (\%) = \left(1 - e^{-kt}\right) \times 100 \tag{3}$$

The rates of release are reported in terms of the rate constant $k$.

### 2.5. Statistical analysis

The data are reported as mean with standard deviation. Statistical analysis was performed using MATLAB (MathWorks, Natick, MA). Statistical significance was determined using a one-way ANOVA test, with a modified Tukey–Kramer multiple comparisons procedure used to compare individual samples. A $p$-value below 0.05 was used as the threshold for statistical significance.

## 3. Results and discussion

### 3.1. Effect of papaverine concentration on acoustic susceptibility of liposomes

The leakage of calcein was used to quantify acoustic susceptibility of liposomes. Papaverine concentration was observed to influence the acoustic susceptibility of liposomes differently depending on lipid phase. In the case of $L_d$ liposomes (Fig. 2A), an increase in papaverine concentration increased the rate of calcein leakage, with a plateau on the effect at approximately 0.3 mg/mL. On the other hand, as shown in Fig. 2B with $L_o$ liposomes, maximum increase in calcein leakage was observed with 0.2 mg/mL; higher concentrations of papaverine were observed to dampen the calcein release profile. (See Fig. 1.)

The effect of papaverine concentration on the acoustical susceptibility of liposomes was consistent with reports that incorporating components into the membrane affect phospholipid chain movement [31]. Cholesterol, for example, is known to reside in the lipid bilayer and interact with lipid tails, partially immobilizing them. This typically makes the lipid bilayer less deformable and less permeable to water-soluble molecules. It would be expected that the release profile of calcein would decrease, if the entire lipid bilayer had become less deformable. However, as shown in Fig. 2A, inclusion of as little as 0.1 mg/mL of papaverine showed a statistically insignificant ($p = 0.55$) but noticeable increase in the release profile of calcein; the addition of 0.2 mg/mL or more papaverine significantly increased the release profile of calcein ($p \geq 0.05$). The data suggests that only scattered regions of the liposome were affected by the presence of papaverine, that the effect of papaverine on lipid chain mobility was a local effect. A previous study showed that inconsistencies in bilayer packing as a result of membrane modification increased calcein leakage in the presence of ultrasound, as the interfaces between regions of mismatching were exploited when under ultrasonic pressure [23]. It would follow then, that if a sufficient amount of papaverine were encapsulated and if it was equally distributed throughout the membrane, the release of calcein would in fact decrease compared to the control (no papaverine) because the entire liposome would be consistently more rigid.

The results presented in Fig. 2B show an increase in leakage rates with 0.1 and 0.2 mg/mL papaverine in $L_o$ liposomes, but 0.3 and 0.5 mg/mL papaverine decreased the overall release rate. However, all liposome formulations with papaverine had a higher leakage rate than the control. $L_o$ liposomes include a higher molar percentage of short-chained, saturated DPPC lipids than $L_d$ liposomes (in this study, 57% compared to 15%) and a higher molar percentage of cholesterol (38% compared to 10%). Consequently, $L_o$ liposomes are more tightly packed and rigid [26,31]. It would seem that $L_o$ liposomes are less forgiving to the inclusion of additional membrane components, as the chains are more tightly packed and there is already a significant amount of cholesterol included. Thus, while 0.2 mg/mL papaverine increased calcein leakage, higher concentrations may have made the liposome more consistently rigid, decreasing the number of mismatching interfaces.

### 3.2. Effect of papaverine concentration on release from echogenic liposomes

Echogenic liposomes are known for their increased sensitivity to ultrasound, specifically useful in the use of ultrasound as an external trigger for the delivery of drugs from liposomes. Papaverine was incorporated into echogenic liposomes to determine if hydrophobic drug content affected the ultrasound-triggered release of hydrophilic

