Atomic force microscopy of nanometric liposome adsorption and nanoscopic membrane domain formation

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Abstract

Scanning probe microscopy studies of membrane fusion and nanoscopic structures were performed using hydrated single lipids and lipid mixtures. Extruded vesicles of DMPC and mixtures at various concentrations of DLPC, DPPC and cholesterol were deposited on freshly cleaved mica and studied in a fluid environment by AFM. The nanostructures formed by these extruded liposomes ranged from isolated unilamellar vesicles to flat sheet membranes and were marked influenced by thermodynamic phase behavior. For DMPC membrane, intact bilayers exhibited a phase transition process in agreement with large bilayer patches. In the DLPC, DPPC and cholesterol mixtures, nanoscopic domain diameters ranged from \( \approx 25 \) to \( 48 \) nm with height differences of \( \approx 1.4 \) nm; all values were lipid composition-dependent. Our data support and extend previous studies of microscopic domains and phase boundaries of the same mixtures in giant unilamellar vesicles determined by confocal light microscopy. Our approach for preparing and utilizing supported membrane structures is potentially relevant to studies of native cell membranes.

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1. Introduction

The energetics and lateral organization of biological membranes are critical to functions at the cell membrane level, such as vesicular trafficking, membrane fusion, signal transduction, and virus or protozoal infection [1–4]. Biological membranes are formed by core molecules including sphingomyelin, phospholipids, cholesterol, glycoposphatidylinositol (GPI)-anchored, and other membrane proteins and are influenced by both intra- and extra-cellular factors. In mammalian cells, many biological functions occur in close proximity to membranes. Biological membranes have traditionally been viewed as a “fluid mosaic” that lacks lateral structures and are merely controlled by energetics related to their morphological shapes at the microscopic level [5]. More recently, the lateral domain organization of biological membranes at the sub-microscopic and
nanoscopic levels (i.e. membrane rafts) has become the topic of extensive study and is now believed to play an active role in many membrane processes. Although biochemical and biophysical data have been accumulated, a unifying theory for membrane rafts and its relation to membrane energetics and states has not yet emerged.

Large unilamellar vesicles (LUVs) (i.e., liposomes with a single lipid bilayer wall) and surface supported lipid bilayers are among the membrane models used for current studies [6,7]. To produce 100 nm LUVs, multiple extrusions of lipid suspensions under pressure through trace-etched polycarbonate filter pores (100 nm pore size) has been adopted. These extruded LUVs have a uniform size distribution, high degree of unilamellar structure, long-term stability, and a controlled response to changes in the environment. The mechanism of LUV formation during extrusion is known [8,9], and can be applied to both single lipid components and mixed-lipid component membranes. For LUVs extruded in isotonic solution, the balance of osmotic pressures results in a reduced internal vesicle volume for the given surface area and the minimal energy shape is approximately prolate for a range of lipid energetic parameters. When such LUVs are dialyzed against hypertonic or hypotonic solutions, water flows across the vesicle membrane to restore osmotic balance and vesicle shape changes with a corresponding adjustment in internal volume. As liposomes contact with each other or interact with surfaces, abrupt vesicle morphological transformations occur as a consequence of changes in membrane mechanical properties and interacting parameters [7]. For example, the area-difference-elasticity (ADE) model [10] describes the vesicle energy as the sum of the integration of the bending curvature over the membrane surface and the curvature-induced area difference between the inner and outer monolayers as

\[ H_c = \frac{K_c}{2} \int dA \left( \frac{1}{R_1} + \frac{1}{R_2} - C_0 \right)^2 + K_t(\Delta A - \Delta A_0)^2, \tag{1} \]

where \( K_c \) is the local bending modulus, \( R_1 \) and \( R_2 \) are the principal radii of curvature at any point on the membrane surface, \( C_0 \) is the spontaneous curvature, \( A \) is the average membrane area, \( \Delta A \) is the difference between the areas of the inner and outer monolayers, \( \Delta A_0 \) is the relaxed area difference, and \( K_t \) is a non-local bending modulus. The minimum energy shape, the shape fluctuation or flickering, and interaction-induced shape changes of the LUVs follow complicated phase-diagrams that can be used to help determine their values [11,12].

