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# Thermodynamic and mechanical properties of model mitochondrial membranes

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**Abstract**

Cardiolipin is a unique four-tailed, doubly negatively charged lipid found predominantly within the inner mitochondrial membrane, and is thought to be influential in determining membrane potential and permeability. To determine the role of cardiolipin in modulating the properties of membranes, this study investigates the thermodynamics of mixed cardiolipin and phosphatidylcholine monolayers and bilayers. Gibbs free energy analysis of mixed monolayers indicates that at low cardiolipin concentrations (5–10 mol%), there is a positive deviation from ideality on a pure water subphase, while at physiological salt concentrations a negative deviation from ideality is observed. The mechanical properties of bilayers containing cardiolipin were measured using micropipette aspiration. Both apparent area compressibility modulus, as well as lysis tension, decrease with increasing cardiolipin content. This destabilization indicates a decrease in the cohesive energy of the membrane. This interplay between interactions of lipids in monolayers and bilayers, suggests cardiolipin plays a dual role in modulating membrane properties. Cardiolipin enhances lateral interactions between lipids within monolayer leaflets, while simultaneously decreasing the cohesive energy of membranes at physiologically relevant concentrations. Taken together, these findings correlate with the decreased permeability and creation of folds in the inner mitochondrial membrane.


*Keywords:* Cardiolipin; Lipid monolayer; Bilayer; Cohesive energy; Lateral interaction

## 1. Introduction

Cells are highly organized with many functional units or organelles defined by one or more lipid membranes. Each membrane is specialized, containing specific proteins and lipid components that enable it to perform the unique role of that cell or organelle. Lipids are very diverse in both their respective structures and functions, and cells exquisitely control membrane composition. One intriguing issue is the specific role of lipids in modulating the physical properties of membranes.

Cardiolipin (CL) is a unique phospholipid found only in membranes of bacteria and mitochondria, i.e. those whose function is to generate an electrochemical potential for substrate transport and ATP synthesis [1,2]. The human inner mitochondrial membrane contains approximately 9.2% by mole CL [3]. Cardiolipin is a dimeric phospholipid in which two phosphatidyl moieties are linked by a central glycerol group. It has four acyl chains of varying lengths and degrees of saturation and a small acidic headgroup [2].

Previously the role of CL in modulating membrane properties and integrity has been probed using biochemical means. Model membrane studies determined that incorporation of small amounts of CL into egg phosphatidylcholine (EPC) vesicles greatly decreased the water permeability of giant unilamellar vesicles in osmotic stress measurements [4]. Complimentary attenuated total reflection IR spectroscopy measurements indicated that the lipids remained mixed and in the fluid state at concentrations up to 20 mol% CL. Taken together with NMR analysis of the headgroup conformations in bilayers [5], these findings suggest that the increased stability of membranes containing CL is due to a change in headgroup packing and hydration.

Similar work has been done on cells by comparing the phenotype and properties of wild-type, mutants with low CL concentrations, and null cells incapable of biosynthetically

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producing CL. In electron micrographs, mutant Chinese hamster ovary (CHO) cells display a drastically altered morphology when the CL content drops to 30% of normal cells. In all cases, the mitochondria appeared greatly enlarged and swollen, and cristae were lacking or disorganized [6]. In similar studies, the ability of yeast *Saccharomyces cerevisiae* to resist shrinkage and swelling under osmotic stress was found to depend greatly on the presence of CL in the mitochondrial membrane [7]. It is possible that proper folding of the inner mitochondrial membrane depends on lateral interactions between lipids and CL. An interesting corollary to this work, are changes in membrane fluidity during temperature acclimation of the protozoa *Tetrahymena pyriformis*. The fluidity of pellicles and microsome membrane fractions increased as the cells adapted to a temperature decrease from 39 to 15 °C. In contrast, mitochondria membrane fractions maintained a constant fluidity even as the saturation of the constituent lipids decreased. When CL was depleted from the membrane, the fluidity decreased suggesting that CL is important for maintaining a consistent fluidity of mitochondrial membranes [8].