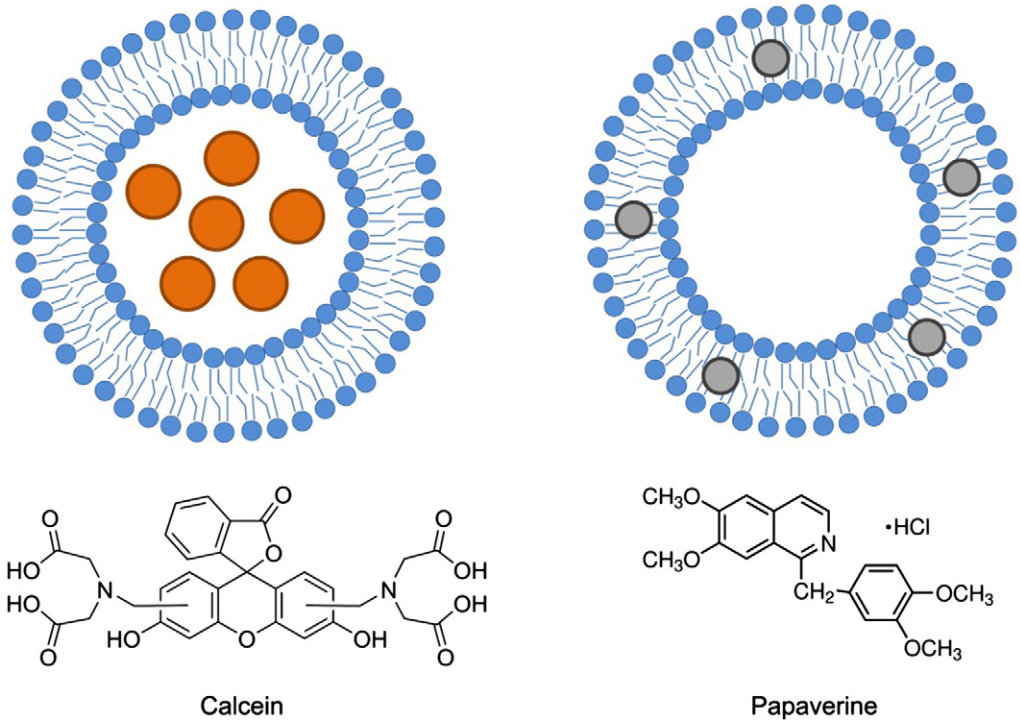


**Fig. 1.** Liposomal storage location and chemical structures of calcein (left) and papaverine (right). Calcein is hydrophilic and would therefore reside in the internal aqueous compartment whereas papaverine is hydrophobic and would likely be entrapped in the lipid bilayer.

encapsulants from echogenic liposomes. Fig. 3A showed that hydrophobic drug content does not have a significant effect on ultrasound-triggered release from echogenic $L_d$ liposomes, whereas papaverine concentration did affect the release rate of calcein from $L_o$ liposomes (Fig. 3B).

The data showed that papaverine concentration may have affected ultrasound-triggered release from $L_d$ liposomes, but the release initiated by the interaction between ultrasound and the encapsulated bubbles of the echogenic $L_d$ liposomes exceeded the changes in release rate due to papaverine presence. This was dissimilar from what was observed from $L_o$ liposomes. It has been established that the leakage from $L_o$ liposomes is lower than from $L_d$ liposomes because of a decrease in bilayer fluidity, even when bubbles are encapsulated into the liposomes [30]. It appears that addition of papaverine further decreased bilayer fluidity.

For another means of comparison, the rate of leakage was modeled according to **(3)** and the calculated rate constants are displayed in Fig. 4.

The trends observed from $L_d$ liposomes (Fig. 4A) have been discussed. As papaverine concentration increased, the number of regions that changed in bilayer fluidity increased, creating an increased number of mismatching interfaces that were exploited during ultrasound exposure. These effects were overshadowed by the mechanism of release from echogenic liposomes in response to ultrasound.

In the case of $L_o$ liposomes (Fig. 4B), on the other hand, 0.2 mg/mL seemed to be a pivotal concentration: inclusion of 0.2 mg/mL papaverine caused the most leakage from non-echogenic liposomes (presumably because it caused the highest number of mismatching regions whereas higher concentrations caused larger but fewer regions of mismatch) and the least leakage from echogenic liposomes.

The release profiles observed from $L_o$ liposomes are not fully understood. Perhaps the data shown is an indication of an energetic preference for hydrophobic molecules to associate with lipids in a fluid phase or with gas bubbles over lipids in an ordered phase. In the presence of a fluid phase, papaverine would readily associate with the phospholipids because the chains were more mobile. When bubbles are present in the lipid bilayer, papaverine would either associate with the phospholipids or the gas bubble. However, when phospholipids are more closely packed due to increased amounts of saturated, short-chain lipids and large amounts of cholesterol (>30% cholesterol gives rise to the $L_o$ phase [32]), papaverine may have had a tendency to associate with the encapsulated air bubble, thereby dampening the acoustic oscillations and bubble growth. At concentrations above 0.2 mg/mL papaverine (Fig. 4B), it would appear that the bubbles had been sufficiently coated and papaverine was forced into the ordered bilayer, causing regions of increased rigidity. This rationale describes the data

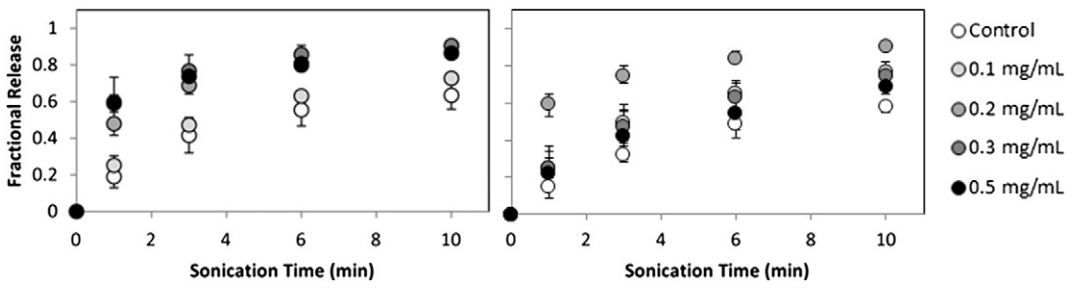


**Fig. 2.** Release profile of the hydrophilic dye calcein from (A) $L_d$ and (B) $L_o$ liposomes with varying amounts of papaverine encapsulated in the lipid bilayer. The controls represent calcein release profiles from liposomes that do not contain papaverine.