Surface supported lipid bilayers are particularly useful for lateral phase separation studies by methods such as X-ray, neutron diffraction, nuclear magnetic resonance, spectroscopic techniques, as well as scanning probe microscopy techniques such as atomic force microscopy (AFM) (e.g. Refs. [13–20]). There are two main approaches to forming supported bilayers: (1) liposome adsorption and (2) Langmuir–Blodgett surface film deposition. The major advantage of the latter method is that bilayer asymmetry can be controlled. However, for symmetric bilayers, the liposome adsorption technique is superior. It allows the direct observation of the liposome adsorption dynamics and nanoscopic to microscopic structure transitions. The curvature energy given by the ADE model is included in the energetic terms that underlay both equilibrium membrane states and dynamic properties that are important to the lateral heterogeneities of biological membranes and membrane rafts. Therefore, studies of the surface application of liposomes and their resulting supported membrane bilayers can provide insights into both membrane energetics and membrane lateral organization properties.

We have reported AFM studies of the phase transition processes of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipid bilayers [19]. In this report, we describe the results of additional AFM studies of adsorbed single bilayer vesicles of DMPC as well as supported bilayer membranes resulted from mixtures of three components, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), and cholesterol (chol.), whose ternary phase behaviors were studied using Giant Unilamellar Vesicles (GUVs) [21,22]. Using extruded LUV(s), we observed membrane structures ranging
from isolated LUVs, to partially fused membrane clusters and flat membrane sheets. For DMPC on a mica support mediated only by monovalent salt (NaCl) solutions and at various temperatures, we observed a significant shift in the main phase transition between membrane states that are sensitive to the environmental variables. For the three-component supported membrane, we observed a coexistence of both microscopic and nanoscopic domains. Quantitative analyses revealed a preponderance of ∼40 nm domains with some domains as small as ∼25 nm. We demonstrate that membrane domains are sensitive to their physical support, which modulates the membrane phase diagram by altered coupling between in-plane and out-of-plane lipid membrane interactions. The observations are relevant to biological membranes in a native environment that undergo a variety of physical and chemical modulations.

2. Materials and methods

2.1. Preparation of lipid vesicles

Phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and cholesterol was purchased from Nu Chek Prep (Elysian, MN). Purity of >99.5% was confirmed by thin-layer chromatography on washed, activated silica gel plates (Alltech, Deerfield, IL) developed with chloroform/methanol/water (65:25:4) for both phospholipids and with petroleum ether/diethyl ether/chloroform (7:3:3) for cholesterol. Phospholipid stock solutions were quantified by phosphate assay. DMPC samples in power form were also obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), and weighted with a precision balance assuming a residual 3% absorbed water.

DMPC dispersion was obtained by mixing a powered sample with salt solutions (50 mM NaCl and 0.3 mM NaN₃) followed by vigorous vortexing to a uniform and metastable suspension. Multilamellar lipid dispersions of DLPC/DPPC/chol mixtures were prepared by rapid solvent exchange, essentially as described by Buboltz and Feigenson [23], but using a simplified procedure. Lipid mixtures dissolved in chloroform, 50–100 nmol in 50–100 µl, were placed in 13 × 100 mm² screw-cap culture tubes. PIPES Buffer (5 mM PIPES, 200 mM KCl, and 1 mM EDTA, pH 7.0), 0.5–1 ml, was added and a vacuum of approximately 23 torr was applied to the tubes for 10 min while vortexing, thereby removing the organic solvent. The tubes were sealed under argon, heated to 50°C for 2 h, and slowly cooled to 23°C over 12–20 h.

Extruded vesicles were prepared in either the same salt solution or PIPES buffer from the hydrated multilamellar lipid dispersions at 0.3% (w/v) of the single DMPC, or the mixed DLPC/DPPC/chol. (∼1 mg/ml total lipid). All LUVs were prepared with a mini-extruder (Avanti, Alabaster, AL) containing two Nuclepore® polycarbonate membranes having a 100 nm pore size (Corning, Corning, NY). Extrusions were performed at a mini-extruder flow rate of about 0.5 ml/s at 49°C until a homogeneous vesicle size was obtained (∼50 cycles). LUVs prepared by this extrusion method have been characterized to be unilamellar, of elongated shape, and of a narrow size distribution centered about the 100 nm pore diameter [8,10,24].

