A New Approach for Investigating the Response of Lipid Membranes to Electrocompression by Coupling Droplet Mechanics and Membrane Biophysics

Supplementary Information

Joyce El-Beyrouthy[a], Michelle M. Makhoul-Mansour[a], Graham Taylor[b,c], Stephen A. Sarles[b],

Eric C. Freeman[a*]

[a]School of Environmental, Civil, Agricultural, and Mechanical Engineering, The University of Georgia, Athens, GA, 30602, United States

[b]Department of Mechanical, Aerospace, and Biomedical Engineering, The University of Tennessee, Knoxville, Tennessee, 37996, United States

[c]The Bredesen Center for Interdisciplinary Research, The University of Tennessee, Knoxville, Tennessee, 37996, United States

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S.1. Lipid Solutions Preparation

Lipid-in solutions were used in all experiments, where lipids are dispersed in the aqueous phase as compared to lipids-out where lipids would be dispersed in oil [1]. Buffer solutions were first prepared by mixing 500 mM of potassium chloride (KCl, ≥ 99.1% – Sigma-Aldrich) and 10 mM of 3-(N-Morpholino) propane sulfonic acid (MOPS, ≥ 99.5% – Sigma-Aldrich) in distilled water. Then, for solutions containing only DPhPC (1,2-diphytanoyl-sn-glycero-3-phosphocholine – Avanti Polar Lipids), the phospholipids stored at -20°C were directly mixed with the buffer solution at a concentration of 2.0 mg/mL. A minimum of six freeze-thaw cycles were used to reduce aggregation in the solution. Extrusion was performed immediately before experiments to ensure uniform liposome dimensions. For solutions containing cholesterol, DPhPC and cholesterol (ovine wool, >98% – Avanti Polar Lipids) were each dispersed in chloroform first, and then volumes of the solutions were mixed depending on the desired cholesterol mole fraction. After mixing the two lipids in a glass vial, the chloroform was evaporated through a gentle stream of argon gas. To ensure total evaporation, the vial was next placed under vacuum for several hours. The lipids were then hydrated with the same buffer solution and stored at -20 °C. The solutions were then subjected to the same six freeze-thaw cycles. At this point, cholesterol may still be in its crystalized form, and since extrusion may remove cholesterol crystals [2], sonication was used instead to break down dispersed lipid aggregates. A probe sonicator (Q55 QSONICA, LLC) was used and sonication was performed in 2-minute cycles to prevent overheating of the solution. The cycles were repeated until the solution was rendered transparent. The sonicator tip, syringes used for extrusion and the O-ring channel were cleaned using isopropanol or acetone, rinsed with DI water then placed under air flow to ensure total evaporation. Oil cuvettes and dishes were cleaned between each experiment using soap (Laboratory detergent, Fisher Scientific) then rinsed with DI. Solutions were prepared with 0, 10, 20, and 30% mole fraction cholesterol in DPhPC. The maximum cholesterol mole fraction adopted in this work was 30% ensuring cholesterol solubility in phospholipids and avoiding cholesterol crystals precipitation [3-5]. Hexadecane (99% – Sigma-Aldrich) was used as the primary oil phase, as it has shown to handle stable bilayers in addition to its relatively large molecule enabling the assumption of a relatively solvent-free model membrane [6, 7].

S.2. Pendant Drop Tensiometry Technique

Monolayer surface tensions were measured using the pendant drop technique [8], which relies on the balance between surface tension and gravitational force. When an aqueous droplet is suspended inside a lower density medium, surface tension attempts to minimize the droplet's surface area while gravity pulls it downwards. This balance of tension and gravitational influence produces a pendant-shaped droplet. Measuring the contour of the droplet and the density of the two phases allows for the estimation of the surface tension at the oil-water interface. Based on this concept, the experimental apparatus consists of an aqueous droplet suspended from a needle inside a 3 mL glass cuvette containing the desired oil. Once the droplet is expelled from the needle into the oil reservoir, lipids molecules migrate towards the water-oil interface forming the monolayer and decreasing the surface tension. The droplet's shape gradually sags from a spherical shape to a pendant shape as the lipid monolayer develops.

This process is recorded using a zoom lens camera (6.5X zoom lenses with a 0.7–4.5× magnification range, Thorlabs). Frames are extracted using MATLAB and then used to obtain the interfacial tension by the open-source tensiometry software OpenDrop [8, 9]. Between experiments and to avoid any contamination, the needles were first washed with DI water, then

isopropanol or acetone, then again with DI water and placed under vacuum to ensure complete evaporation.

