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The Role of Lateral Tension in Calcium induced DPPS Vesicle Rupture

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Abstract

We assess the role of lateral tension in rupturing anionic dipalmitoylphosphatidylserine (DPPS), neutral dipalmitoylphosphatidylcholine (DPPC), and mixed DPPS-DPPC vesicles. Binding of Ca^{2+} is known to have a significant impact on the effective size of DPPS lipids and little effect on the size of DPPC lipids in bilayer structures. In the present work we utilized laser transmission spectroscopy (LTS) to assess the effect of Ca^{2+} induced stress on the stability of the DPPS and DPPC vesicles. The high sensitivity and resolution of LTS has permitted the determination of the size and shape of liposomes in solution. The results indicate a critical size after which DPPS single shell vesicles are no longer stable. Our measurements indicate Ca^{2+} promotes bilayer fusion up to a maximum diameter of ca. 320 nm. These observations are consistent with a straightforward free-energy-based model of vesicle rupture involving lateral tension between lipids regulated by the binding of Ca^{2+} . Our results support a critical role of lateral interactions within lipid bilayers for controlling such processes as the formation of supported bilayer membranes and pore formation in vesicle fusion. Using this free energy model we are able to infer a lower bound for the area dilation modulus for DPPS (252 pN/nm) and demonstrate a substantial free energy increase associated with vesicle rupture.

Introduction

The forces regulating lipid bilayers have important consequences in understanding both real and model biomembranes. The balance between hydrophilic and hydrophobic interactions, entropy, and other forces give rise to the bilayer structure that serves to compartmentalize and define boundaries in cellular organisms. Critical to the viability of these organisms is the ability to transport materials through the bilayer membrane. In nature, this transport is accomplished using both pore-forming proteins and through vesicle fusion.^{1, 2} Phenomenologically, it is believed that the fusion of lipid bilayers is achieved through a series of steps involving the rearrangement of the lipid molecules to minimize the energetic penalties associated with exposing the hydrophobic core to an aqueous environment.^{3–7} The steps involved include an initial contact, followed by the formation of a hemi-fused state where the fusing bilayers each contribute a lipid leaflet to a shared bilayer region. This hemifused state is believed to rupture through the generation of a pore that then rapidly opens generating the fused bilayer membrane. While some have implicated specialized

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Supporting Information Available: A complete derivation of the relationship between free energy and fused vesicle size is provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

proteins, the fusion of proteinless bilayers has also been observed.^{8,9} Additionally, supported bilayer membranes are a common model system for biomembranes. A common method for preparation of these membranes is vesicle fusion, wherein small unilamellar vesicles combine to form a larger vesicle, which then ruptures to form a planar bilayer membrane on the supporting surface.¹⁰⁻¹⁷ Again, the steps involved have been identified but the physical interactions that trigger the rupture of the vesicles are still unclear.

The free energy associated with lipid bilayer bending has been proposed as a factor governing the rupture of lipid vesicles.¹⁸⁻²⁰ It has been shown that the energy associated with bilayer bending is independent of the size of the vesicle and thus does explain the increased probability of rupture with larger vesicles.¹⁶ As the larger vesicle adsorbs, the curved region remains relatively constant. Additionally, in vesicle fusion the formation of high curvature regions can achieve a stable hemi-fused intermediate without driving the fusing bilayer system to completion.^{7,21} Although controversial, while curvature adds to the free energy of a bilayer membrane, other forces seem to be responsible for destabilizing and rupturing lipid vesicles.

Membrane tension has been implicated as the force responsible for the formation of the initial pore in bilayer fusion;^{3-5,18} however, the origin of this force is uncertain. In a previous study using fluorescent lipid mixing, lateral tension was proposed to induce membrane fusion.²² To examine and quantify the role of lateral force interactions for destabilizing these supramolecular assemblies, we have utilized single shell vesicles (SUVs), or liposomes. These model systems provide access to the bilayer structure characteristic of cellular membranes while enabling specific interactions to be straightforwardly probed.²³ In the present report, dipalmitoylphosphatidylserine (DPPS) vesicles were dosed with Ca^{2+} to promote vesicle fusion. Previous results indicate that divalent cations promote lateral interactions between the phosphate moieties of DPPS bilayer lipids. These lateral interactions have been suggested to catalyze the fusion of anionic lipid vesicles.²⁴ The addition of Ca^{2+} to DPPS results in the condensation DPPS- Ca domains. Surface-area-pressure measurements indicate area-per-lipid values for DPPS and DPPS- Ca of 44.2 and 40.2 $\text{\AA}^2$, respectively.²⁵ The condensation effect is substantially more pronounced in DPPS vesicles in comparison to dipalmitoylphosphatidylcholine (DPPC), where the change in area per lipid is reported to be small ($< 1 \text{ \AA}^2$) with the addition of Ca^{2+} .²⁶