For surface application, a 5 µl portion of a freshly prepared LUV suspension was applied onto freshly cleaved mica, incubated for various times for both supported vesicle and multi-layer membrane formation and attachment. Excess fluid was removed and the sample surface was washed with the solutions for AFM studies [19]. The sample was placed in a Multimode AFM fitted with a tapping-mode liquid cell (Digital Instruments (DI), Santa Barbara, CA), and equilibrated in the same solution for 10–30 min prior to imaging.

2.2. Atomic force microscope

A commercially available environmental chamber (Model EC12, Sun Electronic Systems, Inc., Titusville, FL.) was modified to control the temperature of the MultiMode® AFM (Digital Instruments, Santa Barbara, CA) and the sample [19]. Briefly, the chamber uses heaters and liquid nitrogen to maintain a set temperature that is monitored with built-in thermistors (Precision
series 4000 Thermometer and Model 427 pediatric skin thermistor, Yellow Springs Instruments, Yellow Springs, OH, USA) and controlled using the Microsoft Hyperterminal program (Microsoft, Redmond, WA) through an RS-232C link between the chamber and the AFM computer.

All imaging was carried out in liquid Tapping Mode™ using a Nanoscope IIIa controller, a Type D scanner, and standard silicon nitride DNP probes (Digital Instruments, Santa Barbara, CA.) with a cantilever length of 100 μm, a nominal spring constant of 0.58 N/m, and a nominal tip radius of 5–20 nm as previous described [19]. Piezo scanner calibration and probe tip size estimation were performed prior to data collection by imaging a 1.8 μm pitch surface topography reference standard (Model STR2-180, VLSI Standards, Inc., San Jose, CA) and Tobacco Mosaic Virus (TMV). The overall resonance frequency of the liquid cell and probe assembly was approximately 9 kHz. Imaging was performed at a frequency of approximately 8.7 kHz. The tapping force, calculated as the ratio of engaged to free amplitude cantilever oscillations \( A_{sp}/A_o \) [25] was maintained at 0.88 (soft tapping) to minimize sample deformation. Height- and phase-mode images were collected at 512 \( \times \) 128 or 512 \( \times \) 512 pixel resolution at a scanning rate of 1.5 Hz.

AFM images were converted into 8-bit linear grayscale TIF format for further analyses. Intensity and area measurements of the images were made using ImagePro® Plus version 4.5 (Media Cybernetics, Silver Spring, MD). Intensity histograms at each temperature were analyzed with a multi-peak Gaussian model using Origin version 5.0 (OriginLab, Northampton, MA), and data analyses were also performed with custom programs using Mathcad version 8.0 and version 2001 (Mathsoft, Cambridge, MA).

3. Results

3.1. Surface application of LUVs and structure observations

A variety of DMPC membrane structures can form on a physical support, depending upon incubation time and sample preparation conditions. The effect of application time on vesicle morphology is shown in Fig. 1. Fig. 1a shows an example of surface absorbed LUVs immediately after a short incubation time (<1 min). Figs. 1b and c show partially flattened LUVs and membrane patches starting to form at longer incubation times (>1 min); membrane fusions occurred during the formation of the large membrane patches. Fig. 1d is a three-dimensional view of an enlarged LUV adsorption area.

To analyze these images, we consider the physical factors that affect the appearance and reorganization of LUV membrane structures. The forces that attract LUVs onto mica have been described in terms such as hydration state and electrostatic interactions [20]. For our study, the critical factors affecting LUV morphology on mica include the size and shape of the free LUVs and any osmotic imbalances to which the LUVs were exposed during the adsorption process. The osmotic pressures of the NaCl solutions are given by the standard equation [24] as follows:

\[
\pi_{os}(\text{[NaCl]}) = \frac{R \gamma \ln(X_{\text{H}_2\text{O}})}{V_{\text{H}_2\text{O}}} \approx (0.049 \text{ atm}) \frac{\text{[NaCl]}}{\text{mM}},
\]