Micropipette aspiration has been used to examine the general effects of acyl chain characteristics and fraction of anionic lipids on membrane mechanical properties. Previously, it has been shown that the area compressibility modulus of fluid phase diacyl phosphatidylcholine membranes is not affected by alterations in either the acyl chain length or the degree of unsaturation found in the acyl chains [9]. Similarly, varying the amount of charged lipid does not significantly alter the apparent area compressibility modulus, however, lysis tensions were found to decrease with increasing percentages of charged lipids [10]. Lysis tensions have also been found to decrease once at least one of the acyl chains contains two cis- double bonds. Further decreases were shown with three double bonds on each chain [11].

In this paper, we report the results of an investigation to quantify the interactions that occur between various phospholipids and cardiolipin, in the absence of proteins, and thereby provide a simple model for the effect of CL on modulating membrane properties. In the first part, monolayer properties of lipids (EPC, CL, and their mixtures) are presented from a Gibbs free energy analysis framework. In the second part, membrane mechanical properties are evaluated as a function of CL content using micropipette aspiration.

## 2. Materials and methods

### 2.1. Materials

EPC, 1-stearoyl, 2-oleoyl phosphatidylcholine (SOPC), 1-palmitoyl, 2-oleoyl phosphatidylserine (POPS), and 1-stearoyl, 2-oleoyl phosphatidylserine (SOPS) dissolved in chloroform at a purity of >99% were purchased from Avanti Polar Lipids (Avanti Polar Lipids, Alabaster, AL) and used without further purification. Bovine heart cardiolipin dissolved in methanol was purchased from Sigma (St. Louis, MO) at a purity of 98% and used without further purification. Chloroform, HPLC grade, purchased from Acros (New Jersey, USA), was used to dilute and store the lipid solutions. Methanol, HPLC grade, purchased from Fisher Scientific (Fairlawn, NJ) was used in diluting lipid solutions for preparation of vesicles. Ultra grade glucose and sucrose were purchased from Sigma and used in vesicle preparation and resuspension. Sodium nitrate 99.995% was purchased from Aldrich (St. Louis, MO). Bovine serum albumin (BSA) (fraction V, low heavy metals) at a purity of >98% was purchased from Calbiochem (San Diego, CA). The sodium chloride for the ionic subphase was ACS grade and purchased from EM Science (Gibbstown, NJ). Surfasil™, purchased from Pierce (Rockford, IL), was used to passivate both the glass cover slips and the micropipettes during micropipette aspiration experiments.

Lipid solutions for the Langmuir trough were prepared in chloroform at a concentration of 1 mg/ml and varying molar ratios between cardiolipin and EPC. Lipid solutions used to form giant vesicles by electroformation for use in micropipette experiments were prepared at a concentration of 0.5 mg/ml in 2:1, chloroform/methanol. The water used in the subphase was purified with a Barnstead nanopure filtration system (Dubuque, IA) to a resistivity of 18.2 MΩ-cm. For reference, the major lipid components of the human mitochondrial membrane are as follows: phosphatidylcholine (PC), 34%; phosphatidylethanolamine (PE), 43%; cardiolipin, 18%; and phosphatidylinositol (PI), 5% (weight percentages of the total weight of lipids) [12].

### 2.2. Langmuir trough

A Teflon® Langmuir–Blodgett trough with dimensions 20 cm by 30 cm, (Type 611, Nima, UK) was used to measure surface pressure–area per molecule isotherms. The surface pressure was measured using a filter paper Wilhelmy plate. The trough was enclosed in a chamber to prevent external particles from falling onto the surface and to avoid air currents from disrupting the monolayer or the pressure sensor.

