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Direct in situ measurement of specific capacitance, monolayer tension, and bilayer tension in a droplet interface bilayer

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Thickness and tension are important physical parameters of model cell membranes. However, traditional methods to measure these quantities require multiple experiments using separate equipment. This work introduces a new multi-step procedure for directly accessing in situ multiple physical properties of droplet interface bilayers (DIB), including specific capacitance (related to thickness), lipid monolayer tension in the Plateau-Gibbs border, and bilayer tension. The procedure employs a combination of mechanical manipulation of bilayer area followed by electrowetting of the capacitive interface to examine the sensitivities of bilayer capacitance to area and contact angle to voltage, respectively. These data allow for determining the specific capacitance of the membrane and surface tension of the lipid monolayer, which are then used to compute bilayer thickness and tension, respectively. The use of DIBs affords accurate optical imaging of the connected droplets in addition to electrical measurements of bilayer capacitance, and it allows for reversibly varying bilayer area. After validating the accuracy of the technique with diphytanoyl phosphatidylcholine (DPhPC) DIBs in hexadecane, the method is applied herein to quantify separately the effects on membrane thickness and tension caused by varying the solvent in which the DIB is formed and introducing cholesterol into the bilayer. Because the technique relies only on capacitance measurements and optical images to determine both thickness and tension, this approach is specifically well-suited for studying the effects of peptides, biomolecules, natural and synthetic nanoparticles, and other species that accumulate within membranes without altering bilayer conductance.

Introduction

Cell membranes are soft materials that play critical roles in physiological processes both by acting as selectively permeable barriers and by providing a two-dimensional, liquid crystalline bilayer in which transmembrane proteins are anchored. The membrane is involved in physiological processes ranging from homeostasis to vesicle trafficking and many other forms of cellular signaling. For these reasons, methods to quantify physical properties of membranes provide direct insight into how their structures impact their functions.

Further, there is increasing evidence that biomacromolecules, cell-penetrating peptides, and nanoparticles affect the packing and conformations of lipids in the membrane. For example, the bulk hydrophobic region of cholesterol is known to affect the structural order and fluidity of phospholipid bilayers by interdigitating between the acyl chains of neighboring lipids. And because the interactions between lipid bilayers and cholesterol, transmembrane proteins, and membrane active pharmaceuticals can alter the tension of the membrane, methods for quantifying membrane tension can be applied to study the uptake and accumulation of a variety of important species into lipid bilayers.

Motivated by many of the same reasons described above, scientific literature has produced several methods for measuring tension in synthetic model membranes known as lipid bilayers or black lipid membranes (BLM). In many of these studies, the lipid bilayer was formed by spreading a lipid-oil mixture across a small aperture in a hydrophobic solid support submerged in water. As illustrated in Fig. 1A, the suspended BLM consists of the thinned bilayer region of the painted film, which is stabilized at its perimeter by an annulus of excess solvent. It is well known that the bilayer region and the annulus reach mechanical equilibrium through a balance of surface tensions, obeying Young’s equation:

$$\gamma_n = 2\gamma_m \cos \theta.$$  

Equation 1

Specifically, the interfacial bilayer tension ($\gamma_n$) is balanced by the two lipid monolayer tensions ($\gamma_m$) at the annulus-water interface oriented away from the plane of the membrane by the contact angle ($\theta$). Knowledge of the contact angle and monolayer tension allows for direct calculation of bilayer...
cholesterol, BLM tension, which they used to study the effects of Petelska, et al presented a different approach for measuring free energy of formation.

Petelska's method of bilayer tension measurement involves forming a planar bilayer and applying a differential hydrostatic pressure across the membrane, such that the Young-Laplace equation \((\Delta P = 2\gamma_b/R)\) could be used to determine bilayer tension from the radius of curvature of the bulging bilayer. The Young-Laplace equation relates the pressure differential to the radius and tension of the bilayer only. Thus, one drawback of this technique is that it does not allow for direct determination of contact angle, monolayer tension, or free energy of formation.

It is well known that applying voltage across a BLM affects the equilibrium interfacial geometry of the suspended film through a process known as electrowetting. Specifically, an applied electric field increases the external contact angle at the annulus, which drives a subsequent increase in area of the bilayer. The relationship between the contact angle and the applied electric field is described by the Young-Lippmann equation that relates the change in contact angle at the edge of the bilayer to the specific capacitance of the membrane and monolayer tension. When the membrane specific capacitance and monolayer tension are themselves not functions of voltage, the change in contact angle is described by

\[
\cos \theta_0 - \cos \theta_V = \frac{C_M V^2}{4\gamma_m}. \tag{Equation 3}
\]

Equation 3 includes the contact angle measured at a nonzero applied voltage \((\theta_V)\), the zero-volt contact angle \((\theta_0)\), the capacitance per unit area of the membrane \((C_M)\), the monolayer surface tension, and the voltage applied across the membrane \((V)\). Equation 3 shows that if \(C_M\) is known, \(\gamma_m\) can be determined experimentally by measuring the voltage dependent change in the cosine of the contact angle. Requena and Haydon were the first to verify that this relationship could provide access to \(\gamma_m\), though they suggested that either \(C_M\) or \(\gamma_m\) would be known ahead of time from a separate experiment. Their work also highlighted the difficulty in determining an accurate value for the area of a suspended BLM, which is required to precisely determine \(C_M\).

Consequently, rather than using Equation 3 to extract monolayer or bilayer tensions, they used this relationship and separate measurements of \(\gamma_m\) as a way to determine \(C_M\) from the electrowetting response. This approach contrasts the more commonly used discrete measurements of electrical capacitance and bilayer area to determine specific capacitance. Yet, if \(C_M\) and \(\gamma_m\) can be measured in situ, then the Young-Lippmann relationship and Young's equation show that \(\gamma_m, \gamma_b, \) and the free energy of bilayer formation can be comprehensively and simultaneously determined.

The droplet interface bilayer (DIB) method is an elegant technique for assembling planar lipid bilayers to study membrane properties and membrane protein activity. Droplet interface bilayers form spontaneously between lipid-coated aqueous volumes immersed in oil. DIBs offer several advantages to other methods for bilayer formation, including long lifetimes (hours-days), low-volume, tunable bilayer

Figure 1. Common model membranes include the black lipid membrane (A) and the droplet interface bilayer (B). In both cases, the tension of the lipid bilayer that forms is in equilibrium with the vertical sum of the two opposing monolayer tensions.

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control over the composition of each leaflet and of each droplet,\textsuperscript{57} and potential for scale-up by forming multi-membrane networks with many droplets.\textsuperscript{47-53} DIBs have been successfully used as soft functional building blocks for bottom-up synthetic biology, for example in the construction of tissue-like materials\textsuperscript{48} and in the construction of spatially arranged artificial cells.\textsuperscript{60, 61} As illustrated in Fig. 1B, a DIB is energetically balanced by lipid monolayer tensions that oppose bilayer tension in the same manner as a suspended BLM. DIBs also possess the appropriate thickness and amphiphilic, two-dimensional liquid crystalline structure to reconstitute transmembrane proteins and peptides while retaining their natural function.\textsuperscript{58, 62, 63} However, unlike suspended BLMs, the DIB allows for optically tracking both the contact angle and the interfacial area between droplets, in addition to permitting electrical measurements of membrane capacitance.