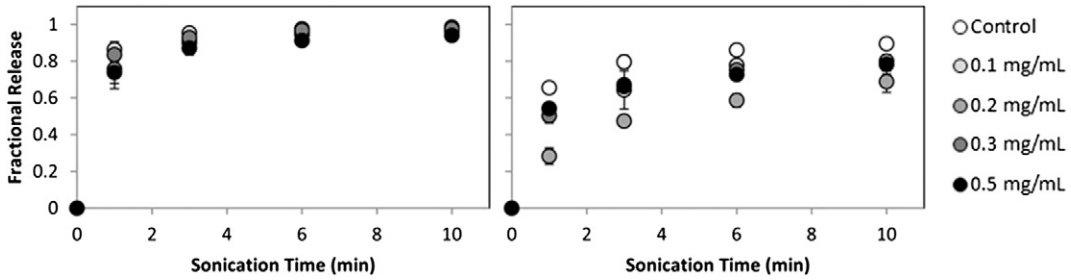


**Fig. 3.** Release profile of calcein from echogenic (A) $L_d$ and (B) $L_o$ liposomes with varying concentrations of papaverine. Controls represent calcein leakage from echogenic liposomes without papaverine.

consistently, but more studies need to be conducted to better understand the system.

## 4. Conclusions

This study monitored the acoustic susceptibility of liposomes by measuring calcein release from liposomes with varying concentrations of papaverine. The effect of papaverine concentration on the acoustic susceptibility of liposomes was found to be dependent on liposome phase ($L_d$ and $L_o$ liposomes were constructed with DOPC, DPPC, and cholesterol) and echogenicity. Our results indicated that papaverine caused a decrease in chain mobility in scattered areas of $L_d$ liposomes, creating numerous interfaces of mismatching lipid packing. Acoustic pressure likely exploited the mismatched interfaces and leakage was observed to increase. The leakage caused by the interaction of ultrasound and encapsulated bubbles of echogenic $L_d$ liposomes seemed to overshadow said effects, as the acoustic susceptibility of liposomes was independent of papaverine concentration.

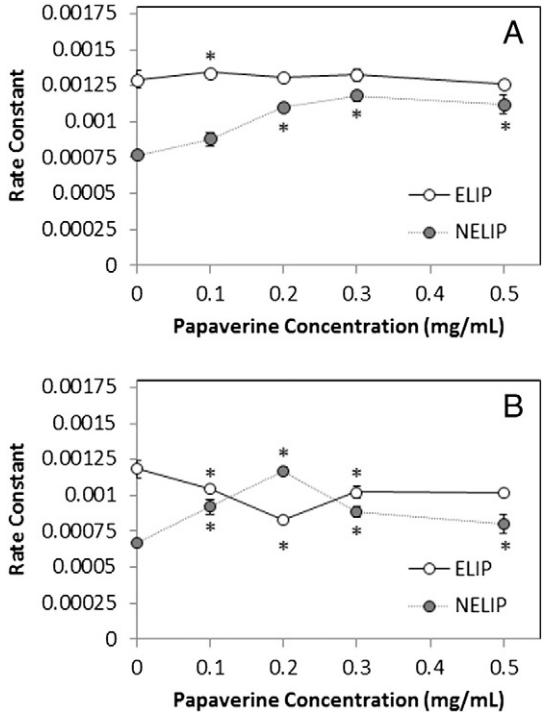


**Fig. 4.** Assuming calcein leakage to be a first order process, rate constants were calculated to represent the release profiles of calcein from (A) $L_d$ liposomes and (B) $L_o$ liposomes. The effects of papaverine concentration (0.1, 0.2, 0.3, and 0.5 mg/mL) on acoustic susceptibility can be compared with a control sample without papaverine. Liposomes of each phase were tested with (ELIP) and without (NELIP) encapsulated bubbles. Asterisks denote statistical significance ($p < 0.05$) when compared to the control sample.

The effect of papaverine concentration on release of calcein from $L_o$ liposomes, although inconclusive, suggested that hydrophobic drugs have a greater affinity towards fluid phases and gas bubbles than to ordered phases. The results from this study suggest that the properties and concentration of hydrophobic drugs have a significant effect on ultrasound-induced leakage. The effects of hydrophobic drug content may be dominated by encapsulating bubbles for ultrasound-triggered release (as in the case of the $L_d$ formulation presented in this article), enabling predictable release regardless of hydrophobic drug content and concentration. On the other hand, some liposome formulations (for example, the $L_o$ formulation studied in this paper) will likely require release studies for every hydrophobic drug and concentration used. Studies investigating the effect of hydrophobic drug size on the acoustic susceptibility of liposomes of comparable lipid phase would be a beneficial future step.

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