The free energy associated with lateral stretch/compression of a lipid bilayer can be modeled according to the relationship in equation 1:

$$G = \frac{K_A}{2} \int \left(\frac{\Delta A}{A_0} \right)^2 dA \quad \text{Eq. 1}$$

Where K_A is the area-stretch modulus, or the amount of energy required to change the area of a bilayer, ΔA is the change in surface area, and A_0 is the initial area. For a uniform tension resulting in the change in area, the equation simplifies to the following:

$$G = \frac{K_A}{2} \frac{\Delta A^2}{A_0} \quad \text{Eq. 2}$$

Typical values associated with K_A are reported to be between $0.1 - 1 \text{ J m}^{-2}$.^{27,28} In this context, it is clear that the decrease in area per lipid associated with DPPS binding of Ca^{2+} will produce a change in the surface area given a fixed number lipids in a vesicle assembly. From this relationship, it is also evident that a change in area will increase the free energy of

the system, destabilizing the integrity of the bilayer membrane. Furthermore, the lateral strain that mediates vesicle fusion builds with increased vesicle size and may result in vesicle rupture.

To quantify vesicle fusion associated with DPPS – Ca^{2+} binding, and its role in destabilizing bilayer vesicles, we have utilized a recently developed measurement technique, high precision laser transmission spectroscopy (LTS).^{29, 30} LTS is based on the wavelength-dependent transmission of light over a broad wavelength range, whereas other nanoparticle sizing techniques rely on diffraction or dynamic light scattering (DLS).^{31–33} The latter techniques use one or more fixed light wavelengths and analyze light scattered at fixed angles. LTS has far greater sensitivity and resolution in the size regime important to these studies ($\sim 50 - 1000$ nm). The sensitivity of LTS in this range is $\sim 10^4$ particles/mL and the resolution is < 5 nm (fwhm). This represents $\sim 10^6$ higher sensitivity and ~ 30 higher resolution compared to the performance of DLS. LTS also provides the absolute particle density (which DLS does not), that is important in establishing the total mass of objects in suspension as reactions occur. The high resolution of LTS enabled the precise determination of vesicle size that was correlated to the free energy associated with rupture.

Materials and Methods

Laser Transmission Spectroscopy

The instrumentation used for LTS has been described previously.^{29, 30} Briefly, LTS characterizes nanoparticles in suspension based on wavelength-dependent light extinction, which is capable of determining the size, shape and density of particles to high precision. As noted above, LTS relies on the measurement of the transmission (at zero angle with respect to the incoming beam) of light through the suspension of liposomes as a function of wavelength. The extinction obtained is inverted to obtain the vesicle size distribution. The present methodology is applicable in the low volume-fraction regime (5×10^{-8} vol.% to 0.5 vol.%). LTS results were obtained by measuring the extinction of a sample and reference solutions in standard 1 cm quartz cuvettes.

For LTS measurements, SUV samples are diluted with de-ionized water. For each sample, 100 μl of the prepared 1% liposome suspension is diluted with 900 μl of de-ionized water, held in a quartz spectrometer cell. The diluted samples are measured with LTS to determine the particle size distribution and particle concentration. De-ionized water is used as the reference to the samples. To study the fusion of these SUVs, Ca^{2+} is added to the samples and excess ethylenediaminetetraacetic acid (EDTA) is then added to all samples to bind to the Ca^{2+} as a test to determine whether the SUVs fused or aggregated.^{9, 34}

Atomic Force Microscopy

Atomic Force Microscopy was performed using a Nanonics MV 4000 instrument, which operates in phase-feedback mode. The tip was a commercial AFM-liquid tip (Nanonics Supertips, LTD). AFM measurements were performed in solution, using freshly cleaved mica for a substrate. Vesicles were added to solution followed by the addition of Ca^{2+} .