where \( R = 8 \pi k T \) is used for a typical membrane [11]. For an extruded liposome having an equivalent spherical radius \( R_s = 50 \text{ nm} \), this bending elastic energy equals the osmotic deformation energy, \( \Delta H = \pi_{os}(\Delta\text{[NaCl]})(4\pi/3)R_s^3 \), when the concentration difference across the bilayer is \( \Delta\text{[NaCl]} = 0.03 \text{ mM} \). Therefore, we can make two observations: (1) nonspherical liposomes must always have their internal NaCl concentration adjusted to that of the dialysis milieu by water permeation across their membranes; (2) fully swollen LUVs can withstand
variations in osmotic concentration change across the bilayer and experience membrane tension expressed by standard Laplace’s law \[26\] as:

\[
\tau = \Delta \pi_{os} r/2.
\]  

When the lateral tension, \(\tau\), exceeds the membrane elastic tensile strength of \(\tau_{lys} = 5.7\), the LUVs should rupture and partially release membrane impermeable ions. Accordingly, we can estimate a membrane tensile strength by:

\[
\tau_{ly} = (0.049 \text{ atmM}^{-1}) r_{\text{max}}/2,
\]  

where \(r_{\text{max}}\) is the largest mean curvature one can detect of the intake LUVs after imposing certain values for \(\Delta [\text{NaCl}]_{ly}\). For a typical membrane with \(\tau_{lys} = 6 \text{ dyn/cm} [8,26]\) and with \(\Delta [\text{NaCl}]_{lys} \approx 50 \text{ mM}\), the largest LUVs that can retain their integrity during surface application is \(r \approx [(2\tau_{lys}/\Delta [\text{NaCl}]_{lys})/(0.049 \text{ atm})] \text{ mM} \approx 46 \text{ nm}\). Smaller vesicles can withstand proportionally higher values of \(\Delta [\text{NaCl}]_{lys}\).

The morphology of the LUVs on a support surface is affected by the deformation of the LUVs under the compression force exerted by the AFM tip. Again the total bending energy is \(\Delta H_B 8\pi k \leq 2 \times 10^{-18} \text{ J}\), for \(k = 10-20 \text{ K}_B T [11]\). Since the force range of the vesicle height, \(h_v \approx r \approx 50 \text{ nm}\), the compression force must be small as \(F_t \leq \Delta H_B / h_v \approx 40 \text{ pN}\). For our MultiMode AFM using soft TappingMode\textsuperscript{MC} imaging, this condition is easily satisfied. Similarly, the attractive force between the LUVs and the mica surface should also be small to avoid excessive flattening of the LUVs. With only single-charge ions and at the total ionic strength of 50–200 mM as in our studies, there is only a weak attraction between phospholipid LUV/membranes and the mica surface [20].

Our AFM data as shown in Fig. 1 allow us to quantify LUV size and morphological distributions on a support surface. Fig. 2a shows that the distribution of the individual LUV heights, \(h_v\), ranged from 10 to 25 nm, to a height limit of close to 60 nm. We set a height cut-off value at 10 nm.
because a supported single bilayer is \( \sim 5 \text{ nm} \) thick. There are two major peaks in the height distribution at 13.9 and 23.9 nm. Fig. 2b shows the scatter plot of LUV height vs. the height-width at half-maximum (HWHM). The HWHM distribution appears fairly broad and centered at \( \sim 155 \text{ nm} \) (\( 155 \pm 5.0 \) (SEM) nm). For flatter vesicles with heights of \( \sim 13 \text{ and } 25 \text{ nm} \), vesicle radii varied between 50 and 350 and 75–300 nm, respectively; the taller vesicles have narrower size ranges. Dividing the vesicle population into three height groups (A: \( h_v < 17 \text{ nm} \), B: \( 17 \leq h_v \leq 30 \text{ nm} \), and C: \( h_v > 30 \text{ nm} \), according to the minima in the population histogram), their morphological shapes can be characterized by the ratio, HWHM/\( h_v \), as shown in Fig. 2c. Height group C has a ratio of \( 4.5 \pm 2.2 \) (SD), whereas the ratios for group B (\( 10.30 \pm 5.25 \) (SD)) and A (\( 7.0 \pm 2.6 \) (SD)) are larger and more broadly distributed. However, it is clear that more vesicles are flatter, thinner, and more stretched out on mica.

