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# Determination of the Liposomal Bulk Modulus by Molecular Acoustics

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## Abstract

In the context of nanomechanics, elasticity is a notable physical property that has garnered recent interest for its roles at the nano-bio interface. This work introduces a novel application of molecular acoustics (ultrasound velocimetry and densitometry) to assess volumetric elasticity modulus or bulk modulus as a tool to characterize liposomal elasticity. One of the main advantages of the presented technique is that the liposomes remain unaltered throughout the entire measurement process, that is, without the artifacts by adsorption onto a substrate and the effect of a probe. Another strength highlighted in our proposal is that the obtained elasticity modulus does not depend on a physical model, such as the Hertz model for atomic force microscopy, and the required considerations, such as assuming the sample is homogeneous and isotropic. We consider that the bulk modulus obtained through ultrasonic velocimetry provides a genuine measure of the liposomal elasticity of the system under study.

**Keywords:** Ultrasound velocimetry and densitometry; Liposomal Elasticity; Volumetric elastic modulus; DPPC; Cholesterol; DHA; Xanthone

## Introduction

The stability of lipidic nanoparticles is closely tied to their elasticity, enabling liposomes to respond dynamically to mechanical stress in their environment. Ultrasound velocimetry and densitometry provide essential data on the speed of sound and density within the liposomal system, which are used to analyze liposomal elasticity by evaluating the membrane's bulk modulus. These techniques are particularly noteworthy compared to other methods for determining the modulus of elasticity, as they allow us to assess changes in the physical properties of the entire bilayer [1,2] through mathematical equations without requiring restrictive sample parameterization. They combine high precision with ease of operation and are non-invasive, as the sample does not need to be supported on a substrate, and no probes are used. Additionally, they require a sample of small volume and low concentration.

In complex systems where no single region is representative of the whole, it is crucial to assess mechanical properties comprehensively, considering the entirety of the nanoparticle. Given the liposome's mechanical properties and anisotropy, a thorough understanding of these properties necessitates investigating membrane deformation in various directions [3].

Young's modulus, frequently used to determine liposomal elasticity, is measured using atomic force microscopy (AFM). AFM enables the imaging and evaluation of the mechanical properties of soft samples. When measuring these properties with AFM, it is relevant to apply appropriate loading forces. Parameters such as indentation, contact area between the tip and the sample, and the applied pressure must be carefully considered when recording and interpreting data. The AFM tip geometry and the stiffness of the cantilever influence these parameters. Generally, Hertz contact models are used to characterize the mechanical properties of soft matter, where the measured stiffness depends on the contact area, applied force, and the elastic modulus of the sample. The tip geometry affects the pressure and mechanical stress generated in the sample. Despite this, the Hertz model remains one of the most widely used contact models. Thus, further investigation into the role of tip geometry in determining Young's modulus is necessary [4].

Lipid bilayers within membranes exhibit lateral heterogeneity, characterized by variations in lipid compositions, chain lengths, and packing densities across different regions. These heterogeneities arise from the dynamic nature of lipid molecules and the coexistence of various lipid species within the bilayer. Additionally, bilayers can contain areas of varying fluidity, reflecting differences in lipid mobility and organization within the membrane. However, the Hertz model and its variants assume a homogeneous and isotropic sample exhibiting a linear response. Regarding biological membranes, the existence of lateral heterogeneity, particularly dynamic heterogeneity, is well-established enough to be considered relevant to the functioning of these structures.

As previously stated, we believe that the bulk modulus obtained through ultrasonic velocimetry provides a genuine measure of the liposomal elasticity of the system under study. This innovative approach opens new possibilities for a broad range of biotechnological applications.