The subphase of the trough was filled with either nanopure water or a solution of 150 mM NaCl. Using a Hamilton microsyringe, 50 μl of a given lipid solution was carefully deposited at the air–water interface with the barrier fully open. After a minimum of 10 min for solvent evaporation, lipids at the air–water interface were compressed at a constant rate of 25 $cm^2$/min, corresponding to a linear barrier speed of 1.25 cm/min, until collapse. Over the course of the compression, the molecular compression rate varies from 1 $Å^2$/molecule-min initially up to nearly 4 $Å^2$/molecule-min upon completion. Compression rates of less than 4 $Å^2$/molecule-min have generally been found suitable for thermodynamic analysis [13–15]. Surface pressure and trough area were recorded simultaneously using software

provided by Nima. Based upon the volume deposited, average molecular weight, and concentration of solution, the average area per molecule was calculated. All experiments were carried out at a room temperature of 22±1 °C.

To check reproducibility of the isotherm analysis between solutions, isotherms were performed on independent mixtures made from separate stock solutions of CL and EPC. Average isotherms were then obtained by arithmetically averaging the independent isotherms from separate solutions for a given subphase.

### 2.3. Micropipette aspiration

Giant unilamellar vesicles with diameters ranging from 20 to 40 μm were made using the electroformation technique developed by Angelova et al. [16]. The interior vesicle solution was composed of 100 mM sucrose with 0.5 mM sodium nitrate. The external vesicle solution was composed of 103–105 mM glucose with 0.5 mM sodium nitrate. Micropipettes were forged from capillary glass tubing (Fredrick and Dimmock, NJ) pulled to a constant inner diameter between 5.5 and 7 μm. Before use, the micropipettes were coated with a 1% SurfaSil™ solution in chloroform. In addition, the micropipettes were filled with a glucose solution containing 0.03 wt.% BSA to reduce vesicle adhesion to the glass surface. The micropipette aspiration experiments were conducted on vesicles composed of SOPC/CL mixtures. SOPC was substituted for EPC in order to prepare more robust vesicles with higher lysis tensions. Three lipid ratios were studied: 99.5% SOPC with 0.5% POPS or SOPS, 5% CL in 95% SOPC, and 9.2% CL in 90.8%SOPC. The addition of 0.5% negatively charged lipid, POPS or SOPS, prevented aggregation of neutral SOPC vesicles in the control experiments. Clean, large unilamellar vesicles were chosen for aspiration. Once the vesicles were captured by the pipette and raised above the bottom surface of the chamber, the chamber was moved around with the microscope stage to dislodge any tethers to other vesicles or to the chamber walls. Additionally, each vesicle was gently pre-stressed to incorporate all attached lipid into the vesicle.

The fundamentals behind application of the micropipette aspiration technique involve relating changes in membrane tension to changes in surface area of the vesicle. The suction pressure, $\Delta P$, applied to a vesicle was determined from the change in height of a water manometer coupled to the micropipette. The membrane tension, $\tau$, was calculated using Eq. (1) based on suction pressure, and the geometry of both the vesicle and micropipette [17].

$$\tau = \frac{\Delta P R_P}{2\left(1 - \frac{R_P}{R_V}\right)} \tag{1}$$

The vesicle radius, $R_v$, and change in length of projection, $\Delta L$, were measured directly using a CCD camera, then converted to microns through calibration with a known grid. The radius of the pipette, $R_p$, was measured by inserting a probe with a known length–diameter relationship, as determined from electron microscopy, into the micropipette. The area strain, $\alpha$, is the change in surface area ($A-A_0$), normalized by the initial area $A_0$. The area strain was determined from the projection length using the approximate relationship given in Eq. (2) [17].

$$\alpha = \frac{\Delta A}{A_0} \approx \frac{1}{2}\left\{\left(\frac{R_P}{R_V}\right)^2 - \left(\frac{R_P}{R_V}\right)^3\right\}\frac{\Delta L}{R_P} \tag{2}$$

Area compressibility measurements were performed by incrementally increasing the suction pressure and measuring the change in the projection length. The apparent area compressibility modulus was determined from the linear slope of the tension versus area strain in the high-tension regime, above $\tau$=0.5 mN/m. Lysis tension experiments were performed by steadily lowering the water manometer at a constant speed until lysis. Care was taken to ensure similar rates of suction pressure application during repeated measurements and experiments.

