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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