Thus, we propose that DIBs could be employed to study the effects of proteins, cell-penetrating peptides, and other biomolecules and lipophilic species on bilayer capacitance, tension, and free energy of formation by combining methods for determining membrane specific capacitance with a technique to measure contact angle. Using a coupled approach, we demonstrate that both monolayer and bilayer tensions can be measured in situ by tracking changes in $C_{ML}$ and $\theta$ with a droplet interface bilayer (DIB) at varying bias voltages. The specific advantages of DIBs for this type of experiment include: 1) control of droplet positions relative to one another allows for direct tuning of the area of the interface,\textsuperscript{58, 59} which allows for accurate determination of $C_{ML}$,\textsuperscript{27, 58} and 2) simple optical imaging of the adjoined pair can be used to determine both membrane area and $\theta$ across a range of applied electric fields.\textsuperscript{22-24}

In the following sections, we demonstrate the accuracy and sensitivity of a multi-step tuning technique that allows for measurement of monolayer and bilayer tension in a DIB. First, mechanical tuning of the interfacial area is used to determine specific membrane capacitance. Then, an electrical tuning routine is performed to determine lipid monolayer tension via $C_{ML}$ and $\theta$ values obtained at various applied voltages. Bilayer tension is subsequently determined from values of $\gamma_{ML}$ and $\theta$ using Equation 1 (Young’s equation). In situ measurements of monolayer tension are compared to separate measurements of monolayer tension obtained via the pendant drop method. After confirming that monolayer tensions can be accurately obtained via measurements of $C_{ML}$ and $\theta$, we showcase the utility of this method for measuring changes in membrane capacitance, monolayer tension, and bilayer tension (via the Young’s equation) caused by the addition of cholesterol, known to affect lipid packing and order and, separately, the incorporation of silicone oil into the oil phase surrounding the droplets. The ability to detect cholesterol-induced changes in thickness help validate the method for future use in studies of the effects of other biomolecules, while measurements using different mixtures of oils confirms what is known about size-selectivity of oil retention versus exclusion in a BLM. These oil mixtures also represent commonly used oils in DIB assembly.\textsuperscript{48, 64}

### Materials and methods

#### Materials

Sodium chloride (NaCl), 3-(N-morpholino)propanesulfonic acid (MOPS), sodium hydroxide (NaOH), agarose (A9539), n-hexadecane (99%), AR 20 silicone oil (product number 10836), acetone, and isopropanol alcohol (IPA) are acquired from Sigma Aldrich. Aqueous buffer (pH 7.4, 100 mM NaCl, 10mM MOPS) is prepared as described previously.\textsuperscript{58} 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) and cholesterol (ovine wool, >98%) are acquired as lyophilized powders from Avanti Polar Lipids, Inc. and stored at -20°C. To prepare solutions of single unilamellar DPhPC liposomes, lipids are first suspended in aqueous buffer at a concentration of 2 mg/mL before being subjected to 5 freeze/thaw cycles to create stock solutions of multilamellar liposomes. Unilamellar DPhPC liposomes are formed by extruding thawed stock lipid solution through 100 nm-pore polycarbonate membranes (Whatman) using an Avanti Mini Extruder. To create liposomes containing DPhPC and cholesterol, the lyophilized (powder) form of each component is dissolved in chloroform to create separate 5 mg/mL stock solutions. Appropriate volumes of each chloroform stock are mixed to obtain the desired DPhPC:cholesterol molar ratio, and the vial containing the mixture is placed under vacuum for several hours to remove the solvent. The resulting films are rehydrated with buffer to achieve a final DPhPC concentration of 2 mg/mL, incubated between 35-45°C to help dissolution, and then sonicated at 45°C using a bath sonicator (FS200D, Fisher Scientific) for several hours or until the solution is completely clear. Sonication is preferred over extrusion with cholesterol-containing mixtures to prevent removal of cholesterol by the polycarbonate membranes during extrusion. Sonicated and extruded liposome solutions are stored at 4°C until further use. Cholesterol-containing solutions are checked for optical clarity before testing and re-sonicated to clarity if there is any evidence of cholesterol demixing.

#### Methods for DIB formation and characterization

DIBs are formed between two aqueous droplets suspended in the oil-filled reservoir of a transparent PDMS substrate, as described elsewhere.\textsuperscript{58} Briefly, aqueous droplets (300nL unless otherwise stated) are pipetted onto agarose-coated, ball-end silver/silver-chloride electrodes made from 50 µm silver wire (Sigma). Suspended droplets “hang” on the gel-coated electrode tips under oil and are intentionally free of contact with either the upper oil/air interface or the PDMS substrate. Optical clarity is improved by injecting a small volume (<10µL) of hexadecane between the substrate and microscope slide.. Each electrode is affixed to a 3-axis micromanipulator (World Precision Instruments, Kite-L and Kite-R models) to allow precise control over the position of each droplet.
dynamically vary bilayer area when determining specific capacitance, one droplet is moved closer to or further away from the other droplet using the micromanipulator. Current measurements are made using an Axopatch 200B patch clamp amplifier and Digidata 1440 data acquisition system (Molecular Devices). All recordings are made with appropriate shielding in place to reduce noise to less than ±5 pA. Nominal capacitance measurements are based on the bilayer’s current response to a 10 mV, 10 Hz triangular voltage waveform output from an Agilent 33210A waveform generator. Membrane capacitance is extracted from sections of the square-wave current response using MATLAB. To induce electrowetting between droplets, custom dc step voltage routines are employed as described previously. Images of DIBs taken from below through the 4X objective lens of an Olympus IX50 inverted microscope are acquired with a QI Click CCD camera controlled using μManager software. DIB images are post-processed using custom scripts in MATLAB to extract bilayer contact length (used for bilayer area calculation) and contact angle (for measurements of tensions and free energies). Bilayer area calculations account for droplet sagging due to differences in density between the aqueous and oil phases (refer to ESI for details). In every DIB test, we carefully ensure droplets are freely suspended under oil and above the substrate surface to reduce error in our calculation of bilayer ellipticity. Practically, an important aspect of measuring DIB length is that the droplets are positioned at the same height such that the waist of each droplet is in focus when viewed from the bottom-up. The height of each droplet is adjusted before specific capacitance and tension measurements to maintain appropriate focus.