## Materials and Methods

### Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA, 22:6, Figure 1A) and cholesterol (Chol) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). DHA was dissolved in ethanol to the final concentration of 6 mmol $L^{-1}$. Chloroform and ethanol of analytical grade were purchased from Sigma-Aldrich. The 3-((3-methylbut-2-en-1-yl)oxy)-9H-xanthen-9-one (3PX), an O-prenylated xanthone derivative (Figure1B) was synthesized, characterized, and purified, in the laboratory of Medicinal Organic Synthesis (UNS) [5,6]. Ultrapure water (pH = 5.5) was provided by the distillation plant (UNS), using a Super Q Millipore system.

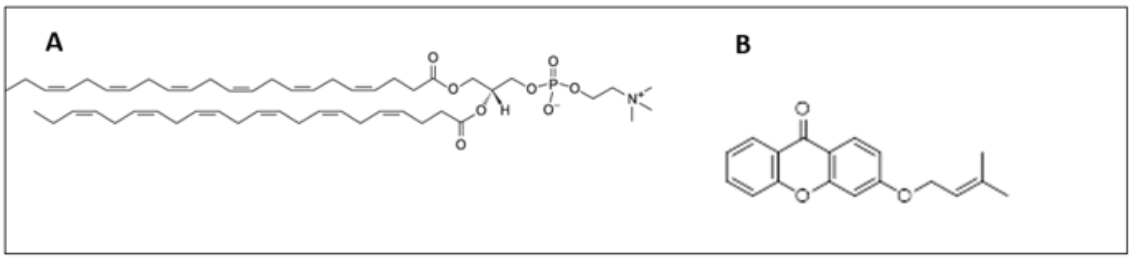


**Figure 1A:** Structure of DHA - B: Structure of 3PX.

### Liposomes Preparation

All liposomes were prepared using the dry film method, with specific variations for each system described below. DPPC or DPPC-dopant mixtures were dissolved in chloroform, which was then evaporated under a stream of $N_2$ to form a dry lipid film. Any remaining solvent was removed using a high vacuum centrifuge (Thermo Scientific Speed Vac SPD11V) for two hours. The resulting dry lipid films were hydrated with 3 mL of Milli-Q water and homogenized through cycles of vigorous vortexing at approximately 10°C above the lipid's transition temperature. This heating-vortexing process resulted in a polydispersed population of multilamellar vesicles (MLVs). The final average concentration of the dispersions was 2.4 mg $mL^{-1}$. Before conducting density and ultrasound velocity measurements, the aqueous vesicle suspension was degassed using a water vacuum pump.

#### *DPPC-3PX and DPPC-Chol-3PX Liposomes*

DPPC-3PX liposomes were prepared with $X_{3PX}$ of 0.3, where $X_{3PX}$ corresponds to the mole fraction of xanthone, excluding the solvent. The amount of dopant was adjusted based on preliminary unpublished studies from our laboratory.

DPPC-Chol-3PX liposomes were prepared using the dried film technique described above, with $X_{Chol}$ of 0.3 relative to DPPC and $X_{3PX}$ of 0.3 relative to the lipid mixture. The 70:30 (DPPC: Chol) ratio, frequently cited in the literature, ensures liposomal stability [7-9].

#### *DPPC-DHA and DPPC-Chol-DHA Liposomes*

DPPC-DHA liposomes were prepared with an $X_{DHA}$ of 0.3. The amount of dopant was adjusted based on preliminary unpublished studies conducted in our laboratory, which align with previous studies by other groups [10]. DHA was dissolved in ethanol and

added to the dry lipid film. We took precautions to minimize oxidation during DHA-containing solutions handling, such as limiting light exposure, using a glove box purged with high-purity nitrogen, and hermetically sealing the cuvettes during measurements. The degree of lipid oxidation was quantitatively controlled by GC analysis at the end of each experiment. Samples were prepared immediately before measuring to minimize oxidation during storage. DPPC-Chol-DHA liposomes were prepared with $X_{Chol}$ of 0.3 relative to DPPC and $X_{DHA}$ of 0.3 relative to the lipid mixture.