Single Unilamellar Vesicle Preparation

Dipalmitoylphosphatidylserine (DPPS, Avanti Polar Lipids, INC) single unilamellar vesicles were prepared by serial extrusion of multilamellar vesicles (MLVs). The MLVs were prepared by rehydrating 10mg of dry DPPS in 1mL of Nanopure water to provide a 1% (weight%) lipid suspension. The lipid suspension was subjected to repeated freeze/thaw cycles where the suspension was heated above the gel-to-liquid crystalline-phase transition temperature of 54°C , vortexed briefly, and then cooled on ice. This was repeated at least 5

times to assure full hydration of the lipid head groups. The solution was then extruded through Polycarbonate Nuclepore Track-Etch Membranes (Whatman) via an Avanti Polar mini extruder. Membrane pore sizes were selected on the basis of desired vesicle size. Liposomes and pore sizes were: 1 μm , 0.4 μm , 0.2 μm , and 0.1 μm . For liposomes below 1 μm in diameter the extrusion was done stepwise by first using a 1 μm pore size and then decreasing pore size to a minimum filter size of 100 nm. Dipalmitoyl phosphatidylcholine (DPPC, Avanti Polar Lipids, INC) was prepared in the same manner; however, it was heated to a temperature above 41 $^{\circ}\text{C}$, the phase transition point for DPPC.

Results and Discussion

Figure 1 shows the LTS results obtained from the sequential addition of 20 mM Ca^{2+} and 100 mM EDTA to DPPS and DPPC vesicles and vesicles with a 3:1 DPPS:DPPC lipid composition. All vesicles were prepared to be 100 nm in diameter. The samples are observed to be monodisperse prior to the addition of Ca^{2+} with diameters of 82, 102, and 114 nm as measured at the peak of the size distribution for DPPS, 3:1 mixture, and DPPC systems, respectively. Upon the addition of Ca^{2+} , changes are observed in the size distribution of the DPPS vesicles. As expected, the vesicles aggregate into larger clusters. The loss of intensity upon addition of Ca^{2+} represents the increase variance in vesicle size and a loss of material observed as a precipitate in the cuvette. The fusion of the SUVs is confirmed by the addition of EDTA to chelate and remove Ca^{2+} from the system. Upon removing the fusogenic agent, non-fused vesicles are known to fall apart to their initial sizes. As shown in Figure 1, the largest fused vesicle observed in these experiments is approximately 320 ± 21 nm. The standard deviation was calculated from the maximum diameter observed over all of our experiments.

Control experiments performed utilizing DPPC and mixed DPPS-DPPC vesicles show the expected insensitivity to Ca^{2+} , as shown in Figure 1B&C. A slight broadening is observed upon addition of Ca^{2+} increasing the most common diameter to 120 nm in both DPPC and DPPS vesicles. A simple surface area calculation assuming fusion of two vesicles indicates the expected diameter should be 140–160 nm. Therefore, the increase in size is likely not due to fusion, but perhaps osmotic swelling or some other slow growth mechanism. The broadening of the peak results in decreased height because the total concentration is the integral under the curve, which is conserved in these experiments.

This observation is consistent with previous light scattering results obtained using Mg^{2+} as the divalent cation, supporting the previous assertion that Mg^{2+} and Ca^{2+} mediate fusion in a similar manner.⁹ In this previous study, infrared spectroscopy indicated segregated domains of DPPC were shown to reorient upon DPPS binding a divalent cation, suggesting membrane tension indirectly influenced DPPC order. In that study, DPPC reorientation appeared to mitigate the membrane tension and impede fusion. DLS results for a 3:1 DPPS-DPPC vesicles in the previous study were inconclusive; however, here we see more clearly the role of DPPC in inhibiting bilayer fusion.

