

Structure of Spin-Coated Lipid Films and Domain Formation in Supported Membranes Formed by Hydration

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An atomic force and fluorescence microscopy investigation of the structure of spin-coated lipid films is presented. In the surface of the dry film, lipids are found to orient in a conformation where acyl chains are pointing outward while laterally the individual layers of the multilamellar film exhibit a dewetting pattern similar to what is found in polymer thin films. Hydration of the film in liquid water promotes detachment of bilayers from the surface while a single membrane remains on the mica substrate. This supported membrane is highly uniform and defect-free as compared to supported membranes prepared by conventional methods. It is further demonstrated that supported membranes of binary lipid mixtures prepared by this method exhibit gel–fluid domain coexistence in accordance with expectations from the phase diagrams.

1. Introduction

Solid-supported lipid membranes represent an important class of model systems in membrane biophysical research. One main reason is that these systems enable high-resolution structural investigations of membrane lateral organization with scanning probe techniques such as atomic force microscopy (AFM)^{1,2} and near-field scanning optical microscopy³ thereby going beyond the diffraction limit of conventional light microscopy. In recent years, studies of lateral lipid organization and domain formation in model membranes have been rapidly increasing. These studies have gained relevance from the hypothesis that specialized domains exist in cell membranes^{4,5} and that these might be correlated with the localization of lipid anchored proteins of importance for cell signaling and trafficking. A central issue in the discussion has been the question of the characteristic size of such functional membrane domains which could be anywhere from nanometers to micrometers. For direct visualization and size determination of small membrane domains, scanning probe microscopy of supported membranes is highly relevant. From an applied perspective, supported membranes are interesting as the basic structural elements in potential biosensors where biomolecular recognition events are translated into a physical readout.^{6,7} It has also been shown that supported membranes have potential use in efforts to control cell adhesion properties of solid surfaces⁸ of relevance for design of biocompatible materials such as implants.

Two common methods exist for the preparation of supported membranes. They are (1) the spontaneous spreading of unilamellar vesicles on the substrate surface and (2) Langmuir–Blodgett (LB) or Langmuir–Schäfer (LS) transfer of two consecutive lipid monolayers to the substrate to form a bilayer. Vesicle spreading was first discovered and used by Brian and McConnell⁹ to study immune response and is now a widely used standard technique. Vesicles are typically prepared by sonication, extrusion, freeze-thawing, or a combination of these, and spreading on the substrate takes place by simply exposing the substrate to the vesicle suspension. The bilayer structure can be equilibrated before transfer to the support, and the procedure allows membrane proteins to be incorporated in the membrane. A limitation is that the success of the spreading process may depend critically on factors such as the membrane composition, substrate material, concentration of salts, pH, temperature, vesicle size, washing procedure, and more.¹⁰ In LB transfer,^{11–13} a lipid monolayer film is spread at the air–water interface from an organic solvent and after compression of the film to a desired pressure it is transferred to the substrate. LB transfer gives control over the film pressure at the air–water interface and allows monolayers and asymmetric bilayers to be prepared. Limitations are that a successful transfer of two monolayers to make up a supported bilayer may depend on several factors including those mentioned above for vesicle spreading. When studying lateral domain organization in supported membranes of lipid mixtures, nonequilibrium domain structures and decoupling of the membrane leaflets has also been observed which presumably resulted from the LB/LS preparation scheme.¹⁴ For these reasons and to take full advantage of supported

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membranes as biological model systems, alternative preparation routes would be desirable.

Spin-coating^{15,16} is a common technique for application of thin, uniform coatings to surfaces. The coating is dissolved in a solvent that wets the substrate, and after placing a droplet on the substrate, rotation of the substrate while the solvent evaporates produces a uniform coating. Recently, spin-coating of lipids has been applied to produce supported highly oriented, uniform multimellar lipid films suitable for investigations with X-ray reflectivity.^{17,18} The number of bilayers in the film was found to scale in a linear fashion with the concentration of the lipid solution. Upon hydration in liquid water, the multilamellar configuration was shown to be unstable and detach from the substrate except for one membrane remaining in the substrate.

In this work, AFM and fluorescence microscopy is used to study the detailed structure of spin-coated lipid films before and after hydration. We use mica as substrate material because it is widely used for AFM studies of supported membranes due to its atomic flatness. Many other substrates are in use as substrate materials including glass, polymer-coated surfaces, and chemically functionalized substrates. The lipid film at varying coverages is studied and, on the basis of the AFM height and phase information a model for the lipid orientation in air is suggested. Upon full hydration, a defect-free single bilayer remains on the substrate, and the structure of this membrane as well as that of the detached bilayers is investigated. Binary lipid mixtures are also found to produce defect-free supported membranes upon hydration, and this may be accompanied by lateral re-organization of the lipids into micrometer-sized domains. In this respect, the method is fundamentally different from other preparation methods in that the lipid domain formation takes place while the membrane is on the substrate. Lipid lateral separation is demonstrated for a binary phospholipid mixture and for a ceramide phospholipid mixture. The lipid domain structures obtained are discussed in relation to alternative preparation protocols.