The success of each experiment was determined by two dimensionless numbers, the Worthington and Bond number. The Worthington number is the ratio of the calculated droplet volume to the maximum volume each needle size can withstand without droplet falling [8]. A value higher than 0.6 indicates acceptably accurate measurements. Since the lipid monolayers measured here are prone to detaching from the needle prior to reaching equilibrium due to the sharp reduction in surface tension, a value as low as 0.4 was also accepted when using needles with a diameter greater than 0.51 mm [8]; however most measurements satisfied the > 0.6 criteria. The Bond number is the ratio of the gravitational force to the surface tension, which must have a value of 0.3 or higher. Any experiment with a Worthington number or a Bond number lower that 0.4 or 0.3, respectively, was not used. The capability to vary the needle dimensions and the magnification provided allows for the assessment of low surface tension cases which otherwise would be problematic.

S.3. Monolayer Surface Tension Results

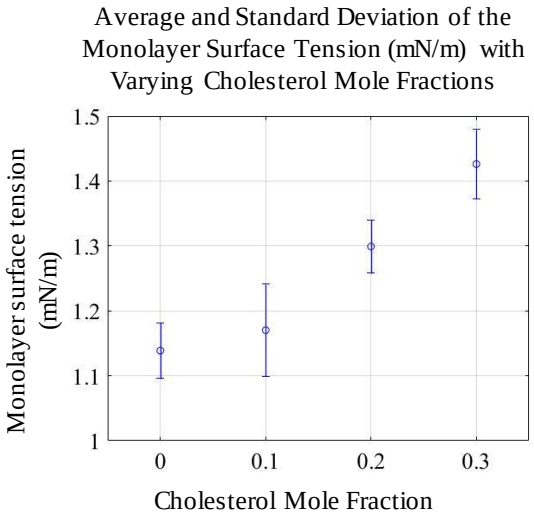

Figure 1: Monolayer surface tension – mean value and standard deviations – with hexadecane oil for different cholesterol percentages.

The monolayer surface tension with varying lipid compositions was measured through the pendant drop tensiometry as discussed in section S.2. Figure 1 shows the interfacial tension average value and interval of error, whereas Table 1 shows all the values considered. Each data point tabulated represents the stable surface tension value calculated from one separate experiment. An experiment is considered successful when the hanging droplet is stable – not shrinking up nor falling down – for more than ten minutes. High resolution frames of the pendant droplet, the exact size of the needle used as well as the exact oil and water densities are key for accurate calculations. Before accepting the tension value, Bond and Worthington numbers were compared to the threshold values. As explained in section S.2, the Bond number must be higher than 0.3 whereas Worthington number depends on the needle size. In fact, a Worthington number higher than 0.6 was accepted for all needle sizes however and for relatively bigger size needles (≈ 0.51 mm in diameter) a value higher than 0.4 was accepted. Any value lower than 0.4 was ignored and the experiment was considered unsuccessful [8].

Table 1: Pendant drop tensiometry experiments for different oil-lipid combinations. Below are all the values used leading to the average and standard deviation presented in Figure 1. These experiments comply with the requirements of a stable droplet while maintaining the recommended Worthington and Bond numbers depending on the needle size used. Monolayer surface tensions shown are in mN/m.

Oil	Hexadecane			
Cholesterol mole fraction	0	0.1	0.2	0.3
Trial 1	1.154	1.192	1.307	1.379
Trial 2	1.196	1.208	1.245	1.478
Trial 3	1.177	1.063	1.352	1.408
Trial 4	1.063	1.120	1.264	1.376
Trial 5	1.145	1.266	1.361	1.474
Trial 6	1.134		1.270	1.456
Trial 7	1.198		1.294	1.400
Trial 8	1.098			1.473
Trial 9	1.091			1.515
Trial 10	1.130			1.396
Trial 11				1.333
Average	**1.139**	**1.170**	**1.299**	**1.426**
STDEV	**0.043**	**0.071**	**0.041**	**0.053**