### Method: Ultrasound Velocity and Density

Densities (ρ) and sound velocities (u) were continuously, simultaneously, and automatically determined by means of Anton-Paar DSA 5000 densimeter and sound velocity analyzer. This instrument is a density and sound velocity meter developed to combine highest precision with easy operation and robust design. It simultaneously determines two physically independent properties within one sample. The two-in-one instrument is equipped with a density cell and a sound velocity cell thus combining the proven Anton Paar oscillating U-tube method with a highly accurate measurement of sound velocity. Both cells are temperature-controlled by a built-in Peltier thermostat ($\pm 10^{-2}$ K). The sample volume (1mL) to be measured is usually filled manually by syringe.

The determinations were performed at progressively decreasing temperatures (50°C to 30°C), because of the liposome preparation method. Density and sound velocity measurements were highly reproducible ($\pm 5\times10^{-6}$ g cm$^{-3}$ and $5\pm10^{-1}$ m s$^{-1}$, respectively). Reported data are the average of three different batches of each sample.

The measurement of ultrasound velocity enables to assess the elastic properties of aqueous media and suspensions like vesicles [11-13], using a straightforward relationship:

$$\beta_s = 1/u^2 \rho \quad (1)$$

whereby $\beta_s$, $\rho$, and u are the adiabatic compressibility, the density, and the sound velocity of the suspension, respectively. By measuring the changes in sound velocity and density, variations in adiabatic compressibility can be calculated [14].

In physics textbooks, it is noted that bulk elasticity parameters are closely related to the equation of state and fundamental thermodynamic potentials like Gibbs free energy. To link the macroscopic (thermodynamic) and the microscopic (molecular) features of a fluid-like medium characterized by short-range molecular interactions, it is necessary to use the language of partial physical parameters that describe the individual contributions of specific types of molecules to a measurable property. Therefore, in molecular acoustics, due to the additive nature of all system components, the adiabatic compressibility of the lipid is commonly used, which is given by

$$\beta_{lipid} = \beta_0 \left( 2 - \frac{[u]}{\varphi_v} \right) \quad (2)$$

whereby $\beta_0$ is the adiabatic compressibility of the solvent, $[u]$ is the concentration increments of the sound velocity, and $\varphi_v$ is the apparent specific partial volume of the sample.

$$[u] = \frac{u - u_0}{u_0\, c}\,;\ \varphi_v = \left[ 1 - \frac{\rho - \rho_0}{c} \right] \frac{1}{\rho_0} \quad (3)$$

where c is the solute concentration (lipid) in mg/ml, $u_0$ indicates sound velocity of the solvent (milliQ water) and $\rho_0$ is the density of the solvent.

Finally, the volumetric compressibility modulus $K_{lip}$ is obtained by calculating the inverse of apparent adiabatic compressibility of the lipid:

$$K_{lip} = 1/\beta_{lipid} \quad (4)$$

## Results and Discussion

The volumetric modulus of elasticity was studied for these liposomes: DPPC, DPPC-Chol, DPPC-Chol-DHA, and DPPC-Chol-3PX. DHA is an omega-3 fatty acid with a 22-carbon chain and six double bonds. It is well known that DHA and cholesterol have a mutual aversion, driving the lateral segregation of DHA into highly disordered domains away from cholesterol [15,16]. 3PX is a planar structure compound that together with cholesterol does not cause significant structural modifications to the lipids. Our group's experience with these effectors guided this choice.

Ultrasound velocity determinations allow the evaluation of the elastic properties of aqueous suspensions of lipid vesicles. Based on molecular acoustics and calculations with equations in section Method: Ultrasound Velocity and Density, $K_{lip}$ values were obtained and are shown in Figure 2.

It is worth mentioning that, although $K_{lip}$ data is not found in the literature—as far as we know—it can be inferred from adiabatic compressibility data that the obtained values agree in magnitude for this type of system.