2. Experimental Section

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and brain ceramide (BC) were purchased from Avanti Polar Lipids, and 1,1'-dioctadecyl-3,3',3'',3'''-tetramethylindocarbocyanine perchlorate (DiIC₁₈) was from Molecular Probes. BC contains a mixture of ceramide chain lengths¹⁹ with C₁₈-ceramide (46%) being the most abundant component. Solvents (hexane, methanol) were all HPLC grade quality, and for hydration experiments Milli-Q water was used throughout. The main requirements to the spin-coating solvent are that it should wet the hydrophilic substrate and at the same time be able to dissolve the lipids of interest. Moreover, we have found by AFM controls that the uniformity of the film on a micrometer scale is also influenced by the solvent. Several solvents were tested for their suitability, and we have found hexane to be the most optimal candidate in this respect. To promote the solubility of BC and DPPC, 2–5% of methanol was also added. Freshly cleaved muscovite mica (Plano GmbH, Germany) with a size of 8 × 8 mm² was used for all coating experiments. Spin-coating was performed using a Chemat Technology, KW-4A spin-coater that was accelerated immediately

to 3000 rpm for 40 s upon application of the lipid solution. A volume of 15–20 μL was applied, which was sufficient to cover the surface completely before starting the rotation. Provided that the droplet wets the entire surface area, the actual amount of the lipid suspension applied is not critical because the excess liquid volume is removed during rotation. The critical parameter for controlling the film thickness is the concentration of the coating solution. For experiments with lipid mixtures, a total lipid concentration of 7 mM was used for spin-coating, which is typically sufficient to produce complete coverage of the substrate. Both hexane and methanol have been reported^{20,21} to form molecular thin films on mica when placed in vapor or liquid. To ensure maximal evaporation of solvents, the samples were after rotation placed under a vacuum in a desiccator for 10–15 h. Separate AFM control experiments using the pure solvents (without lipids) did not reveal any sign of a molecular thin film after this procedure. Immediately after storage in a vacuum, the samples were measured with the atomic force microscope using a PicoSPM (Molecular Imaging, Tempe, AZ) operated in conventional tapping mode (AAC). The relative humidity during AFM measurements was 20–30%. For tapping mode measurements on lipid films in air, we used Si cantilevers (NanoSensors, NCL-50) with a resonance frequency in air of 150 kHz and a force constant of $k_{\text{NCL50}} = 48$ N/m (nominal). For measurements of the hydrated samples in fluid water, a homemade fluid cell was used and the AFM was operated in magnetic tapping mode (MAC) using MAC levers operated around their resonance frequency of 25 kHz (in water) with a force constant of $k_{\text{MAClever}} = 2.8$ N/m (nominal). The scan rate used for imaging was in all cases in the range of 1.2–1.9 Hz. The free amplitude for tapping mode imaging was in all cases around 8.5 V, and the amplitude on scanning was around 6.5 V. Note that MAC mode imaging used for hydrated membranes in water is generally much more gentle for the sample than conventional tapping mode imaging. We have never observed scanner-induced damage to supported membranes when operating in MAC mode.

Force–distance measurements were performed in an MFP-3D system (Asylum Research, Santa Barbara, CA), and force curve analysis was done in Igor Pro (Wavemetrics). The silicon nitride cantilevers used for force measurements were of the triangular type (MSCT-AUHW, F-lever, Veeco Instruments) with a nominal spring constant of 0.5 N/m and a resonance frequency of 120 kHz. The same cantilever was used for comparison of results on the mica and POPC coated samples in Figure 4B. The measured force constant of this cantilever was $k = 0.825$ N/m and was calibrated using the thermal fluctuation method. The relative humidity during force curve measurements was 50–52%. Hydration of the binary lipid mixtures included an annealing procedure where the fluid cell was heated to above the miscibility phase transition temperature for about 1 h and re-cooled to room temperature to bring the membrane close to equilibrium. Confocal fluorescence microscopy (Figures 2B and 5A) was done in an inverted microscope system (Zeiss LSM 510 META) with the sample placed in a microscope chamber (Lab-tek Brand Products, Naperville, IL) with the lipid side facing the microscope objective. Water immersion and air objectives (40× and 10× respectively, Zeiss, Germany) were used, and the excitation wavelength for DiIC₁₈ was 543 nm. Epi-fluorescence imaging of domains in the hydrated membrane (Figure 7) was done in a Nikon TE2000 inverted microscope using a G-2A filtercube (Nikon), mercury lamp excitation, and a 40× ELWD air objective. A coolsnap of color camera (Photometrics, Roper Scientific, Tucson, AZ) was used for acquiring the fluorescence images. The sample was placed in the same type of sample holder as that used for confocal imaging.