Pendant drop measurements of monolayer tension
Interfacial tension of lipid and lipid-cholesterol monolayers formed at various oil-water interfaces are measured via the pendant drop method with a Model 590 goniometer and DROPimage Advanced software (Ramé-Hart Instrument Co.). The method involves forming a pendant drop from one of the liquid phases (i.e. aqueous solution) at the tip of a needle submerged in the other phase (i.e. oil). A horizontally mounted camera acquires images of the droplet profile at a frequency of 1 Hz, which are used by the software to compute the surface tension versus time. Refer to the ESI for additional details regarding pendant drop measurements.

Results and discussion
I. Specific capacitance, monolayer tension, and bilayer tension measurement with a DIB
Fig. 2A illustrates the three-part process for measuring $C_m$, $\gamma_m$, and $\gamma_o$ on a DIB. In Part 1, a DIB is formed between two aqueous droplets suspended on agarose tipped ball-ended electrodes in oil. The initial thinning of the bilayer occurs generally within 1-2 minutes of initial contact and results in a rapid increase in the amplitude of the capacitive current signal.

![Figure 2](image-url)
due to the formation of a thinned bilayer region between droplets. The area of the thinned region and the angle between droplets stabilize to constant values when the total energy of the connected droplets reaches a local minimum.\textsuperscript{48}

Part 2 of the process involves incrementally changing the bilayer area, and thus the nominal capacitance of the interface, by moving one electrode in a stepwise fashion relative to the other. As others have shown,\textsuperscript{27} returning the mobile electrode to its original position results in a reversible change in the capacitive current and bilayer area. The size of the bilayer at each position stabilizes within about 30 s of the electrode positioning. At each level of contact area (typically >5 contact areas are prescribed), the steady-state amplitude of the square-waveform current signal is recorded and an image of the DIB is acquired simultaneously. Nominal capacitance is computed using $C = i/(4Af)$, where $i$ is the amplitude of the current waveform measured at steady state and $A$ and $f$ are the amplitude and frequency, respectively, of the applied triangular waveform voltage. Specific capacitance is obtained simply from the slope of a linear least squares regression of nominal capacitance versus area data from all electrode positions (Fig. 2A, center).\textsuperscript{27, 56}

Part 3 requires an applied dc transmembrane voltage that increases in a stepwise fashion in addition to the ac triangular voltage required for capacitance measurement. This procedure begins by fixing the electrode (i.e. droplet) positions, setting the bias voltage to zero, and allowing the DIB to equilibrate for several minutes to ensure the droplets reach a steady contact area and angle. Images are taken at the 0 mV equilibrium point to allow measurement of $\theta_0$ before voltage is increased in a stepwise fashion. The bilayer is held at each new voltage for 15-30 seconds, during which electrowetting causes the external bilayer contact angle to increase as predicted by the Equation 3 (Young-Lippmann equation). Images ($n \geq 10$) of the contact angle ($\theta_v$) are obtained at the end of each voltage step and the process is repeated at successively higher voltage levels. This part of the experiment takes only a few minutes to perform. Using the average contact angle from each voltage level, the change in the cosine of the contact angle is plotted versus the square of the applied bias voltage (Fig. 2A, right). As noted above with Equation 3, the change in the cosine of the contact angle is recorded with respect to the cosine of $\theta_0$.

Fig. 2B shows the square current waveform induced by the ac triangular voltage applied to a DPhPC DIB formed in hexadecane during all three parts of the experiment. Fig. 2C shows the corresponding bilayer capacitance, computed from the same current response, versus time. During Part 1 of the experiment, the amplitude of square current waveform stabilizes at ~240 pA. Images of the connected droplet pair show that $\theta_0$ is 29.3° for this type of lipid and oil. The second and third parts of the measurement process result in stepwise changes to bilayer capacitance, caused by mechanical and electrical tuning, respectively. During Part 2, the measurement shows that at each successive electrode...
position (i.e. farther apart), the square wave current stabilizes to smaller amplitude, which corresponds to smaller area of contact and thus capacitance. Conversely, the application of an increasing dc bias (of either polarity) drives the square wave current and bilayer capacitance to successively higher levels in Part 3.

The micrographs of a DPhPC DIB in hexadecane in Fig. 3A show that increasing levels of electrowetting lead to increases in the external contact angle, \( \theta \), as well as the projected length of the interface, \( a \). Fig. 3B presents the capacitance versus area data obtained in Part 2 of the same test on this DIB, and the change in the cosine of the contact angle versus voltage data from Part 3 are shown in graphical form in Fig. 3C. Typically, tests to measure \( \gamma_m \) conclude when the bilayer ruptures at voltages between [200–350] mV. In this test, \( \theta \) increased from 28.8° at 0 mV to 35.9° at +225 mV. The change in cosine of the contact angle is well represented by a linear least squares regression with respect to \( V^2 \) across the range from 0 to 225 mV with an R-squared value >0.98. From Equation 3, it can be seen that the slope of the regression (\( m \)) is related to both \( C_M \) and \( \gamma_m \), as given by

\[
 m = \frac{C_M}{4\gamma_m} \quad \text{Equation 4}
\]

Equation 4 is rearranged to solve for monolayer tension using the value of \( C_M \) obtained in Part 2 and the value of \( m \) obtained in Part 3. The specific data shown in Fig. 3C yield a slope of \( m=1.372 \) which, combined with the \( C_M \) value (slope from Fig. 3B) of 0.673 μF/cm², yields a computed value of monolayer tension, \( \gamma_m \), equal to 1.23 mN/m. For a group of eight DPhPC DIBs formed in hexadecane, this method results in an average (± one standard deviation) monolayer tension of 1.18±0.136 mN/m, which is in excellent agreement with prior results²⁵ and the results of our own independent pendant drop measurements of DPhPC monolayer tension (1.19±0.067 mN/m, \( n=3 \)). A student t-test confirms that there is no significant difference (p<0.005) between the values obtained with either tension measurement method, which serves to validate an approach that leverages the electrowetting response of DIBs to determine the tension state. In summary, the measurement method introduced herein is capable of determining lipid monolayer tensions at the surfaces of the droplets that are consistent with those obtained using accepted techniques such as pendant drop goniometry.

After computing monolayer tension, the bilayer tension at zero volts is readily computed for each trial using Young’s equation (Equation 1) along with the measured contact angle at 0 mV.¹⁸,²¹,⁶⁶ For the example shown in Fig. 3, the calculated monolayer tension (1.197 mN/m) and zero-volt contact angle of 28.7° results in a calculated value for \( \gamma_b \) of 2.10 mN/m. For the pool of eight different DPhPC DIBs in hexadecane that are tested, we obtain an average value of 2.04±0.222 mN/m for the tension of DPhPC bilayers in hexadecane. Prior studies obtained values of 1.62 mN/m and 1.9±0.3 mN/m for planar lecithin bilayers¹⁹,⁶⁷ and DPhPC DIBs,²⁵ respectively. The latter value, provided by Dixit et al., was obtained using Equation 1 along with the monolayer tension determined via independent goniometer measurements and the contact angle estimated using images of connected droplets. It is possible to calculate bilayer tension via Equation 1 using the data herein: the average value of monolayer tension obtained from the goniometer and the average contact angle in DIB measurements (29.3°, see Table 1) yields an estimate of 2.08±0.198 mN/m for bilayer tension. All of these results are in strong agreement with one another, and there is no significant difference (p<0.005) between bilayer tensions computed from monolayer tensions obtained via the pendant drop and DIB methods.