## 3. Results and discussion

### 3.1. Langmuir trough

The surface pressure–average area per molecule ($\Pi$–$A$) isotherms for mixed cardiolipin/EPC monolayers at various molar ratios on a pure water subphase are shown in Fig. 1A. The error bars indicate±one standard deviation from the average values reported. The results are a compilation of three compressions each from three independent sets of experiments. Due to the large sampling, there is a fairly large standard deviation. However, there is a systematic trend for the molecular area results, which facilitates the analysis. As expected, the area per molecule of pure cardiolipin is nearly a factor of two larger than that of pure EPC, due to its four acyl chains as compared to the two of EPC. Variations in collapse pressures between the mixed isotherms indicate miscibility between the two components [18,19].

As shown in Fig. 1B, the isotherm results of the mixtures on the ionic subphase are shifted to slightly smaller areas per molecule. With an ionic subphase the charges carried by the cardiolipin are screened due to the decreased Debye length, which allows the molecules to pack closer together. A more detailed method of determining miscibility between the two components is to calculate the excess area as compared to ideal mixing. For a given surface pressure the expression for excess area is given by [20]:

$$A_{ex} = A_{12} - (x_1 A_1 + x_2 A_2) \tag{3}$$

where $A_i$ and $x_i$ are the molecular area and mole fraction of the two components and $A_{12}$ is the average area per

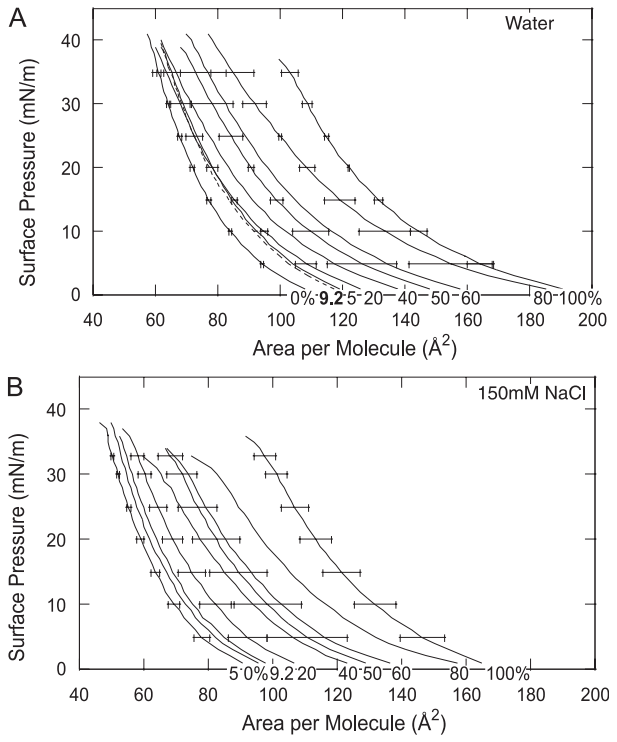


Fig. 1. Pressure versus area ($\Pi - A$) isotherms for mixed CL/EPC monolayers at various molar ratios on (A) pure water subphase, dashed line corresponds to 9.2% CL, (B) 150 mM NaCl. Selected error bars shown are $\pm 1$ standard deviation.

molecule of the mixture. A negative excess area per molecule indicates there are attractive forces, while a positive excess area suggests that there are repulsive forces acting between the molecules. A zero excess area indicates that either the two components are immiscible or that an ideal mixed monolayer has formed [21].