3. Results

3.1. Dewetting Patterns in Dry Spin-Coated Films.

The morphology of the spin-coated lipid film is investigated with respect to a variation of the lipid concentration in the coating solution. We have chosen POPC because it is

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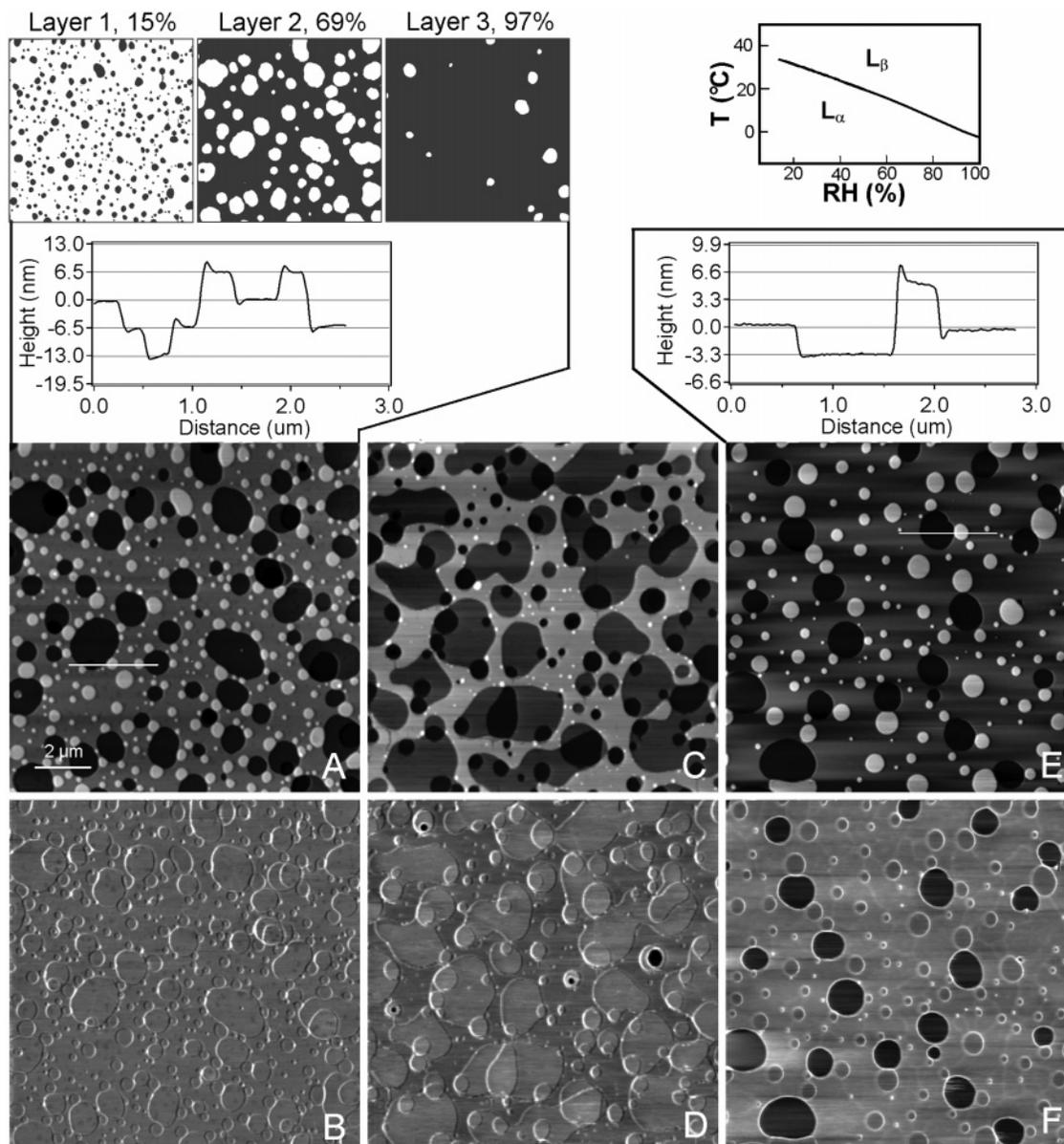