For these DMPC vesicles, \( \Delta [\text{NaCl}]_{\text{lys}} \) is the maximum outward osmotic stress imbalance they experience on a mica surface. Any osmotic stress with higher extra-vesicular solute concentration would shrink the vesicles and be less likely to induce lysis. Assuming group C represents isolated pre-lysis LUVs and group A and B are consist of ruptured and fussed vesicles, we can estimate the lysis strength from Eq. (4). Since the LUVs in height group C have a maximum height of 60 nm (Fig. 2a), the simplest consideration of the geometrical constrains sets an upper limit for \( r_{\text{max}} = \frac{h_v}{2} \geq 30 \text{ nm} \). Therefore, this gives a corresponding
lower limit of $t_{\text{lys}}^\text{low} \approx 4 \text{ dyn/cm}$. The most probable value for $t_{\text{max}}$ is larger than $h_v/2$ by a geometric factor for the LUVs in the height group C (Figs. 2b and c), and therefore, $t_{\text{lys}}$ for DMPC should be a few times larger than the above lower limit of $t_{\text{lys}}^\text{low}$. A precise estimate of this geometric factor depends on the non-spherical shapes of the LUVs both in free space and on mica surfaces during the surface application process dictated by the reduced LUV internal volume and an interplay between membrane energetics and surface interaction parameters (cf. Eq. (1)). Nevertheless, our result of $t_{\text{lys}}^\text{low} \approx 4 \text{ dyn/cm}$ is in good agreement with the reported value $t_{\text{lys}} \approx 6 \text{ dyn/cm}$ [8,26] for DMPC. Therefore, our AFM approach yields reasonable values for the viscoelastic properties of membranous vesicles. From the formation process reported above, we now have insights regarding the factors leading to surface supported membranes.

### 3.2. Phase transition on a supported DMPC membrane

In an earlier report [19], we showed that isolated vesicles on a mica surface can undergo membrane fusion to form large single bilayer membrane patches, nearly continuous bilayer sheets, or multi-layered arrays. In addition, we demonstrated that the membrane structures responded to temperature changes and revealed structural transitions correlated to intrinsic membrane properties. We now show that nearly complete bilayers respond similarly (Fig. 3). The AFM height images provide clear contrast between two states of DMPC membrane sheets near the acyl-chain melting transition temperature. As temperature increased, the area fraction of the thinner membrane increased from nearly 0% at 23°C to nearly 100% at 32°C and a boundary between the thinner and thick membrane evolved (Fig. 3). Cross-sectional analyses (Fig. 4a) confirmed that a single membrane layer is present on the mica surface, which is visible through small membrane holes. Estimates of membrane heights are $\sim 4.2$, $\sim 3.6$, and $\sim 0.49$ nm, respectively, for the thick membrane, thin membrane and for the difference in their thickness. As temperature increased, the

Fig. 3. Supported bilayers for structural observation DMPC vesicles form single bilayer membranes to multiple bilayer membranes over a mica surface. A series of AFM images ($7.4 \mu m \times 1.85 \mu m$) acquired during a heating ramp at the rate $0.1^\circ C/\text{min}$ shows that the flat DMPC bilayer with small amount of uncovered surface underwent phase conversion near the DMPC main transition temperature. The liquid-crystalline phase began at the edge of the membrane and expanded towards the center. Height images show that the fraction of thinner, liquid-crystalline bilayer increased with temperature (white letters, °C). The expansion of the bilayer at higher temperature is evident from the shrinkage of the membrane holes reaching down to mica surface.
membrane holes decreased in size corresponding to an increase in area of the lipid upon conversion from the thick phase to the thin phase. In an earlier report [19], we identified a similar phenomenon in isolated DMPC bilayer patches and associated this phenomenon to DMPC undergoing temperature-induced phase transitions between the low temperature gel-phase (thick membrane) and the high temperature liquid-crystalline phase (thin membrane). Here, we compare directly (Fig. 4b) the phase transition properties of a nearly intact bilayer membrane (Fig. 3) with properties reported previously for micrometer size bilayer patches. For large membrane patches with temperature increases at the rate of \( \sim 0.1^\circ C/\text{min} \), the lipid molar ratio showed good agreement with our nanoscopic domain model equation [19],