It is also possible to use Equation 1 to discretely compute bilayer tension as a function of bias voltage using the measured values of \( \gamma_m \) (fixed) and DIB contact angle at each voltage applied during Part 3 of the experiment. Additionally, the average bilayer tension can be empirically projected in a continuous fashion versus voltage using experimentally determined values of \( C_M \gamma_m \), and \( \theta_0 \). This second method evaluates bilayer tension versus voltage using a rearranged form of Equation 3,

\[
\gamma_b(V) = \gamma_{b,0} - \frac{C_M}{2} V^2, \quad \text{Equation 5}
\]

along with the bilayer tension at 0 mV (\( \gamma_{b,0} \)) computed using Equation 1. Fig. 4 compares \( \gamma_b \) as a function of voltage computed discretely and continuously using the values of \( \theta \), \( C_M \gamma_m \), and \( V \) obtained for the data presented in Fig. 3B-C. Clearly, \( \gamma_b \) decreases with increasing voltage, which illustrates that the stored electrical energy across the bilayer acts to mechanically relax the interface. Further, close agreement between the discrete data points and the continuous curve show that the measured contact angles and thus the computed bilayer tension, conform to the Young-Lippmann relationship for bilayer tension at voltages between [0-175] mV. This agreement suggests that bilayer tension can also be computed accurately at a non-zero voltage provided the contact angle is measured at that potential and the monolayer tension is known. Monolayer tension is assumed to be independent of applied potential due to the fact that the

![Figure 4. Bilayer tension (\( \gamma_b \)) as a function of applied voltage (V) for a DIB, calculated using the representative data presented in Fig. 3.](image-url)
thinned bilayer contributes the dominant electrical impedance between droplets. This occurs because of the presence of nonconductive oil in the Plateau-Gibbs border that is much thicker and has a lower capacitance per unit area than the bilayer. As a result, the applied voltage produces an electric field predominantly across the membrane.

For further comparison of the DIB Young-Lippmann equation with prior studies, it is possible to evaluate the reduction in free energy obtained upon droplet adhesion (Equation 2).

For a DPhPC DIB formed in hexadecane, the calculated free energy obtained upon droplet adhesion (Equation 2) is $0.301 \text{ mJ/m}^2$. As a result, the applied voltage produces an electric field predominantly across the membrane.

Uncertainty of capacitance and tension measurements

Error in determining specific capacitance and tensions arises from uncertainties in measuring capacitance and area (for $C_{M}$) and in determining the contact angle (for $\gamma_{m}, \gamma_{b}$), respectively. Nominal capacitance is determined directly via electrical measurement as described previously where it is shown that there is <1.3% error in any C versus A data point used for $C_{M}$ determination.

Therefore, we focus instead on the accuracy and repeatability of bilayer area and contact angle measurements, which are obtained via a two-step process: 1) an image acquisition step, and 2) image processing (MATLAB) to determine the angle and projected length (diameter) of the bilayer. To determine uncertainties introduced from image acquisition, we look at repeatability of measurements taken from 10 images of a DIB at a fixed equilibrium condition. The images are acquired rapidly within a few seconds (10Hz). Estimation of bilayer area begins in MATLAB by extracting the projected diameter of the bilayer from images taken through the inverted microscope objective. These “bottom-view” images are analyzed via a custom image processing routine to calculate the horizontal distance, $a$, between the intersection points of the two droplets and the external half angle (i.e. contact angle), $\theta$, that exists at the waist of the interface (as shown in Fig. 3A). Area estimation requires an assumption regarding the shape of the interface. Dixit, et al outlined the geometrical relationships that govern DIB formation for spherical droplets of identical size in an equally dense oil phase. This analysis predicts a perfectly flat, circular interface. However, the shape of the interface becomes more elliptical when gravitational effects cause the water droplets to sag vertically from the wire-type electrodes in a less dense oil phase (Refer to ESI, Fig. S4). With droplets hanging on electrodes, the minor axis of the elliptical interface ($a$) is the horizontal distance obtained from microscope images whereas the longer major axis ($b$, ESI Fig. S4) is oriented vertically and cannot be determined from a bottom-view image. Analysis of bilayer shape under increasing levels of sagging enables estimation of correct bilayer area using only $a$.

The average and standard deviation of bilayer area and contact angle are calculated from the 10 images acquired with zero applied voltage to assess the repeatability. Furthermore, the average group standard deviation of bilayer area using images acquired from 11 unique DPhPC bilayers (110 images total) is $458 \mu\text{m}^2$. Similarly, contact angles measurements from 14 different DPhPC DIBs (140 images total) provide an average group standard deviation of only $0.763^\circ$. With a typical zero-volt area of $30000 \mu\text{m}^2$ for a DPhPC DIB in hexadecane, the average standard deviation in measured bilayer area corresponds to an error of less than $0.002\%$. For the average contact angle of $29.3^\circ$, the average standard deviation represents an error of 2.6% for the angle measured at a given area step. These results present a lower standard deviation than previously reported for contact angle measurements in DIBs in DIBs in DIBs. The comparison here shows that the automated image processing routine and the use of multiple images at each voltage level provide an order of magnitude lower variation in average angle measurements. Separate analysis for quantifying error propagation within the multi-step technique (refer to ESI for additional details) yields uncertainties of ± 0.32 mN/m and ± 0.51 mN/m in monolayer and bilayer tension measurements, respectively.