Figure 1. AFM imaging of spin-coated POPC multilamellar films in air with all images covering $10 \times 10 \mu\text{m}^2$. Images in parts A, C, and E are topographical maps whereas parts B, D, and F show the corresponding phase images. Lipid concentrations used for spin-coating the three films were 5 mg/mL (A, B), 3 mg/mL (C, D) and 2 mg/mL (E, F). Complete coverage of the substrate has been achieved in part A whereas for films in parts C and E, the substrate is partly exposed in the areas where the phase image shows dark contrast. The film surface is layered with a terrace height for full coverage of $62 \pm 5 \text{ \AA}$ (line scan indicated in part A). Each layer in the film exhibits a dewetting pattern as illustrated for the three layers visible in part A (top left). For the boundary layer that neighbors the substrate surface, the layer height is reduced to almost half, indicating that this is a monolayer (line scan of part E). The lyotropic phase diagram for POPC bilayers was adapted from Binder and Gawrisch²² (top right).

abundant in nature and regarded as a good model for fluid biological membranes. Figure 1A shows the topology in air of POPC that is spin-coated on mica in a concentration of 5 mg/mL. The surface has a lamellar structure with completely planar layers and an interlayer spacing which is determined by line scans to be $62 \pm 5 \text{ \AA}$. This thickness is considerably higher than for a hydrated fluid POPC membrane. However, POPC is known to undergo a lyotropic chain melting transition from the solid L_β to the fluid L_α state upon hydration, and the repeat distance in L_β POPC multilayers has previously been measured to be around 58 \AA by Binder and Gawrisch.²² Because our spin-coated films have been stored under vacuum and the relative humidity (RH) during AFM measurements was

between 20 and 30%, we conclude that the POPC films in Figure 1 are in the solid L_β state.

The phase image corresponding to Figure 1A is shown in part B, and this image contains basically no contrast except at the boundary lines of the lipid layers. The absence of contrast in extended areas is a clear sign that the substrate is completely covered by lipids and none of the holes in the lipid layers extend all the way to the substrate. The outer individual lipid layers exhibit partial coverage as illustrated (top left) for each layer in Figure 1A. The characteristic dewetting patterns that are formed in each lipid layer are closely resembling theoretically and experimentally observed dewetting patterns observed in thin polymer films.^{23–25} These patterns are most likely

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initiated by the thinning of the lipid film during the spin-coating process which leads to rupture of the outer lipid layers, hole formation, and eventually film breakup. Once the solvent has evaporated, the patterns in the spin-coated dry films are found to be fairly rigid and stable over time. The outer layers of the film exhibit different degrees of film breakup depending on their proximity to the air interface. The outermost layer is a noncontiguous layer made up of nearly circular droplets and covering 15% of the surface area. When interpreted in terms of the standard thin film dewetting picture, this corresponds to a late stage in the dewetting process where holes in the film have merged and the rims between holes are broken up into droplets by a classical Rayleigh instability. Successive layers beneath the outermost layer contain holes, but these layers are more contiguous and could be considered to correspond to earlier stages in the dewetting process. Recently, Perino-Gallice et al.¹⁸ have speculated on spinodal dewetting to be a possible mechanism for the breakup of multilamellar spin-coated lipid films upon hydration in water vapor. Although this could possibly be the case, we would like to point out that a dewetting pattern is also present even in the dry, nonhydrated spin-coated film. The images in Figure 1 do suggest that there is a characteristic length scale of a few micrometers associated with the breakup patterns. On the other hand in none of our sample have we observed evidence of an undulated breakup pattern with a characteristic wavelength usually associated with spinodal dewetting.²⁶

For POPC films that have been coated from lower lipid concentrations, the substrate eventually becomes exposed in the holes of the lipid film. This is expected because the number of lipid layers should scale roughly linearly with the lipid concentration, as reported previously.¹⁷ The onset is clearly seen in Figure 1C,D (3 mg/mL) where the phase image exhibits a few dark areas corresponding to areas where the substrate is exposed. The substrate (mica) is a hard crystal and will have a different viscoelastic response to the cantilever motion than the lipid film, and this difference gives rise to contrast in the phase image^{27,28} whenever both surfaces are in the scan window. When the lipid concentration in the coating solution is reduced further (2 mg/mL, Figure 1E,F) the holes exposing the substrate grow in size as evident from the larger dark regions in Figure 1F. Also, in this case a line scan of the topography reveals that the lowest lipid layer adjoining the mica substrate has a reduced height of 33 ± 5 Å. This is slightly more than half the thickness of the remaining lipid layers in the film that are not neighboring the substrate, leading to the interpretation that the lowest layer in Figure 1E is a lipid monolayer. Even in the dry samples, a water film may possibly exist between the mica and the lowest lipid film, thus, accounting for the observation that the monolayer is measured slightly higher than expected.

3.2. Supported Membrane Formation. The hydration behavior of a spin-coated POPC film made from a 5 mg/mL solution is demonstrated in Figure 2. This concentration was chosen because it is above the approximate level where the substrate is fully covered by the lipid film, as shown in Figure 1A. Thus, the effect of holes in the lipid film on the hydrated structures should be minimized.