To examine whether 320 nm was indeed the largest vesicle diameter that would result from Ca^{2+} induced fusion, or merely an arbitrary value, we performed sequential additions of smaller amounts of Ca^{2+} . Results from this Ca^{2+} titration are plotted in Figure 2. In Fig. 2A, we see that upon addition of 3 μL of 20 mM CaCl_2 solution, a new peak appeared at 116 nm that exactly matches the diameter expected for the fusion of two 82 nm vesicles. Upon addition of an additional 3 μL , a large aggregation of vesicles was observed with a maximum diameter of 304 nm (correlating to ca. 14-mer fused vesicle). The addition of an additional 3 μL resulted a shift in the observed maxima to 102 nm, consistent with a mixture comprised of single and fused dimer vesicles. The addition of an additional 3 μL of CaCl_2

(Fig. 2D) evinced a large maximum at 142 nm (fused trimer) and a smaller maximum near 312 nm. Additional CaCl_2 additions showed decrease in the 142 nm peak and increase in intensity near 300 nm.

The titration data illustrates two phenomena. First, that ca. 320 nm appears to be a real maximum diameter for fused DPPS vesicles. The measurement is sensitive to particles up to 1000 nm in size and no particles were formed at these larger dimensions. Additional measurements, including differential dosing with Ca^{2+} , failed to produce a stable product larger than the 320 nm maxima. Interestingly, the discrete sizes detected in the LTS measurements are not resolvable using dynamic light scattering.

Second, there appears to be differential aggregation behavior in the system. In the initial system, there appeared to be mass accumulation with low concentrations of added Ca^{2+} . Additional Ca^{2+} resulted in the observance of smaller vesicles with slower aggregation kinetics. This differential behavior suggests the rupture of large vesicles removes lipids from solution, decreasing the overall concentration of vesicles remaining to react.

To verify this behavior we repeated the Ca^{2+} titration on DPPS as well as on 3:1 mixed DPPS:DPPC and DPPC vesicles as shown in Figure 3. The DPPS (Fig. 3A) shows the expected increase in size and decrease in density upon the addition of Ca^{2+} . The other two systems show interesting Ca^{2+} dependent behavior. At low concentrations of Ca^{2+} , broad size distributions are observed in both, but as the concentration of Ca^{2+} increases fluctuations are observed tending back to smaller distributions in agreement with the data in Figure 1. At the highest Ca^{2+} concentrations, the smaller sizes appear more stable. These changes in size distribution suggest possible aggregation events, possibly related to the electrical charge on the vesicle surfaces. Calculations of the surface area associated with particle size and density indicates the amount of material in solution is unchanged in these two systems.

Figure 4 plots the total surface area of vesicles in solution (equivalent to lipid concentration) as a function of Ca^{2+} addition. The decay fits well to a single exponential correlating Ca^{2+} activity to removal of lipid from solution. Experiments performed on DPPC and a vesicle with mixed lipid composition did not show this exponential decrease in lipids from solution as discussed above.

To assess the fate of the lipids removed from solution, we performed AFM analysis of DPPS lipids on a mica surface as shown in Figure 5. A flat surface is observed in the absence of Ca^{2+} , suggestive of bare mica (Fig. 5A). In the presence of Ca^{2+} , features appear in the AFM images including terraces with a height change of 4–5 nm on the mica surface (Fig 5B). The observed height change is consistent with the formation of a DPPS bilayer. Experiments performed in the presence of Na^+ showed no evidence of vesicle rupture or fusion. These results further support our assertion that Ca^{2+} ruptures the DPPS vesicles. Previous studies have reported that the addition of Ca^{2+} to DPPS liposomes results in the formation of cochleate structures.^{35, 36} One characteristic of the cochleates is dehydration of the PS lipids upon complexation with Ca^{2+} .^{36, 37} A solid precipitate was observed to form upon increased addition of Ca^{2+} to our DPPS vesicles, which may indicate formation of these structures following rupture at high Ca^{2+} concentrations. LTS is only sensitive to vesicles remaining in suspension and did not detect cochleate particles; had cochleate structures formed, we would have expected to see cigar shaped clumps rather than large terraces in the AFM image.