Effects of mechanical manipulation on electrowetting and tension measurements

In analyzing images obtained during specific capacitance measurements of DIBs (Fig. 2A, Part 2), we observe that the zero-Volt contact angle at equilibrium is directly affected by mechanical manipulation (i.e. positioning) of the droplets, which is used to intentionally vary the area of the bilayer. To explore this relationship, Fig. 5A-B shows the results of a representative experiment in droplets were successively pulled apart in 3 steps and then sequentially pushed back together in 3 area steps (refer to Fig. S7 in the ESI for sequential presentation of area and angle data). The data in Fig. 5B shows that the contact angle reversibly increases by as much as $5-6^\circ$ as 300 nL droplets are pushed together to yield a larger interfacial area. To further test this response, Fig. 5C shows zero-Volt contact angle versus bilayer area obtained from $n = 115$ various measurements made via Part 2 of the method on 13 different DPhPC DIBs. The larger population of data confirms that contact angle increases with mechanically driven increases in bilayer area. While the precise mechanism for this change in angle remains undetermined, the data suggest that pushing or pulling the droplets in a direction perpendicular to the interface reversibly alters the equilibrium tension balance that establishes the contact angle.
The fact that changing the electrode separation distance alters droplet contact angle begs the question: how much does mechanical manipulation required for Part 2 affect the accuracy of the electrowetting-based method in Part 3 for determining monolayer tension in a DIB? To examine this possibility, we consider the role of the zero-Volt starting contact angle, which was shown in Fig. 5A-B to be a function of droplet positioning. Based on Equation 3, tuning or altering the initial wetting condition \( \theta_0 \) should not affect the magnitude of the change in the cosine of the contact angle across a range of voltage assuming fixed \( C_M \) and \( \gamma_m \). However, the nominal change in contact angle does depend on \( \theta_0 \) since the cosine of an angle varies nonlinearly. Such behavior is observed experimentally as portrayed in Fig. 5D and Fig. 5E, which show the theoretical and experimentally observed values for \( \cos(\theta_0) - \cos(\theta_V) \) and \( \theta_V \), respectively, with increasing applied voltage. The data portrayed is from two different electrowetting trials where the zero-Volt contact angle is varied (\( \theta_0 = 29^\circ \) and \( \theta_0 = 25^\circ \)) by mechanical manipulation between trials. The data in Fig. 5D provides slope values of \( m = 1.548 \) and \( m = 1.579 \). With a specific capacitance value of 0.652 \( \mu F/cm^2 \), the resulting calculated monolayer tensions are 1.05/1.03 mN/m for \( \theta_0 = 29^\circ/25^\circ \) and the Young equation is used to calculate bilayer tension of 1.84/1.87 mN/m. The variation in \( \theta_0 \) causes <2% change to the slope, monolayer tension, and bilayer tension. Thus while mechanical manipulation can affect the zero-Volt contact angle, it does not significantly affect the ability to correctly determine monolayer and bilayer tension in the electrowetting-based method used in Part 3.

While varying \( \theta_0 \) does not affect the magnitude of the change in the cosine of the contact angle, the magnitude of the nominal voltage-dependent change in angle is expected to increase with decreasing zero-Volt contact angle due to the nonlinearity of the cosine function (Refer to ESI, Fig S8). This behavior is seen with both predicted and experimentally observed values for \( \theta_0 \), shown in Fig. 5E, which show that contact angles for DIBs starting at different contact angles appear be on converging trajectories at increasing voltage. These data also confirm that DIB contact angle follows the Young-Lippmann equation up to 175 mV, regardless of starting angle. At higher voltages, the data obtained from the DIB with \( \theta_0 = 29^\circ \) indicates that the change in the contact angle saturates. The saturation issue will be discussed later, but the immediate point to be made is that the lower initial \( \theta_0 \) incurs a larger nominal angle change as voltage increases. Thus, while a given amount of electrical energy (for fixed values of \( V, C_M \) and \( \gamma_m \)) produces a fixed change in the cosine of the contact angle, the corresponding change in the nominal value of \( \theta \) is greater when starting from a lower \( \theta_0 \) (closer to unity on the cosine curve, refer to ESI Fig. S9). Because we demonstrated that our method for measuring monolayer and bilayer tensions in a DIB is limited most by the resolution of contact angle measurement, the nonlinear relationship between angle change and applied voltage suggests that achieving a small \( \theta_0 \) (e.g. via mechanical manipulation or even through oil selection) could be used to maximize the nominal change in contact angle and improve the accuracy of this technique. This approach is also common to other electrowetting-based...
applications, including single droplet electrowetting on dielectric (EWOD) experiments, where tuning the initial wetting condition to make $\theta_0$ small can be used to enhance angle change in response to voltage.\textsuperscript{68}

Nonetheless, it is necessary to remember that the nominal contact angle is used in the Young Equation to compute bilayer tension. Thus, while the two membranes characterized in Fig. SD-E exhibited bilayer tensions within 2% of each other, the amplitude of error in determining bilayer tension also varies nonlinearly with starting contact angle. Thus, it is advisable to initiate Part 3 of the method at a location where the droplets are minimally deformed by electrode separation in order to reduce the effects of mechanical manipulation on the accuracy of determining bilayer tension.

For completeness, we also investigated the effect of starting contact angle on the voltage-induced change in membrane capacitance (Refer to ESI, Fig. S10). This analysis showed that the sensitivities of nominal bilayer capacitance and area to voltage are affected by the starting angle. However, the linear relationship between capacitance and area (Fig. 3B), and thus the determination of $C_M$, holds across a wide range of areas obtained via mechanical manipulation as we\textsuperscript{56} and others\textsuperscript{27} have shown previously. In a following section we examine if specific capacitance varies under an applied dc voltage.

**Limits of electrowetting in DPhPC DIBs**

Numerous studies of electrowetting have shown that the change in the wetting angle saturates at high voltage, falling short of the expected angle change predicted by the Young-Lippmann equation.\textsuperscript{58-71} With a single sessile droplet placed on a dielectric, common in many EWOD systems, the saturation limit is believed to be the result of dielectric charging or breakdown, charging of the insulating fluid surrounding the droplet, formation of instabilities and/or microdroplet ejection, or reduction of interfacial tension to zero.\textsuperscript{69} The zero-tension theory, which states that the maximum angle change occurs at a voltage where the interfacial tension is zero,\textsuperscript{69, 71} is capable of predicting the saturation angle for single droplet EWOD cases.\textsuperscript{69} Applying this concept to a DPhPC DIB, the Young-Lippmann equation predicts that an applied voltage of nearly [800] mV is required to reduce a bilayer tension to zero (calculated using Equation 5 and values of $C_M$ and $\gamma_{b,0}$ for DPhPC DIBs in hexadecane, Table 1). However, because the typical rupture potential of DPhPC DIBs occurs at [200-300] mV,\textsuperscript{56} it is unlikely that saturation of the contact angle could be caused by reducing tension to zero. Rather, the observed contact angle saturation at voltages above [175] mV is likely due dielectric breakdown and electroporation which typically precede bilayer rupture.\textsuperscript{56, 72}

**Effect of increasing voltage on $C_M$**

When using specific capacitance to determine monolayer tensions in Equation 3, it is important to understand how much $C_M$ varies when an electric field is applied to induce changes in the contact angle. The effect of voltage on $C_M$ has been shown previously to follow the experimental relation\textsuperscript{27, 32, 40}

$$C_M = C_{M,0}(1 + BV^2),$$

Equation 7

which involves the specific capacitance at 0 mV ($C_{M,0}$), the magnitude of applied dc bias ($V$), and a parameter ($B$) describing the voltage dependence of $C_M$. While electrostriction of the membrane is a possible mechanism for voltage affecting $C_M$, prior works have shown that planar lipid bilayers formed in the presence of solvents can exhibit voltage-dependent specific capacitance due to the electric field exerting a compressive force that excludes trapped oil from the membrane, thereby reducing the thickness of the hydrophobic core.\textsuperscript{27, 32, 40} This effect is particularly significant when membranes are formed in the presence of organic solvents with high solubility in the hydrophobic region of the bilayer, and less in “solvent-free” membranes formed in the presence of large-molecule solvents that are more easily excluded from the hydrophobic region.\textsuperscript{18, 73, 74}