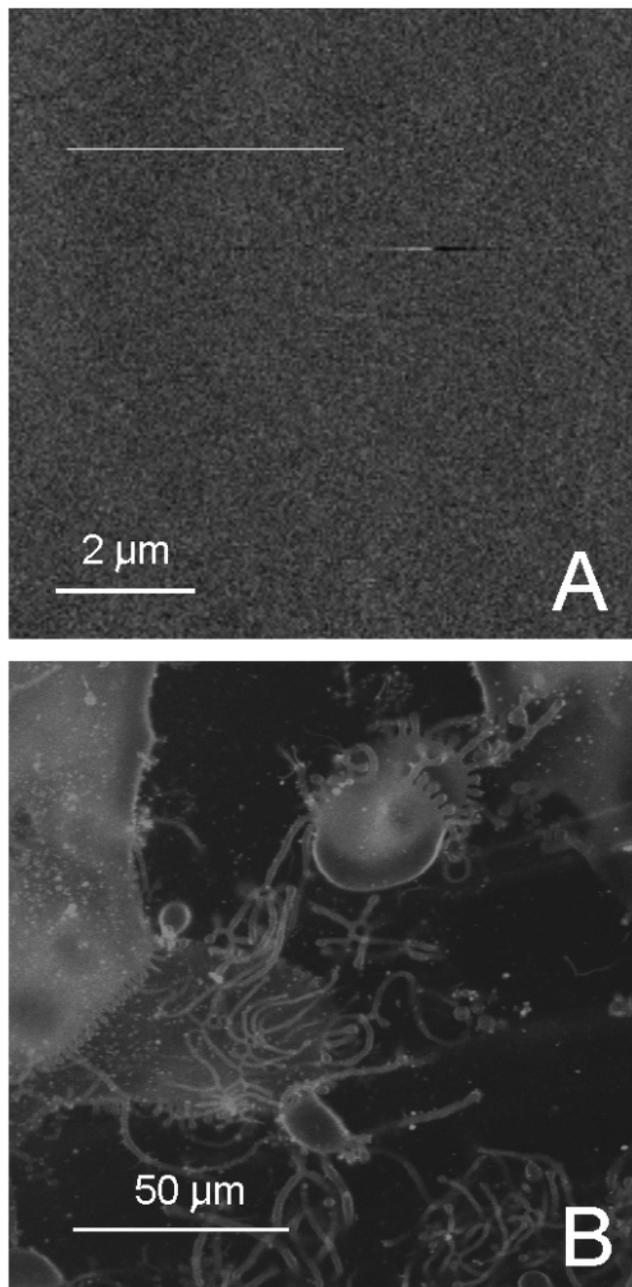
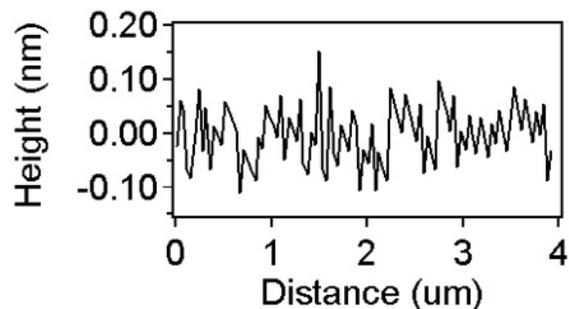


Figure 2. Structure of a fully hydrated POPC film spin-coated from a stock solution of 5 mg/mL. The fluid cell AFM imaging of the film after hydration for 30 min (A) shows a perfectly smooth sample surface with height variations of 1–2 Å. This situation corresponds to a defect-free supported membrane. Confocal fluorescence imaging of the surface region of an equivalent sample containing the probe (0.5% DiIC₁₈) is shown in part B. It reveals highly complex three-dimensional lipid bilayer structures that are extending outward from the substrate surface.

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Figure 2A shows an aqueous AFM scan after hydration at 20 °C for 30 min. The sample topography is completely flat and smooth with only random variations below 2 Å corresponding to sample fluctuations and instrument noise. As previously indicated from data by Mennicke et al. for DMPC spin-coated on glass substrates, full hydration in liquid water produces detachment of all lipid layers except for one single membrane that remains on the substrate. The flat topography observed in Figure 2 shows that this supported membrane is highly uniform and defect-free. This uniformity of the supported membrane is a general property observed for the supported membranes that we have prepared by this procedure. In contrast, membranes prepared by alternative techniques such as vesicle spreading¹⁰ or LB transfer²⁹ are often observed to contain defects in the form of holes that are distributed over the membrane area.