S.4. Membrane Specific Capacitance Measurements

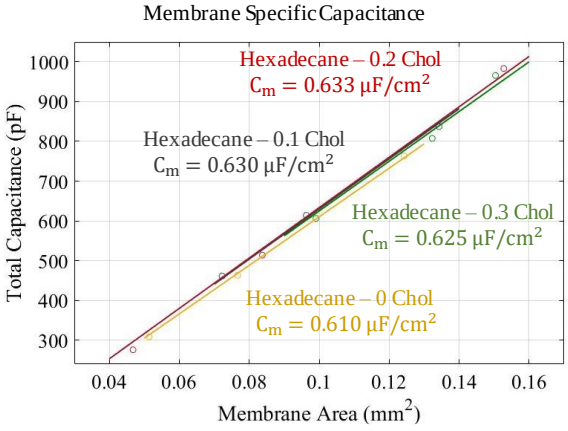

Figure 2: One example from each cholesterol composition showing the linear fit of the total capacitance with respect to the membrane area. The slope indicates the membrane's specific capacitance.

Table 2: The values of the specific capacitance used to obtain the average and standard deviation presented in the manuscript. Units are μF/cm².

Oil	Hexadecane			
Cholesterol molefraction	**0**	**0.1**	**0.2**	**0.3**
Trial 1	0.628	0.624	0.633	0.616
Trial 2	0.623	0.631	0.608	0.613
Trial 3	0.611	0.631	0.648	0.634
Trial 4	0.613	0.630	0.642	0.643
Trial 5	0.622	0.646	0.618	0.625
Trial 6	0.610			
Average	**0.618**	**0.632**	**0.630**	**0.626**
STDEV	**0.007**	**0.007**	**0.015**	**0.011**

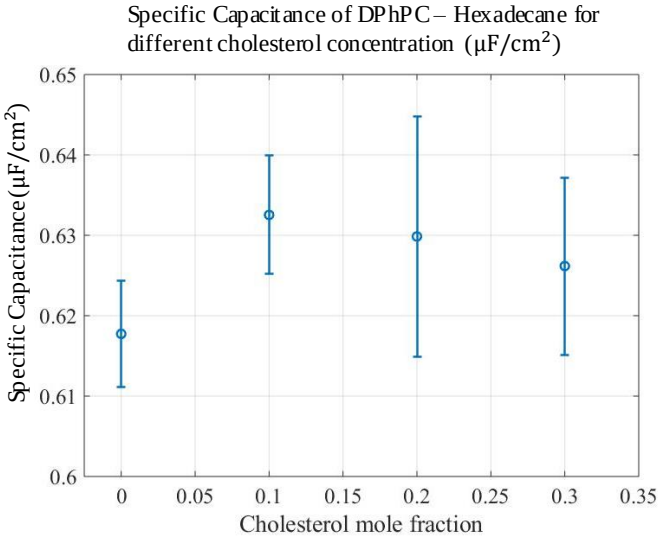

Figure 3: Mean and standard deviation of the membrane's specific capacitance (μF/cm²) with hexadecane for different cholesterol mole fractions. These values correspond to the last two rows of Table 2.

Figure 2 shows a few examples of the linear fit regression method used to calculate the specific capacitance for each oil-lipids combination. As discussed in the manuscript, the specific capacitance was obtained by plotting the membrane's total capacitance versus its area [2]. The latter was modified manually by pulling the micromanipulators slightly apart for a few times - 3 to 4 steps, depending on the membrane's initial size - until the droplets are separated completely. Thus, each experiment consisted of 3 to 4 data points that were fit into a straight line passing through the origin. Any linear fit that showed an R^2 value of less than 0.97 was assumed problematic with compensating the electrode capacitance and the experiment was repeated. The slope unit (pF/mm²) was adjusted to the conventional unit of μF/cm².

Table 2 shows all the values used to obtain the average and the standard deviations, which are plotted in Figure 3.

S.5. Membrane Area Ellipticity

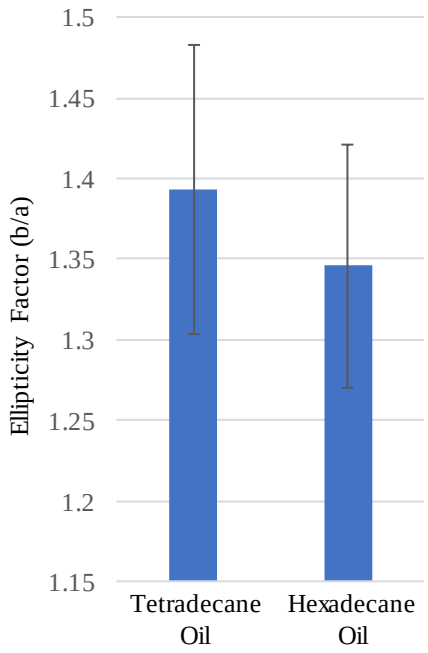

Figure 4: Ellipticity factor – ratio of the major radius of membrane area to the minor one – for tetradecane oil (ρ = 764 kg/m³) as well as for hexadecane oil (ρ = 773 kg/m³). The average value shown is the mean of the ellipticity factor when the droplets are at rest – no electrical field applied.