Fig. 6 shows values obtained for $C_M$ normalized by $C_{M,0}$ at each voltage step with a DPhPC DIB in hexadecane. Discrete values of $C_M$ at each voltage step are calculated (refer to ESI, Fig. S11) using the capacitance and area at that voltage step (as opposed to dynamically varying area at each voltage step to determine $C_M$). Slight nonzero offset (Fig. S11) in the total measured capacitance is obtained from mechanical tuning and is used to correct the instantaneous calculated $C_M$. From Equation 7, a linear least squares regression of $C_M/C_{M,0}$ as a function of $V^2$ yields a straight line with an intercept of 1 and a slope of 0.27 V$^{-2}$ representing the value for parameter $B$. The resulting value of $B$ indicates that $C_M$ would only increase by 0.01 $\mu$F/cm$^2$ at a voltage of [200] mV, a deviation that is approximately 1.6% of the value of specific capacitance measured at zero volts for DPhPC DIBs in hexadecane (0.67 $\mu$F/cm$^2$). Compared to the larger $B$ values measured for planar bilayers formed with decane as the oil-phase (4 V$^{-2}$),\textsuperscript{71} our result is much lower as expected due to the decreased solubility of hexadecane in the bilayer. Our results are
agreement with previously reported values of $B$ in tests with solvent-free supported bilayers.\textsuperscript{20, 27, 41} As a result, small values of $B$ in solvent-free membranes allow for monolayer and bilayer tensions based on contact angle and specific capacitance to be accurately determined using a value of specific capacitance obtained with zero bias applied. This approach is supported by previous reports that the Young-Lippmann equation accurately predicts the response of lipid bilayers subject to applied voltage when using a constant value for $C_{MM}$.\textsuperscript{20, 27, 75}

II. Application of capacitance and tension measurements

Until now, the multi-part experiment for determining monolayer and bilayer tensions for DPhPC DIBs formed in a mixed solvent phase containing silicone oil (AR 20) and hexadecane. Prior to performing contact angle measurements versus voltage, values of specific capacitance are measured separately for DPhPC DIBs formed in pure hexadecane, in a 1:1 (vol:vol ratio) AR 20:hexadecane mixture, and in a 9:1 mixture of AR 20:hexadecane. Values obtained for $C_M$ (average ± standard deviation) are shown in Fig. 7A as a function of volume percentage of silicone oil. These values are then used in calculations of monolayer tension obtained via Part 3 of the experiment (Fig. 7B) and are combined with measured $\gamma$ values to estimate bilayer tension at 0 mV (Fig. 7C).

First, tests are performed to determine monolayer and bilayer tensions for DPhPC DIBs formed in a mixed solvent phase containing silicone oil (AR 20) and hexadecane. Prior to performing contact angle measurements versus voltage, values of specific capacitance are measured separately for DPhPC DIBs formed in pure hexadecane, in a 1:1 (vol:vol ratio) AR 20:hexadecane mixture, and in a 9:1 mixture of AR 20:hexadecane. Values obtained for $C_M$ (average ± standard deviation) are shown in Fig. 7A as a function of volume percentage of silicone oil. These values are then used in calculations of monolayer tension obtained via Part 3 of the experiment (Fig. 7B) and are combined with measured values of $\theta_0$ for each to estimate bilayer tension at 0 mV (Fig. 7C).

Table 1 presents average values, standard deviations, and the number of trials included for each case tested, as well as for DPhPC DIBs assembled in decane.

It is well established that $C_M$ varies strongly with the amount of solvent in the hydrophobic region.\textsuperscript{27, 32, 73, 74} The solubility of solvent in the bilayer region generally increases with decreasing solvent molecule size.\textsuperscript{18, 73, 74} As a result, techniques for measuring the thickness of the hydrophobic region are used to probe the amount of solvent trapped or dissolved in the bilayer.\textsuperscript{27, 42} $C_M$ is related to the thickness of the hydrophobic region ($L_C$) and permittivity ($\varepsilon_r$) of the hydrophobic region of the bilayer, as well as the permittivity of vacuum ($\varepsilon_0$) by

\[ C_M = \frac{\varepsilon_r \varepsilon_0}{L_C} \quad \text{Equation 8} \]

The minimum value of $C_M$ for the three oil cases tested is obtained for DIBs formed in pure decane (0.488 $\mu$F/cm$^2$), and the result is in close agreement with the value of 0.445 $\mu$F/cm$^2$ obtained elsewhere.\textsuperscript{27} $C_M$ increases by 33% to 0.65 $\mu$F/cm$^2$ upon changing the solvent from decane to hexadecane, and the result is again consistent with previously reported values of 0.64 $\mu$F/cm$^2$.\textsuperscript{27, 76} Compared to DIBs in hexadecane, $C_M$ increases by 3.1% to 0.67 $\mu$F/cm$^2$ for 1:1 AR 20:hexadecane and by 7.7% to 0.70 $\mu$F/cm$^2$ for 9:1 AR 20:hexadecane. The increase in $C_M$ with increasing silicone oil in the oil phase indicates a thinner, more solvent-free bilayer, while the reduced value of $C_M$ in decane indicates a thicker, oil-rich bilayer. Assuming a constant dielectric of $\varepsilon_r = 2.2$ for the hydrocarbon-rich hydrophobic region of the bilayer, our values of $C_M$ yield thickness values of 29.9, 29.2, and 27.8 Å for DPhPC DIBs in 1:0, 1:1, and 9:1 mixtures of silicone oil:hexadecane, respectively. The 9:1 mixture thus results in bilayers that are 7.3% thinner than those in pure hexadecane. Gross et al. reported that the hydrophobic region of DPhPC-hexadecane DIBs is 10% oil by volume, leading us to believe that with DIBs formed in the 9:1 mixture, the bilayer hydrophobic region consists of only 2.7% (10%-7.3%) solvent by volume. The 1:1 mixture inverts a similar effect, although the thickness reduces by only 2.7% leading to hydrophobic regions containing approximately 7.3% (10%-2.7%) oil by volume. These estimates assume that the changes in volume, based on $C_M$ measurements, are equal to the changes in membrane thickness (i.e. oil distributes evenly across the membrane area and the permittivity is unchanged). Converting percent volume oil to molar volume ratio provides a rough estimate of 6:1 for lipids:hexadecane in the hydrophobic region (refer to ESI for calculation details). The average thickness of DPhPC DIBs in decane is 39.9 Å. Based on the calculated thickness values, DIBs formed in decane are 33.4% thicker than those formed in hexadecane as a result of the increased solubility of small decane molecules in the hydrophobic region.\textsuperscript{27} DPhPC bilayers formed in decane are expected to contain around 43% (10% +33.4%) oil by volume which is in close agreement with the estimate of 38% by Gross and Wallace.\textsuperscript{27} Conversion of the volume ratio of lipid:decane to a molar volume ratio suggests that there are 1.7 decane solvent molecules for every lipid molecule present (refer to ESI).