Multilamellar vesicles (MLV) are well-known to form when a dry lipid film is immersed in water or buffer³⁰ above the main phase transition temperature. Swelling of the lamellar film is accompanied by detachment from the surface of membrane structures in the form of tubes and vesicles in addition to highly complex membrane geometries. Figure 2B confirms that such complex bilayer structures are also released from a spin-coated lipid film. The swelling and formation of membrane structures is a dynamic process that evolves over many hours³¹ and depends on system parameters such as temperature and osmotic pressure that may be applied to the membrane.³² When AFM imaging is performed on the fully hydrated film, the cantilever will during approach and scanning apply forces and generate a stirring motion in the surface region. We find that these forces are sufficient to penetrate and possibly wipe away the detached bilayer structures that are in the 0–100 μm region away from the support surface. Thus, the AFM image in Figure 2A reveals the structure of the supported membrane remaining on the substrate only. Furthermore, it is possible by washing of the fluid cell to remove detached bilayers when the hydration is completed analogous to the washing procedures employed when supported membranes are prepared by vesicle spreading to remove excess vesicles. This is especially an advantage for quantification purposes because it makes the total membrane area in the cell more well defined and corresponding to the area of the support surface.

3.3. Structure of the Dry Lipid Film. In the dry film, the thickness of the lipid layer adjoining the substrate suggests that this is a monolayer. As proposed in Figure 3A the lipid headgroups will in this case be oriented toward the hydrophilic substrate while the acyl chains are directed outward to the air/vacuum space. The only possibility for the next bilayer to be compatible with the boundary monolayer is if it has an inverted bilayer configuration, as shown in Figure 3A. The total film structure is stable in air because all lipids in the outer surface will have their acyl chains pointing outward while the polar headgroups are interacting either with the polar substrate or with opposing lipid headgroups. When a thicker lipid film is applied to the substrate by increasing the concentration of the coating solution, the structure will be formed by extending with additional inverted bilayers, as

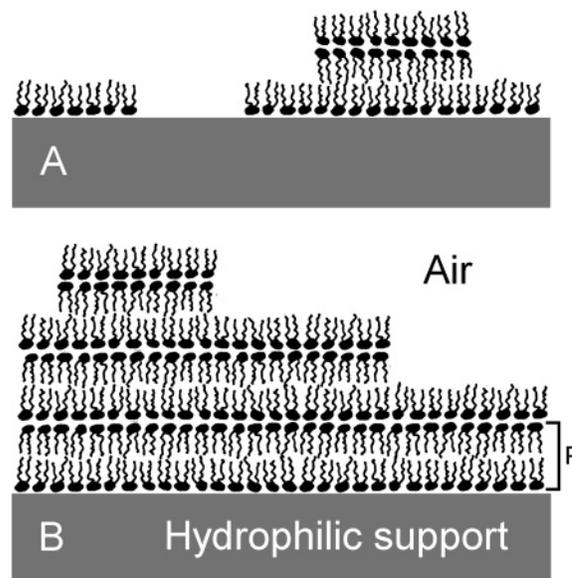


Figure 3. Schematic illustration of the proposed lipid orientation in spin-coated films on hydrophilic substrates. At low lipid concentration (A) the substrate will only be partially covered. The layer adjoining the substrate is a lipid monolayer with headgroups oriented toward the hydrophilic substrate. Additional layers on top of this are inverted bilayer structures with the acyl chains pointing outward. At higher lipid concentration (B), the substrate surface will be fully covered by lipids. In this case, a noninverted (normal) bilayer membrane denoted P is neighboring the substrate.

shown in Figure 3B. Such a thick multilamellar film will appear as a sequence of normal bilayer membranes resting on the support surface while the outermost surface will have the lipid acyl chains pointing outward due to the hydrophobic nature of the air/vacuum space. Upon hydration in liquid water, a gradual detachment of membrane structures from the surface is observed that eventually results in only a single membrane, denoted P in Figure 3B, remaining on the substrate.

The hydrophobic nature of the lipid-coated surface as compared to mica was further probed and confirmed by force–distance measurements as shown in Figure 4. A typical force curve for these surfaces is shown in Figure 4A. The total attractive force existing between the silicon nitride tip and the surface is composed dominantly of capillary forces and van der Waals forces, but for a hydrophobic surface the capillary force will be absent, because a water meniscus cannot be formed.³³ This will generally give rise to a lowered adhesion force. On the other hand, when a water meniscus is absent, the van der Waals force will typically increase as a result of an increase in the Hamaker constant.³³ An accurate quantitative prediction of the total change in the adhesion force when going from a hydrophilic to a hydrophobic coated surface does not exist, and the specific forces measured will also depend strongly on air humidity and the curvature radius of the tip. In our case, the force–distance curves in Figure 4B were measured at a relative humidity of RH = 50–52%, and they show a decrease in the adhesion force on the lipid-coated mica surface of ~40% relative to the pure mica surface. For a rough comparison, measurements by Xiao and Qian³³ on SiO₂ and on silanized SiO₂ (hydrophobic) show a similar decrease in the adhesion force on the hydrophobic surface of approximately 45%, at the same relative humidity. On the basis of this, we conclude that the force–distance data presented here support the model