\[
\rho_G = \frac{1}{1 + e^{\Delta H N(T - T_m)/RT_m^2}} \\
\approx \frac{1}{1 + (A_L/A_G)(h_L/h_G)},
\]

yielding an intrinsic DMPC domain size, \( N \), of 18–75 lipid molecules per leaflet (Fig. 4b, ●). The measured gel/liquid-crystalline area ratio \( A_G/A_L \) and the membrane height ratio \( h_G/h_L \) are deduced directly from AFM images; we also used the estimates of \( T_m = 28.5^\circ C \) and the enthalpy value range of \( \Delta H = 2–8 \text{ kcal/mol} \) [27]. As the surface area per DMPC molecule is \( \sim 60 \text{ Å}^2 \) [28] and the area per lipid domain is \( \sim 1380 \text{ Å}^2 \) when \( N/2 = 23 \) lipid molecules per leaflet, the domain diameter \( d \) is \( 2(13.8/\pi)^{1/2} \text{ nm} \approx 4.2 \text{ nm} \).

For the data reported here from our membrane (Fig. 3), we find similar membrane area changes associated with the phase conversion process (Fig. 4c, △). The agreement with the model is excellent near the middle of the transition at \( T_m = 28.5^\circ C \), but not as good at temperatures more than two degrees above or below the \( T_m \). Hence, our previous estimate of 23 lipid molecules per leaflet with a domain diameter of \( \approx 4.2 \text{ nm} \) also applies to nearly intact membrane sheets. The slight deviation at lower and higher temperatures may be due to factors such as uncertainties in area measurements for high aspect ratio phase boundaries (Fig. 4c, △).

The data reported here on complete membrane sheets amplifies our earlier report that the intrinsic domain diameter derived from quantitative phase conversion measurements is about 5 nm. The phase transition for supported lipid membranes is broad over \( \sim 10^\circ C \). Consequently, the gel and liquid crystalline phases coexist throughout the transition process.

### 3.3. Nanoscopic domains in Chol/DLPC/DPPC membranes

Lateral domain separations also occur in mixed lipid membranes and we have used similar techniques to study supported bilayers of DLPC/DPPC/
chol. mixtures. A comprehensive giant unilamellar vesicle (GUV) phase diagram based on confocal florescence microscopy (CFM) and florescence resonance energy transfer (FRET) measurements of this mixture system has been published [22]. Since a mica surface modulates membrane energetics, the phase behavior of the supported membrane may differ significantly from that of a GUV membrane. Here, we compare the structural features of single DMPC lipid membrane with that of a three-component membrane that better mimics the naturally occurring biological membranes.

Our AFM observations reveal that nanoscopic and microscopic domains can coexist under a range of composition mixtures at room temperature with or without cholesterol (Fig. 5). For example, we detected clear nanoscopic domains at $\chi_{\text{DPPC}} = 0.7$ and $\chi_{\text{chol}} = 0.135$ (Fig. 5b). The nanoscopic domains are $\sim 1.4$ nm higher than the majority of surrounding lower membrane sheets. Since DLPC lipids with shorter 12-carbon double chains are known to be in a liquid crystalline state at room temperature, and DPPC lipids with 16-carbon double chains are known to be in a gel state, we can identify the higher domains as a DPPC-rich phase and substantially more ordered than the surrounding membrane composed of a DLPC-rich phase. To elucidate the relationship between lipid mixture ratio and domain formation, we quantified the nanoscopic domain size distribution and used a multi-peak Gaussian model to obtain a peak domain size of 48.4 $\pm$ 0.2 nm (Fig. 5c). The majority of the domains were nanoscopic at $<100$ nm in diameter and no micron-size domains were observed. Such domain structures at $\chi_{\text{DPPC}} = 0.7$ and $\chi_{\text{chol}} = 0.135$ observed by AFM would appear homogeneous at the light microscopy level.