The excess area, as a function of CL content, was calculated using Eq. (3) and isobars of 5 mN/m increments are shown in Fig. 2. In the case of pure water subphase (Fig. 2A) the excess area is predominately negative indicating attractive forces between the molecules. The exception to this is at very low mole fraction of CL (<9.2 mol%) where the excess area is positive indicating repulsive forces are present between the molecules. Additionally the 80% CL isotherm exhibited positive excess area at low pressures, up to $\Pi$=10 mN/m. The most negative excess area occurs at a mole fraction of 40% CL indicating the strongest attraction as compared to the other ratios.

On 150 mM NaCl, the trends are similar (Fig. 2B). Over the entire range of mole fractions, the excess area per molecule is negative. Notable changes from the isotherms on pure water include a negative excess area at low mole fractions of EPC as opposed to the positive excess area that was observed on the water subphase. Additionally at 80% CL the excess area is never positive, even at low pressures.

Thermodynamic stability of the mixed monolayers, as compared to that of pure monolayers, was determined by analysis of the excess Gibbs free energy and the Gibbs free energy of mixing [22]. The expression for $\Delta G_{ex}$ is:

$$\Delta G_{ex} = \int_0^{\Pi} [A_{12} - (x_1 A_1 + x_2 A_2)] d\Pi \quad (4)$$

Where $A_i$ and $x_i$ are as previously defined and $\Pi$ is the surface pressure. Since the results for the excess Gibbs free energy are similar to the excess area, they are not shown.

Gibbs free energy of mixing, $\Delta G_{mix}$, is defined as the combination of the excess free energy of mixing and the ideal free energy of mixing [22].

$$\Delta G_{mix} = \Delta G_{excess} + \Delta G_{ideal} \quad (5)$$

From thermodynamics, the ideal free energy of mixing is given as

$$\Delta G_{ideal} = kT(x_1 \ln x_1 + x_2 \ln x_2) \quad (6)$$

where $k$ is the Boltzmann constant and $T$ is absolute temperature.

As shown in Fig. 3A, on a pure water subphase the only instance at which the mixing is thermodynamically less stable is at a ratio of 5% CL and a surface pressure above 20 mN/m. In all other situations, mixing is more favorable than separation between the two species. The most thermodynamically stable composition occurs at a mole fraction of 40% CL on a pure water subphase.

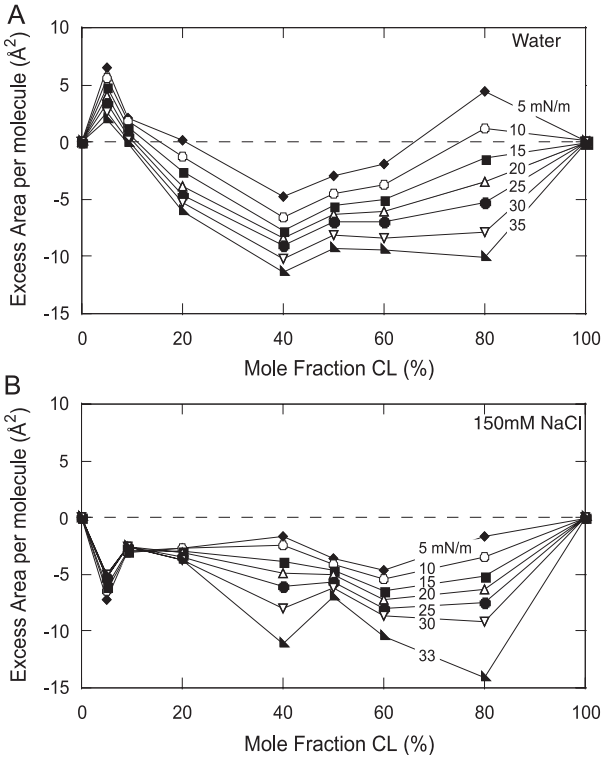


Fig. 2. The excess area as a function of CL content on (A) pure water subphase, (B) 150 mM NaCl. Isobars for 5 mN/m increments are shown.