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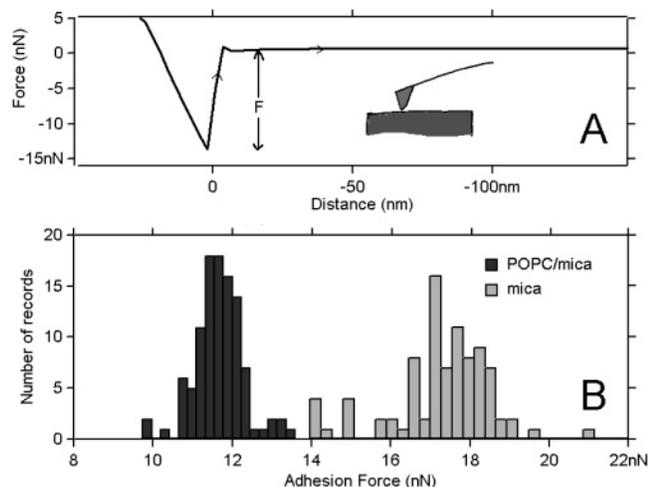


Figure 4. Measurement of adhesion forces between the AFM tip and mica and mica spin-coated with POPC (5 mg/mL). A typical force curve for retraction of the tip from the surface (A) exhibits adhesion until unbinding occurs at $F \sim 10\text{--}20$ nN. The distribution of adhesion forces (B) shows a decrease by $\sim 40\%$ in the average adhesion force for the lipid-coated surface as compared to pure mica.

for the lipid orientation proposed in Figure 3 and that they comply with the resulting hydrophobic nature of the lipid-coated surface.

The uniformity of a spin-coated lipid film can be compared with the nonuniform structures obtained by simple drying of a droplet of the lipid suspension that is placed on mica. Lipid films made by simple or modified drying procedures have often been used in preparation of samples for scattering experiments. Figure 5A shows fluorescence imaging of the drying pattern formed in a POPC film by this procedure. The ring-shaped structures, typically referred to as “drying rings”, are generally observed whenever a droplet of solid suspended in a liquid dries out on a substrate.^{34,35} Material is deposited at the outer ring of the droplet when the contact line is pinned to the substrate by defects and material is transported radially outward to the region of the contact line. Figure 5A also shows drying features that are oriented radially to the rings (“spokes”), in support of this mechanism. The AFM topography of the dried lipid film shown in Figure 5B exhibits height variations of several hundred nanometers but also demonstrates that a lamellar structure of the dry lipid film on the substrate exists in the non-homogeneous film. Thus, for experiments with multilamellar lipid films on substrates where lateral uniformity of the film is important, the use of a spin-coating procedure for applying the film is clearly an advantage.

The model proposed in Figure 3 might shed new light on the so-called vapor pressure paradox put forward by Rand and Parsegian.³² This describes the common experimental observation that a dry lipid film will have a tendency to take up less water when hydrated in 100% RH water vapor than if hydrated in liquid water. This effect has also been observed in spin-coated lipid films.¹⁸ Several interpretations of this observation exist including a theory³⁶ by Parsegian and Podgornik describing how thermal undulations in the outer lipid surface control the degree of swelling and experiments by Katsaras and Nagle indicating that the paradox is merely a technical problem