In addition to thickness, the oil used to form a DIB affects the tension state of a DIB.\textsuperscript{18} Of the oils studied, bilayer tension is highest for DPhPC DIBs formed in decane. The data in Fig. 7 and Table 1 show that the presence of silicone oil has the opposite effect. The average monolayer tension decreases by 17% from 1.18 mN/m in pure hexadecane to 1.03 mN/m in the 1:1 mixture. Meanwhile, bilayer tension decreases by 29% from 2.04 mN/m in hexadecane to 1.54 mN/m in the 1:1 mixture. Note that for 0:1 and 1:1 AR 20:hexadecane mixtures, the monolayer tension values obtained via the multi-step technique introduced herein are not significantly different than the values obtained via pendant drop measurements with a goniometer. Tension values are not calculated for DIBs formed in the 9:1 AR 20:hexadecane mixture, because these bilayers consistently ruptured at voltages near 100mV which prevented us from recording the increases in contact angle as applied bias voltage increases.
The free energy of formation is also a metric that can be used to understand the same phenomenon of oil-exclusion. The free energy of formation is also a metric that can be used to be a valid approach for measuring rich DIBs. Collectively, the DIB-electrowetting method appears which itself has a adhesion. Our results show that more solvent-free bilayers exhibit higher free energies of

Figure 7. A) Values obtained from measurements of specific capacitance for bilayers as a function of the fraction of silicone oil added to the hexadecane oil phase. Error bars show ± one standard deviation. Two lipid compositions are tested in the various oil mixtures: pure DPhPC, and DPhPC containing 20% (mol %) cholesterol. B) "DIB-YL" -monolayer tensions measured by monitoring Young-Lippmann related changes in DIB contact angle as voltage is increased. "PD" - monolayer tensions measured using the pendant drop method with a goniometer. C) Bilayer tensions for each lipid-oil combination, calculated using average observed contact angle (case specific) and the monolayer tension measured via the DIB-YL method.

The average value of \( C_M \) is higher for DPhPC with 20% cholesterol compared to pure DPhPC in both oil cases tested. Assuming the effective dielectric constant of the interface is unchanged by the incorporation of cholesterol \( (\varepsilon_r = 2.2) \), Equation 8 shows that the increase in \( C_M \) must instead be attributed to thinning of the bilayer hydrophobic region. While numerous studies have identified lipid bilayer thickening upon incorporation of cholesterol, the effect depends greatly on the length (relative to cholesterol) and degree of saturation of the lipid being used. For instance, McIntosh reports that bilayers comprised of phospholipids with up to 16-carbon acyl chains are thickened by the introduction of cholesterol, whereas 18-carbon chain bilayers in the solid phase (i.e. \( T < T_m \)) exhibit a reduction in thickness upon incorporation of cholesterol. The effects of cholesterol on bilayer thickness are explained by comparing the hydrophobic lengths of the cholesterol and lipid chains. Similar biphasic behavior regarding the effects of cholesterol on lipid transition temperature is also attributed to hydrophobic mismatch. The cholesterol-induced decrease in thickness (Fig. 7A) measured herein suggests that a length mismatch exists between the DPhPC acyl chains and the embedded cholesterol. As encountered by McIntosh with 18-carbon acyl chains, the mismatch between rigid DPhPC tails and cholesterol hydrophobic lengths may create voids near the bilayer midplane that prompt the free ends of the acyl chains to bend or kink around embedded cholesterol molecules in an attempt to fill this space. The result is a net shortening of the lipid length and thinning of the bilayer hydrophobic region. This effect is also supported by molecular dynamics simulations showing flexible lipid acyl chains packing tightly around cholesterol molecules.

The fact that \( C_M \) increases with the addition of cholesterol in both pure hexadecane and the 1:1 AR20:hexadecane mixture shows that DPhPC bilayers containing cholesterol are thinner
than those without cholesterol in each solvent case. Equation 8 allows calculation of bilayer thickness, again using \( \varepsilon_r = 2.2 \) as the dielectric constant, which reveals that bilayers containing 20\% cholesterol possess a hydrophobic thickness of 29.7 Å or 27.3 Å when droplets are submerged in hexadecane or 1:1 AR20:hexadecane, respectively. Cholesterol reduces the thickness of bilayers in hexadecane by 0.14 Å (<0.5% of nominal value without cholesterol), however when DIBs are formed in a 1:1 AR20:hexadecane oil phase, cholesterol decreases bilayer thickness to 1.88 Å (a 6.4\% reduction). This finding is consistent with prior studies that observed increased sensitivity of membrane specific capacitance to small molecules when bilayers are more solvent free, i.e. using solvents that are well-excluded from the hydrophobic region.\(^9\),\(^12\).

Additionally, our technique allows us to determine that doping a DPhPC bilayer with cholesterol results in an increase in both monolayer and bilayer tension. This result is consistent with the observations of previous studies\(^{67, 81}\) and is explained by the fact that cholesterol interdigitation decreases packing in gel-phase bilayers,\(^{81}\) such as those formed from DPhPC, which would drive increased interaction between water and the solvent or hydrophobic region of the bilayer. Incorporation of 20\% cholesterol increases monolayer and bilayer tension by 0.24 mN/m and 0.21 mN/m in pure hexadecane and 1:1 AR20:hexadecane, respectively. The nominal changes to monolayer tension represent a 20.3\% spike, while bilayer tension changes by 22.6\% or 24.7\% in hexadecane or the 1:1 AR20:hexadecane mixture. As with specific capacitance described above, bilayer tension is more significantly affected by cholesterol when the membrane is solvent-free.

Free energy of adhesion (\( \Delta F \), Equation 6) also increases nominally through the incorporation of cholesterol into the bilayer. Raising cholesterol content to 20\% increases \( \Delta F \) of DPhPC DIBs formed in hexadecane by 0.010 mN/m (+3.3\%). For DPhPC DIBs formed in the 1:1 AR20:hexadecane mixture, inclusion of 20\% cholesterol causes \( \Delta F \) to increase by +0.028 mN/m (+5.4\%). It is interesting to consider the mechanism by which cholesterol increases free energy of adhesion. Evidenced by the equation for free energy (Equation 2), \( \Delta F \) increases with increases in either tension or contact angle. Our measurements demonstrate the cholesterol increases bilayer and monolayer tensions while causing the contact angle to decrease. To achieve such changes in all three parameters, Equation 1 verifies that the ratio \( \gamma_m/2\gamma_m \) must in fact increase in response to the addition of cholesterol. Based on the experimentally obtained values in Table 1, increasing cholesterol content is accompanied by an increase in the ratio of \( \gamma_m/2\gamma_m \) (0.853 and 0.880 at 0 and 20\% cholesterol, respectively). The data here suggests that cholesterol causes less distortion of lipids positioned in the monolayers around the droplets compared to lipids in the bilayer interface, possibly due to the presence of bulk solvent molecules near the monolayers that fill voids between the sterol and lipid molecules. These results simultaneously support the notion that cholesterol affects bilayer tension more than monolayer tension through the same mechanism that results in membrane thinning: i.e. kinking and bending of the lipid acyl chains to “wrap” cholesterol molecules and fill sterol-induced voids in the hydrophobic region.