The curves for the systems containing cholesterol show no significant change in the liquid crystalline-solid crystalline transition temperature. The bulk modulus values differ between vesicles containing xanthone and those with DHA. The minimal impact of xanthone on lipid structuring is reflected in the elasticity modulus, showing practically no differences for liposomes in the presence or absence of xanthone. Liposomes with DHA and cholesterol exhibit bulk modulus higher than other systems do. That issue is presumably attributable to lipid domains presence with higher packing density, which raises the average value in the determination of $K_{lip}$ and this implies a decrease in the volume adiabatic compressibility. Other authors [17] previously explored the relationship between total volume adiabatic compressibility and the presence of domains. They found that in their studied system (including DPPC, Chol and Galactocerebroside), the formation of microdomains decreases the total volume adiabatic compressibility of the multilamellar vesicle assemblies. However, cholesterol disrupts the galactocerebroside domains, leading to a slight increase of this parameter in the lipid assemblies.

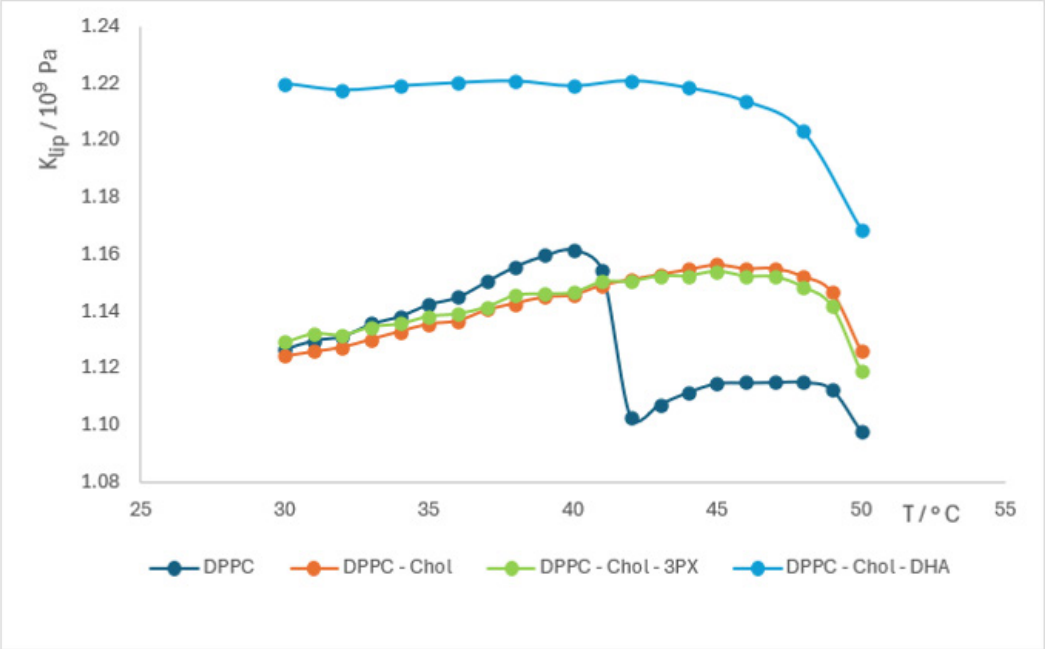


**Figure 2:** Lipidic volumetric compressibility modulus, $K_{lip}$, vs temperature for MLV liposomes. In all cases, the standard deviation was lower than 5%, not shown for better viewing.

Both kinds of observations suggest that adding cholesterol to a system does not always have the same effect on liposome compressibility. Thus, the mechanical impact of cholesterol seems to depend on the composition of the system where is incorporated.

## Conclusion

This work demonstrates the relevance of molecular acoustics as a tool in nanomechanics. In this regard, the use of the bulk modulus emerges as a simple, sensitive, non-invasive, low-cost instrument, mainly free from parameterizations and any type of artifacts on the sample. This allows obtaining genuine data from the system under study, with very good prospects in the biotechnological field.

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## Conflict of interest

None.

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