Studies of vesicle rupture, such as the formation of supported bilayer membranes by vesicle fusion, suggest that small vesicles coalesce into a larger vesicle and then rupture onto the surface.^{10–17} While the mechanism of rupture is not known, these observations suggest that

vesicle size plays a role. The relationship for the energy associated with lateral strain (Eq. 2) can be reformulated to show the change in energy associated with increasing vesicle size. The surface area of a vesicle is approximately equal to the area per lipid (A_{lipid}) times the number of lipids (N_{lipid}) as shown in equation 3:

$$SA_{vesicle} \approx N_{lipid} \cdot A_{lipid} \quad \text{Eq. 3}$$

The number of lipids in a vesicle is thus the $SA_{vesicle}/A_{lipid}$. Modeling the surface area of a vesicle as a sphere, the lateral strain energy can be correlated to vesicle diameter and the change in lipid area associated with DPPS binding Ca^{2+} as shown in equation 4:

$$G = \frac{K_A}{2} \frac{\left[\frac{4\pi R_{vesicle}^2}{A_{lipid}} \Delta A_{lipid} \right]^2}{4\pi R_{vesicle}^2} = K_A 2\pi \left(\frac{\Delta A_{lipid}}{A_{lipid}} R_{vesicle} \right)^2 \quad \text{Eq. 4}$$

Using values reported in the literature for DPPS and the DPPS- Ca^{2+} complex one can plot the trend for the increase in energy with increasing vesicle size as shown in Figure 6. The quadratic increase in energy supports the idea that a critical size exists that, once exceeded, results in vesicle rupture.

A key parameter in Equation 4 is the area-stretch modulus (K_A) of the lipid bilayer. For a series of PC lipids, the value of K_A has been shown to be invariant with respect to acyl chain composition and equal to 230 pN/nm.²⁷ The classic method for determining K_A is by aspirating a vesicle into a pipette.³⁸ The small vesicles that are stable for DPPS are not amenable to this characterization; however, it has been shown that pressure induced tension correlates linearly with K_A . The area compression of lipid bilayers upon complexation with Ca^{2+} has been well studied and the change in lipid area for DPPS and DPPC is known.^{25, 26} Assuming that binding Ca^{2+} induces an instantaneous compression, before osmotic equilibrium, the force associated with this aerial compression can be calculated. K_A is reported to have the following linear relationship to bilayer tension²⁷:

$$K_A = \tau \frac{A}{\Delta A} \quad \text{Eq. 5}$$

Where τ is the bilayer tension, A is the initial bilayer area, and ΔA is the change in area. Using the Young-Laplace relation, the tension is related to the pressure change across a bilayer and the curvature (R) of the vesicle:

$$\Delta P = \frac{2\tau}{R} \quad \text{Eq. 6}$$

Combining Eqns. 5 and 6, one derives an expression for K_A in terms of pressure across the membrane:

$$K_A = \frac{\Delta P \cdot R \cdot A}{2 \cdot \Delta A} \quad \text{Eq. 7}$$

The pressure change can be expressed in terms of the initial pressure (P_0) and calculating the volume change of the vesicle associated with the decrease in area per lipid attendant to the interaction with Ca^{2+} . By assuming ideal behavior, $P_0 V_0 = P_F V_F$, the change in pressure (ΔP)

can be expressed in terms of the initial pressure and literature values associated with lipid area (A) and the Ca^{2+} induced change in area (ΔA):

$$\Delta P = P_0 \left(\frac{1}{\left(\frac{A - \Delta A}{A} \right)^{3/2}} - 1 \right) \quad \text{Eq. 8}$$

Substituting Eq. 8 into Eq. 7, and then using the result in Eq. 4, gives rise to an expression for the free energy associated with the increase in vesicle size resulting from Ca^{2+} induced fusion:

$$G = P_0 \left(\frac{1}{\left(\frac{A - \Delta A}{A} \right)^{3/2}} - 1 \right) R^3 \cdot \frac{\Delta A}{A} \quad \text{Eq. 9}$$

Assuming DPPC and DPPS vesicles have the same internal pressure in the absence of Ca^{2+} , P_0 can be determined from the $K_A = 230$ pN/nm literature value of PC lipids.²⁷ Eq. 9 represents the change in free energy associated with area compression, such as that induced by lateral compression resulting from Ca^{2+} interactions with PS lipids (see supporting information for complete derivation).

The plot of free energy vs. vesicle diameter (Fig. 6) shows the simple difference between the area of DPPS lipid and DPPS complexed to Ca^{2+} gives rise to a free energy of 332 nN nm ($\sim 81 \times 10^3 \text{ k}_B T$) at a vesicle size of 320 nm. The rupture of DPPS vesicles is readily explained by this substantial increase in free energy. For DPPC vesicles to attain a similar free energy, the vesicle would exceed 10 μm in diameter. From the data for DPPS, it is possible to back out an effective K_A for DPPS of 252 pN/nm. This value likely represents a lower bound for K_A , as lipid bilayers are semi-permeable membranes and the diffusion of water across the bilayer will also act to minimize the pressure difference. Incorporating the changes in osmotic pressure are beyond the scope of these measurements.