Collectively, results from our measurements of \( C_M, \gamma_m, \gamma_b \), and \( \Delta F \) confirm that the multi-step process is suitably capable for measuring changes to bilayer thickness and tension caused by

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Table 1. Values obtained for DIBs formed at 25°C.

<table>
<thead>
<tr>
<th>Lipid Type</th>
<th>( C_M ) [( \mu F/cm^2 )]</th>
<th>( D_C ) [Å]</th>
<th>( \gamma_m ) [mN/m]</th>
<th>( \gamma_b ) [mN/m]</th>
<th>( \theta_{adv} ) [°]</th>
<th>( \Delta F ) [mJ/m^2]</th>
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<td></td>
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<tr>
<td>DPhPC</td>
<td>0.667 (0.022)</td>
<td>1.03 (0.115)</td>
<td>1.01 (0.041)</td>
<td>1.54 (0.198)</td>
<td>41.63 (2.21)</td>
<td>0.520</td>
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<tr>
<td>1:1 AR20:hex</td>
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<tr>
<td>DPhPC</td>
<td>0.701 (0.027)</td>
<td>1.01 (0.095)</td>
<td>1.03 (0.043)</td>
<td>1.40 (0.111)</td>
<td>2.50 (0.095)</td>
<td>0.312</td>
</tr>
<tr>
<td>9:1 AR20:hex</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DPhPC</td>
<td>0.488 (0.043)</td>
<td>1.42 (0.051)</td>
<td>1.40 (0.045)</td>
<td>1.92 (0.056)</td>
<td>38.85 (2.34)</td>
<td>0.548</td>
</tr>
<tr>
<td>decane</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DPhPC</td>
<td>0.655 (0.030)</td>
<td>1.24 (0.056)</td>
<td>1.15 (0.045)</td>
<td>1.92 (0.056)</td>
<td>38.85 (2.34)</td>
<td>0.548</td>
</tr>
<tr>
<td>20% Chol.</td>
<td></td>
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<tr>
<td>hexadecane</td>
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</table>

*: DIB-electrowetting/Young-Lippman method
**: Pendant drop with goniometer

\( D_C \): hydrophobic thickness, \( D_C = (\varepsilon_f \varepsilon_0)/C_M \)
changes in membrane composition or oil content. Interestingly, solvent-free DIBs formed in 1:1 AR20:hexadecane displayed a heightened sensitivity in terms of the effects of cholesterol on $C_M$, $D_C$, $\gamma_B$, and $\Delta F$. These takeaways provide guidance for future studies aimed at investigating the effects of biomolecules, peptides, pharmaceutics, and other species on lipid bilayer thickness and tensions. Specifically, such studies could use 1:1 AR20:hexadecane mixtures to amplify the effects on membrane thickness and tension caused by accumulation of a species of interest.

**Conclusions**

The Young-Lippmann equation relates changes in contact angle to the applied voltage which affects the interfacial tension. In the DIB platform, where mechanical manipulation can be used to accurately measure $C_M$, and applied bias voltage induces measurable changes in contact angle, the Young-Lippmann relation provides a framework for simultaneous measurements of both monolayer and bilayer tension. The method is simple and involves measuring changes in the contact angle between droplets as applied bias voltage is increased. Contact angle alone is not sufficient for tension calculations as the Young-Lippmann equation includes membrane specific capacitance. However, specific capacitance is easily and precisely determined during the same test for tensions with droplets suspended on agarose-tipped electrodes. Knowledge of the shape (i.e. the circularity) of the interface is crucial to the accuracy of the measurement, particularly in light of observed droplet deformation due to gravity when there is a difference in densities of the oil and aqueous phases. Such density differences are encountered here with ≥ 200 nL droplets in tests using pure hexadecane or mixtures of hexadecane and silicone oil. Further, we learned that the zero-Volt contact angle between droplets increases with increasing droplet area (as droplets are pushed together). However, while it was shown that values of monolayer tension are not affected by manipulation-induced changes to $\theta_0$, the nominal contact angle does affect the calculation of bilayer tension in both theory and practice, larger angle changes are obtained for lower $\theta_0$. This conclusion allows tuning of the system (targeting $\theta_0 \leq \theta_{\text{max}}$) to achieve greater angle changes for the same voltage, which increases the signal-to-noise ratio of angle measurement.

With appropriate understanding of the shape of the interface, we demonstrated that this procedure exhibits comparable or better accuracy in measuring values of specific capacitance, bilayer tension, monolayer tension, and free energy of formation to methods described in prior literature. Further, all four physical properties can be measured in a single test over the course of 30 minutes or less. There are several advantages of the DIB-Young-Lippmann approach over supported or painted lipid bilayer techniques: 1) forming bilayers between lipid coated droplets allows the area of the bilayer to be easily manipulated, which has been shown to enable accurate determination of specific capacitance; 2) bilayer formation in a liquid environment (versus on a solid support) provides direct visual access to bilayer contact length and contact angle.

Measurements of $C_M$ (and thus $D_C$, $\gamma_B$, and $\Delta F$ support the conclusion that DPhPC DIBs formed in silicone oil:hexadecane mixtures are more solvent-free than DIBs formed in pure hexadecane or decane. Further, the solvent-free bilayers formed in silicone oil:hexadecane mixtures show an increased sensitivity to the effects of cholesterol on $C_M$, $D_C$, $\gamma_B$, and $\Delta F$. This conclusion suggests that, in future works, tuning oil composition to yield solvent-free bilayers could improve the ability to detect and quantify the effects of biomolecules, nanoparticles, peptides, or other species accumulating in lipid bilayers. The proposed and validated method of measuring capacitance and tensions in a DIB opens the door to being able to study a wide variety of lipidic and non-lipidic species and their effects on membranes. A key application of this procedure is the ability to characterize changes in bilayer physical properties driven by the incorporation of species (e.g. cholesterol) that, unlike transmembrane ion channels, do not elicit changes in membrane conductance. The initial embodiment of the method is ready for studies aimed at characterizing, monitoring, or sensing the interactions of a number of small molecules, peptides, pharmaceutic and therapeutic agents, or other lipophilic species with droplet interface bilayers.

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**References**