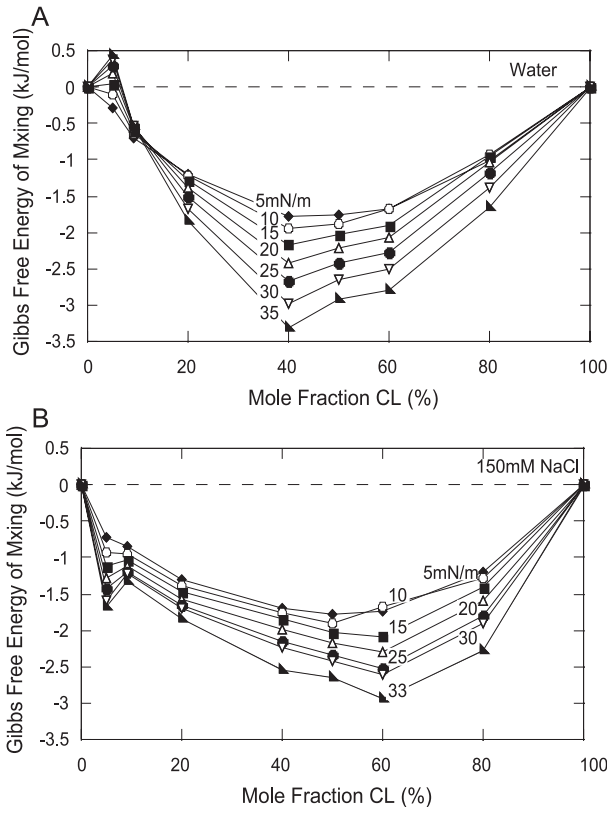


Fig. 3. Gibbs free energy of mixing as a function of CL content on (A) pure water subphase, (B) 150 mM NaCl.

At the unique concentration of 5%CL on a subphase of pure water, repulsive interactions between the lipid molecules dominate the entropy increase due to the mixing of the two species. Despite the large standard deviations in the isotherms, the unique behavior at 5% was shown consistently. This positive change in Gibbs free energy suggests that mixing is not spontaneous between the two species. In this case, miscibility cannot be precisely determined from the small variation in collapse pressures at 5% CL. Fluorescence microscopy was used to observe the monolayer over a range of surface pressures at 5% CL. While fluorescence microscopy can only detect phase separation when the domains are larger than 1 μm, there was no visible evidence of phase separation between CL and EPC.

The change of free energy with pressure suggests a dependence of stability on pressure. At a mole fraction of 9.2% CL there is only a very small change in free energy with increasing pressure, suggesting little dependence at that concentration. At 5% CL, the Gibbs free energy of mixing increases with increasing pressure, indicating a decrease in stability as the molecules are more closely packed together. Conversely at all other mole fractions examined, increasing surface pressure corresponded to a decrease in the Gibbs free energy of mixing indicating additional stability as the molecules became more condensed.

On the salt subphase, the Gibbs free energy of mixing (Fig. 3B) is always negative with a relative minimum occurring at a low percentage of CL, 5%. The overall minimum indicates the most favorable state; this is 60% CL on the salt subphase. The mixing behavior of EPC and CL is quite similar on both a pure water subphase and a salt subphase with two main differences. One difference is the changed behavior at 5% CL; on pure water the repulsive forces dominate the lipids whereas on the ionic subphase the intermolecular forces are attractive. Additionally, the absolute minimum in the Gibbs free energy of mixing is shifted from 40% CL on pure water to 60% CL on the salt subphase. This indicates a change in the thermodynamically optimal mixture.