The droplets used in this study were approximately 250 nL in volume (800 μm in diameter). The difference in densities between the aqueous droplet and the oil medium as well as the reduction in surface tension enhanced by surfactants are two primary causes for the droplet distortion from a spherical shape. The ellipticity factor was calculated as the ratio of the major radius of the membrane area to the minor one. For hexadecane oil (773 kg/m³), the ellipticity factor is 1.35 (± 0.075), whereas tetradecane (764 kg/m³) showed a slightly higher value of 1.39 (± 0.089).

S.6 Contact Angle Measurements

Contact angle measurements used in this study were obtained using from the inverted microscope and the findcircles() algorithm in MATLAB. The angle of contact is defined at the monolayer-bilayer-monolayer intersection. When viewed from the side the droplets are distorted due to gravitational influences, and extracting the exact contact angle is prohibited by the resolution of

the camera and artifacts from lighting, where the angle must be defined by some point on the droplet surface and the selection of this point greatly influences the measurement. These difficulties are alleviated when using the inverted microscope view since the entire droplet contour may be used to determine exact intersection points and calculate the angle of contact accordingly. This measured angle of contact is assumed to reflect the true angle of contact for the side view camera and remain constant all around the bilayer perimeter as necessary for equilibrium of the areas assuming constant interfacial tension in each interface.

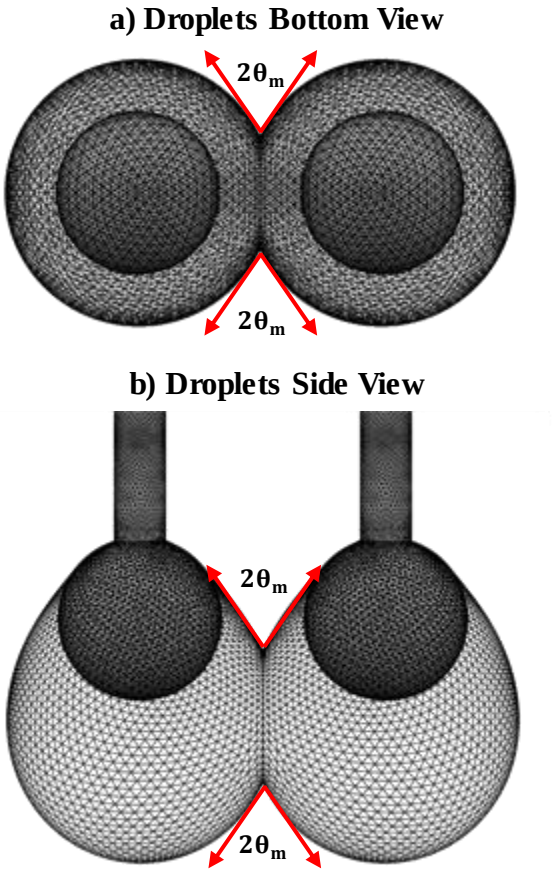

Figure 5: Surface Evolver bottom and side view images of a DIB at steady state, where the droplets are suspended from electrodes. Values for the monolayer and bilayer surface tensions as well as for the specific density were obtained assuming hexadecane oil and only DPhPC lipids – no cholesterol. Comparing the bottom and side view, one can see how the contact angle at the annulus is consistent all over the ring. The angle between the two red arrows is the same as measured geometrically.

To illustrate this, surface evolver was used to generate the predicted steady state dimensions for the DPhPC and Hexadecane case – no cholesterol. Here the surface is discretized into individual facets, reducing the immediate changes at the monolayer-bilayer-monolayer intersection. As seen in Figure 5, the upper and lower contact angles from the side and bottom views are exactly the same. The angle between the two red arrows is the same measured angle. The principle of least total energy requires the droplets to be separated by the same contact angle all over the elliptical circumference of the symmetric bilayer.

S.7 Lipids-in-Oil Monolayer Surface Tension Measurements

Initial experiments for this project were conducted with lipids dispersed in the oil phase rather than in the aqueous solution. These results are not included in the study, but it is of interest to show the dissimilar way cholesterol affects the monolayer surface tension depending on the phase it is dispersed in.