Domain appearance changed between $\chi_{\text{chol}} = 0$ (Fig. 5a) and $\chi_{\text{chol}} = 0.135$ (Fig. 5b), showing that the presence of the cholesterol caused a dispersion of larger domains in favor of a larger number of nanoscopic domains. These data confirm our observation that membrane structural features exist at the nanometer level. Our collective evidence suggests that the supported membrane $\chi_{\text{DPPC}} = 0.7$ and $\chi_{\text{chol}} = 0.135$ may correspond to region D of the GUV phase diagram between $\chi_{\text{chol}} = 0.16$ and $\chi_{\text{chol}} = 0.25$ [22]. Comparing such observed data for supported membranes to previous data for free GUV membranes, we conclude that membrane phase transitions and properties are sensitive to surface support and other parameters.

4. Discussion

4.1. LUV extrusion mechanism and size distribution

Our observations confirm and extend existing knowledge of liposomes and lipid membranes. Both DMPC and mixed lipid suspensions can be
extruded through polycarbonate filters to produce LUVs. The pressure extrusion behavior is consistent with the mechanism proposed by Clerc and Thompson [9] (Fig. 6a). For DMPC LUVs extruded in 50 mM NaCl (DMPC sample) and for DLPC/DPPC/chol. LUVs extruded in the PIPES buffer, the initial vesicle morphology on a mica surface suggests that the LUVs when in a bulk suspension were in the 100 nm size range (Fig. 6b). The reduction in height to about 50 nm as compared to the horizontal dimensions of ~200 nm diameter for the surface DMPC vesicle population agrees with the following facts: (1) LUVs undergo a large internal volume reduction (about 50%) related to their proposed vesicle membrane area; (2) LUV morphology both in solution and on a mica support follows energetics described by the ADE model; (3) large LUVs are destabilized through osmotic lysis and can fuse into larger membrane patches, single membrane sheets, and multiplayer membrane stacks.

### 4.2. Domain structures resulting from temperature and lipid composition modulations

We demonstrate that intrinsic membrane domains are as small as ~5 nm based on temperature induced melting transitions of mica-supported DMPC membranes. We also directly visualized the coexistence of nanoscopic domains with microdomains in a three-component DLPC/DPPC/chol. mixed lipid membrane on a mica surface. The nanoscopic domain size distribution is unimodal and centered at a diameter of ~40 nm.

It is reasonable to question the connection between intrinsic membrane domains and the nanoscopic domains as visualized directly by AFM. It is also useful to question the relationships between the above domain sizes and other measurements of membrane lateral structures such as the differential scanning calorimetry (DSC)-derived cooperative unit size [29], cellular membrane patch size [15,30], and other membrane “rafts” sizes [1,31].

The data reported here add new insight into the complexity of supported lipid membrane behavior. For DMPC in particular, an unusual signature at about 28°C in probe diffusion by fluorescence recovery after photobleaching (FRAP) has been reported for a multi-layer membrane stack on a glass substrate [32]. The AFM images presented here are in good agreement with that study. With the high resolution of the AFM, lateral membrane organizations in the nm spatial range have now been seen both in a single lipid system near chain orientation-order transitions and in a mixed component system over a broader temperature range. The phase diagram shift on a supported membrane we observed by AFM supplements CFM studies of free GUV membranes, and furthers the concept that membrane states and lateral organizations leading to rafts must be carefully assessed in the context of interactions with neighboring entities.

### 5. Biological implications and conclusion

In biological systems, cell membranes functionally interact with cytoskeletal components, neighboring membrane stacks, extracellular matrices, and other elements. These interactions alter native lipid phase behaviors, as do proteins, alcohols, and certain drugs [33–35]. Obviously, cellular membranes are more complex than model lipid
mixtures, and the resulting membrane domains or "rafts" may depend on such things as the net negative charge of the membranes, strength of interactions, membrane composition, and external effects similar to those we observed using a mica-support. The exact nature of these modulations, however, must be sought within the cells or the biological system. Nevertheless, nanoscopic domains appear to be an intrinsic and prevalent feature of membrane lateral organization. We have demonstrated an approach for preparing nanometric surface supported membrane structures and their utilization for probing membrane bilayer lateral organizations from nanoscopic domains to microscopic phase behaviors. Our data provide new insights into the marked differences between free vesicle systems and supported membrane bilayers that are useful toward understanding the native biological membranes.

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