In summary, there are attractive lateral interactions between CL and phospholipids in monolayers and an increase in monolayer stability under physiological conditions of a high salt concentration. Moreover, from excess area analysis, repulsive interactions occur at physiological concentrations of cardiolipin on a pure water subphase, which suggests that screening of the electrostatic charges of CL is necessary to observe lateral attraction.

### 3.2. Micropipette aspiration

Membrane mechanical properties as a function of CL content were measured using micropipette aspiration. In humans fetal mitochondria contain 3% CL but by the first year the concentration reaches adult levels of 9% CL [23]. Both area compressibility modulus and lysis tension of membranes containing 0, 5, and 9.2 mol% CL were determined by monitoring the change in projection length of giant vesicles as the aspiration pressure was increased.

The area compressibility modulus and lysis tension of SOPC membranes containing 0, 5, and 9.2 mol% CL were determined by monitoring the change in projection length of giant vesicles as the aspiration pressure was increased by micropipette aspiration. The lateral tension, $\tau$, and area strain, $\alpha$, were calculated using Eqs. (1) and (2). The apparent area compressibility modulus, $K_{app}$, is given by the slope of $\tau$ versus $\alpha$. A representative plot for a 95:5 mol% ratio of SOPC:CL is shown in Fig. 4. Apparent area compressibility

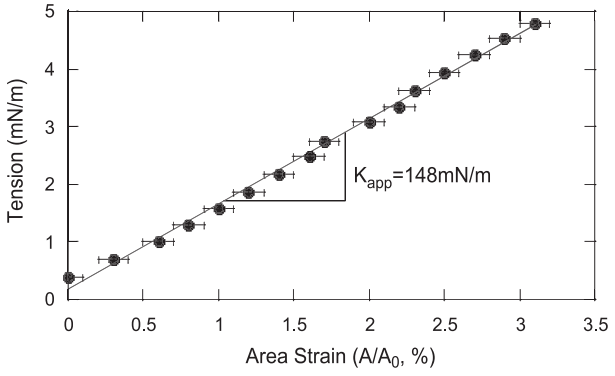


Fig. 4. Representative plot of tension versus area strain for 5%CL–95%SOPC. The apparent area compressibility modulus in this measurement is 148 mN/m with a lysis tension of 4.8 mN/m.

and lysis tension results for each concentration studied are listed in Table 1. Control experiments with SOPC in 0.5 mM sodium nitrate, gave a $K_{app}$ of 153±22 mN/m. Previously, Ly et al. [24] found the apparent area compressibility of SOPC in aqueous solution containing only glucose and sucrose to be 186 mN/m. Similarly, a value of $K_A$=180 mN/m was obtained by Discher et al. [25] in an aqueous sucrose solution. A study of the mechanical properties of POPC/POPG mixtures using micropipette aspiration found no significant variation in area compressibility dependent on the fraction of anionic lipid. However, in the presence of 1 mM monovalent salt, area compressibilities of POPC with between 4% and 40% POPG were reduced to143 mN/m [10]. Our findings of a $K_{app}$=153±22 mN/m for SOPC in 0.5 mM monovalent salt match well with previous measurements in the presence of a weak electrolyte. As reported in Table 1, the area compressibility modulus for SOPC was nearly unchanged when 5% CL was incorporated into the vesicle. With 9.2% CL the area compressibility modulus was significantly reduced. Decreases in area compressibility modulus indicate that a lower stress is required to achieve a given change in membrane area and suggest a weakening of the cohesive strength of the membrane.