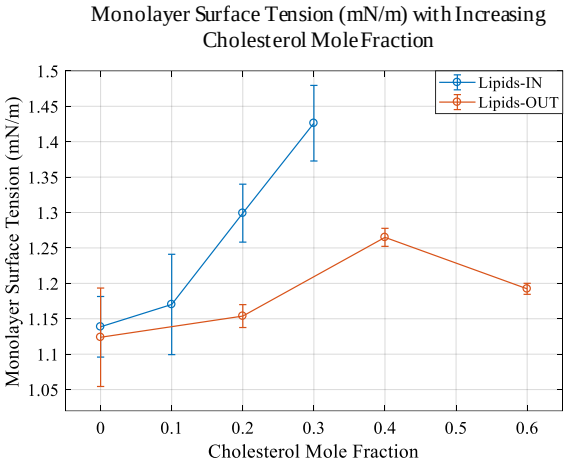

Figure 6: Monolayer surface tension (mN/m) with varying cholesterol mole fraction. In both cases, cholesterol is mixed with DPhPC phospholipids. In the lipids-in scenario, hexadecane oil was used. Whereas in the lipids-out scenario a 1:1 mixture of hexadecane and silicone oil was used.

Figure 6 shows the monolayer surface tension with varying cholesterol mole fractions with respect to DPhPC. In this case, the value is compared between lipids-in scenario – lipids are dispersed in the aqueous phase – and lipids-out scenario – lipids are dispersed in the oil phase. Note that the oil phase is not the same in these two cases (hexadecane for lipids-in; 1:1 hexadecane:silicone oil AR20 for lipids-out) which produces a difference even without cholesterol, but we are more interested in comparing the influence of cholesterol. As observed in the figure, cholesterol's effect on the surface tension is much more significant when lipids are dispersed in the aqueous phase. In fact, increasing the cholesterol from 0% to 20% increases the tension by 2.6% when dispersed in the oil phase compared to 14.1% in the aqueous phase. This behavior can be explained by how the solutions are prepared. For the lipids-out cases, both cholesterol and the lipids are dispersed directly into the oil solution and sonicated, enabling the formation of individual micelles. For lipids-in, the lipids and cholesterol are dispersed in chloroform, evaporated, and hydrated and filtered/sonicated generating unilamellar liposomes. The lipids-out approach does not guarantee that the interfacial composition matches the composition dispersed within the oil, while the lipids-in approach does. This is the reason the lipids in water technique was adopted in all of the experiments.

References

1. Venkatesan, G.A., et al., *Adsorption Kinetics Dictate Monolayer Self-Assembly for Both Lipid-In and Lipid-Out Approaches to Droplet Interface Bilayer Formation.* Langmuir, 2015. **31**(47): p. 12883-93.
2. Taylor, G.J., et al., *Direct in situ measurement of specific capacitance, monolayer tension, and bilayer tension in a droplet interface bilayer.* Soft Matter, 2015.
3. Huang, J., J.T. Buboltz, and G.W. Feigenson, *Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 1999. **1417**(1): p. 89-100.
4. Hung, W.-C., et al., *The condensing effect of cholesterol in lipid bilayers.* Biophysical journal, 2007. **92**(11): p. 3960-3967.
5. Parker, A., et al., *Lateral distribution of cholesterol in dioleoylphosphatidylcholine lipid bilayers: cholesterol-phospholipid interactions at high cholesterol limit.* Biophysical journal, 2004. **86**(3): p. 1532-1544.
6. Gross, L.C., et al., *Determining membrane capacitance by dynamic control of droplet interface bilayer area.* Langmuir, 2011. **27**(23): p. 14335-14342.
7. Tarun, O.B., M.Y. Eremchev, and S. Roke, *Interaction of Oil and Lipids in Freestanding Lipid Bilayer Membranes Studied with Label-Free High-Throughput Wide-Field Second-Harmonic Microscopy*. Langmuir, 2018. **34**(38): p. 11305-11310.
8. Berry, J.D., et al., *Measurement of surface and interfacial tension using pendant drop tensiometry*. Journal of Colloid and Interface Science, 2015.
9. Lin, S.-Y., et al., *An examination on the accuracy of interfacial tension measurement from pendant drop profiles.* Langmuir, 1995. **11**(10): p. 4159-4166.