Previous reports suggested that the Ca^{2+} needed to induce asymmetric tension across the bilayer to promote fusion.²² In our experiments the vesicles are prepared free of calcium; thus Ca^{2+} is only added from the outside. Lipid bilayers are largely impermeable to large cations; however, our experiments cannot rule out the possibility of ion leakage as Ca^{2+} interacts with the vesicle. The change in PS lipid area with Ca^{2+} binding should induce lateral tension in the bilayer whether the condensation occurs in the inside or outside leaflet, contracting the bilayer, and generating the required change in free energy. This is contrary to what was suggested previously; however, our model system is significantly less complex. We do not have fluorescent labels or an ionophore interacting in our bilayer membrane to shuttle Ca^{2+} across the membrane. These additional components will likely modulate the elastic properties of the membrane and complicate the free energy model developed here.

It has been reported that addition of cholesterol to PC vesicles increases K_A of the bilayer membrane.³⁸ Cholesterol is reported to have a condensing effect on saturated lipids, which maintains a gel phase configuration.³⁹ The interaction between DPPC and cholesterol is expected to be substantially smaller than that of DPPS and Ca^{2+} . In the presence of cholesterol, DPPC will still exhibit a gel-to-liquid crystalline phase transition; however, the gel-to-liquid crystalline transition for DPPS and Ca^{2+} is suppressed beyond the boiling point of H_2O .⁴⁰ This suggests that the K_A of the DPPS- Ca^{2+} complex is substantially larger and can produce a sizable energy penalty with increased vesicle size.

These calculations show that the change in area per lipid associated with Ca^{2+} binding imparts a substantial destabilization force that can explain the rupture of bilayer vesicle assemblies. LTS results enable us to determine with high precision the critical vesicle size and extract the free energy of rupture. Lateral tension thus appears to be a key parameter regulating bilayer stability relevant to vesicle rupture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Laser Transmission Spectroscopy results are shown for DPPS (A), a 3:1 DPPS-DPPC mixture (B), and DPPC (C). Upon the addition of 20 mM Ca^{2+} (pink curves), the observed particle size for DPPS is observed to increase dramatically, while the mixed and DPPC vesicles shows little effect. The systems were treated with EDTA (dotted line) to assess for vesicle fusion. The pure DPPS and mixed vesicle initial curves has been scaled down as indicated for clarity.

Figure 2.

The serial addition of 3 μL of 20 mM Ca^{2+} is plotted (A–E). Upon the addition of 6 μL of 20 mM Ca^{2+} , mass aggregation is observed. Addition of additional increments of Ca^{2+} results in rupture of the large vesicles and kinetically slower aggregation of the remaining small vesicles.

Figure 3.

LTS size distributions are plotted for (A) DPPS, (B) 3:1 mixture DPPS-DPPC, and (C) DPPC vesicles that result from the addition of the indicated amounts of 20 mM Ca^{2+} . In the plots for DPPS (A) and in the 3:1 mixture (B), the intensity of the initial distribution has been scaled as indicated for clarity.

Figure 4.

The total surface area detected in the sample is plotted as a function of the amount of Ca^{2+} added. The dots are the experimental data points for pure DPPS (red), a 3:1 DPPS-DPPC mixed vesicle (blue), and pure DPPC (green). The solid red line is a single exponential fit to the DPPS data. Neither the mixed system nor pure DPPC fit to an exponential and a linear fit is provided as a guide to the eye.

Figure 5.

The AFM image of a mica surface in a solution of DPPS vesicles is shown before (A) and after (B) the addition of Ca^{2+} . In both images the height profile is shown across the line (1) in inset. In the presence of Ca^{2+} , a terrace with a 5 nm step is observed, consistent with a supported bilayer membrane.

Figure 6.

The calculated free energy associated with increased vesicle diameter according to equation 9 is shown for DPPS and DPPC vesicles. The dashed line represents the maximum size vesicle that was observed in the LTS measurements utilizing DPPS. The plot clearly shows the tension induced by Ca^{2+} condensation incurs a substantial energy penalty.