In addition, incorporation of charged CL lipids dramatically decreased the lysis tension of the vesicles. The lysis tension is a measure of critical strength of the vesicle; results are reported in Table 1. Addition of 5% CL decreased the lysis tension by 3.5 mN/m and the addition of 9.2% CL caused an overall decrease of 5.1 mN/m, corresponding to a 38% reduction in the critical lysis tension. Again, control experiments with SOPC agree with literature values of 7.1 mN/m for POPC with 4% POPG in 1 mM monovalent salt [10]. Repulsive electrostatic interactions between lipids in vesicles may render them unable to withstand high stress. A similar reduction in lysis tension with charged lipid concentration was found upon increasing the fraction of POPG within POPC lipid vesicles [10]. However, the addition of CL not only introduces charge to the vesicles, but also alters the acyl chain characteristics. SOPC is monodisperse with a single unsaturation on one acyl chain with a saturated second chain, whereas CL is a mixture predominantly containing lipids with four chains of 18 carbons with two unsaturated bonds on each chain. As a result, addition of cardiolipin increases the amount of unsaturation present in the vesicle. A similar decrease in lysis tension of PC bilayers upon addition of doubly unsaturated lipids has been documented [11].

Table 1
Mechanical properties for SOPC:CL membranes

| Lipid composition | $K_{app}$ (mN/m) | $\tau_{lysis}$ (mN/m) | $C_s^{-1}$ (mN/m) with EPC at 30 mN/m |
|---|---|---|---|
| SOPC[a] | 153±22 (23) | 8.2±0.9 (26) | 100.±1. |
| 5%CL–95%SOPC | 149±21 (50) | 4.7±1.4 (22) | 85.7±2.7 |
| 9.2%CL–90.8%SOPC | 115±16 (28) | 3.1±0.9 (33) | 92.6±2.2 |

Error is one standard deviation; parentheses indicate number of vesicles averaged.
$C_s^{-1}$ is the elastic moduli of area compressibility obtained from the isotherms at a surface pressure of 30 mN/m where EPC was used in place of SOPC.
[a] $C_s^{-1}$ of pure SOPC is 123 mN/m [13].

Isothermal compressibility measurements obtained from the monolayer work lead to another measure of elasticity and compressibility of the membrane, shown in Table 1. $C_s^{-1}$ is the inverse of the isothermal compressibility, known as the elastic moduli of area compressibility, therefore a larger $C_s^{-1}$ value indicates a less compressible membrane.

$$C_S = -\frac{1}{A}\frac{dA}{d\Pi}$$

The values shown for the elastic moduli of area compressibility indicate the lipid monolayers are in the fluid liquid-expanded phase. From literature, at 30 mN/m $C_S^{-1}$ is 123 mN/m for SOPC [13]. In comparison, for EPC we found $C_S^{-1}$=100±1 mN/m. The increased compressibility is most likely due to additional hydrocarbon chain unsaturation. Upon addition of CL into the EPC monolayer $C_S^{-1}$ is further reduced, indicating the monolayer becomes more fluid with CL. These results are consistent with observations linking mitochondrial membrane fluidity with CL incorporation [9].

## 4. Conclusion

The presence of cardiolipin strongly affects the thermodynamic properties of lipid monolayers and bilayers. Monolayer analysis favors mixing between EPC and CL with specific mixed ratios and pressures being most stable. Results indicate an increase of stability with increasing pressure and an optimal mixture of 40% CL on water as compared to 60% CL on a 150 mM salt subphase. In the physiological range of 0–9.2% CL, a relative minimum occurs at 5% CL on the salt subphase, whereas a relative maximum is observed at 5% CL on pure water indicative of the important role of electrostatic interactions on monolayer stability. In bilayer structures, both apparent area compressibility modulus as well as lysis tension decrease with increasing cardiolipin content. Both suggest a decrease in the lateral interaction between the monolayer leaflets, requiring less energy to stretch the membrane. Such an effect would likely decrease the energy required to create folds in the inner mitochondrial membrane, aiding in their formation. In contrast, area compressibility has not been found to decrease with increased unsaturation or charged lipid concentration, and therefore highlights the unique effect of CL on modulating membrane properties [9,10]. Similarly, the elastic moduli of area compressibility from monolayer analysis is reduced upon incorporation of CL which correlates with the unusual fluidity of membranes containing CL.

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