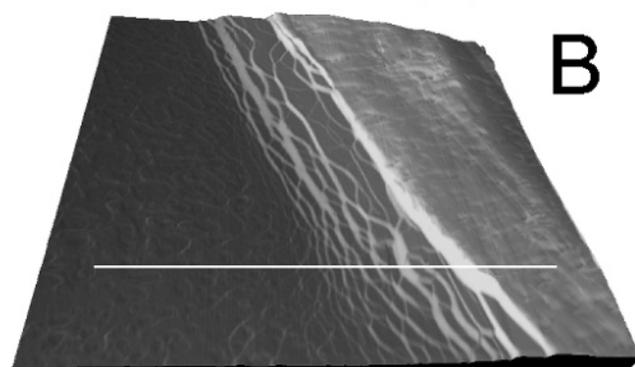
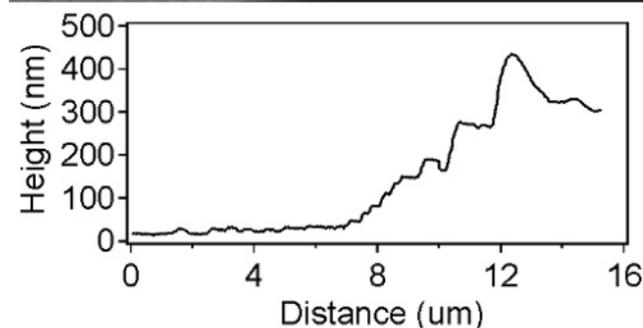
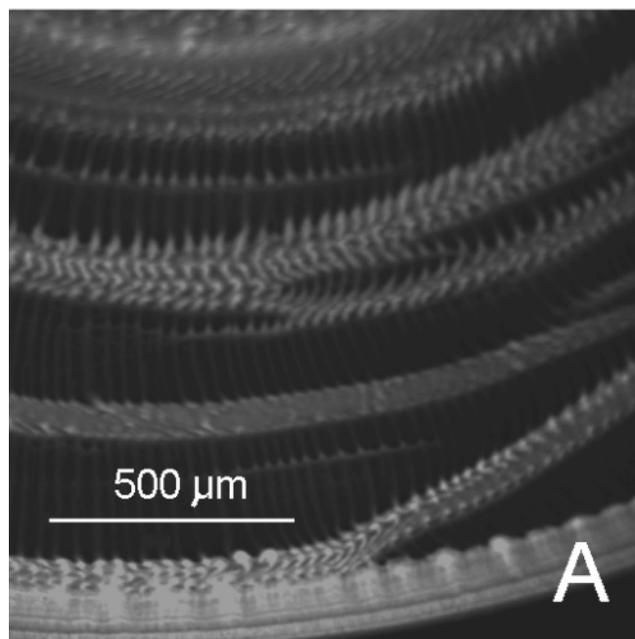


Figure 5. Structures in a POPC film on mica formed by simple evaporation of the hexane solvent in a $10\text{-}\mu\text{L}$ droplet (5 mg/mL containing 0.5% DiI_{C18}). Fluorescence imaging (A) shows a pattern of circular drying rings and radial spokelike features. AFM imaging of the sample (B) reveals height variations of hundreds of nanometers and shows the lamellar structure of the dry film.

and fundamentally does not exist.^{37,38} Our results points to the possibility that, when a dry lipid film is exposed to water, an interface is created between water and the hydrophobic surface presented by the lipid acyl chains. As recently shown for water in contact with hydrocarbon monolayers³⁹ or polystyrene,⁴⁰ this class of interfaces may

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lead to depletion of water in a region close to the hydrophobic surface. In the case of a lipid film, such a region depleted of water could be speculated to act as a kinetic barrier toward hydration with water vapor. In liquid water the lipid surface appears to be unstable and lipid bilayers start to detach from the substrate.

3.4. Domain Formation in Supported Membranes upon Hydration. To further examine the quality and behavior of supported bilayers prepared by spin-coating and hydration, we have investigated domain structures formed in supported membranes of binary mixtures exhibiting phase coexistence. More specifically, this serves to further confirm that the highly ideal planar surface imaged in Figure 2 indeed corresponds to a single supported membrane, and it also demonstrates the ability of the membrane to rearrange laterally.

Membranes exhibiting gel–fluid phase coexistence with micrometer-sized domains have been observed with several binary lipid mixtures in both free-standing giant unilamellar vesicle (GUV)⁴¹ membranes and supported membranes.^{3,42,43} As a natural extension of our results for pure POPC membranes, we have worked with the two binary mixtures POPC–DPPC and POPC–BC. These mixtures have been chosen for their physiological relevance and because some previous information on phase separation is available. Thus, the system of POPC–DPPC is known to phase separate over a large range of compositions as evident from the phase diagram⁴⁴ and as observed recently in GUVs.^{45,46} We have investigated a sample with a 1:1 mixture, for which a membrane should be located centrally in the coexistence region of the phase diagram at 20 °C. A dry spin-coated film of this mixture is shown in Figure 6A. The dewetting pattern in this film appears to be qualitatively similar to what was observed for pure POPC films, and the step height between neighboring bilayers is around 60 Å. This film was now hydrated in liquid water accompanied by annealing for 1 h (60 °C) above the gel–fluid phase transition temperature and finally re-cooled to 20 °C. This procedure accelerates the hydration and release of bilayers from the lipid film and promotes equilibration of the supported membrane that remains on the substrate. As shown in Figure 6B, the supported membrane has phase-separated into a characteristic gel–fluid pattern as evident from the irregular boundaries of the bright (tallest) regions. These gel domains have a size of several micrometers and height relative to the fluid phase of 12–15 Å. This number is in quite good agreement with a height difference of 11 ± 2 Å reported for membranes composed of DOPC–DPPC.⁴³

Important physiological functions have been associated with the presence and generation of ceramide in cell membranes. These effects include the role of ceramide as a messenger in apoptosis^{47,48} and as a main structural component in the lamellar lipid structures found in the outer skin (stratum corneum).⁴⁹ Several of these specific biological functions are speculated to be linked to formation of ceramide-enriched domains or patches in the bilayers.

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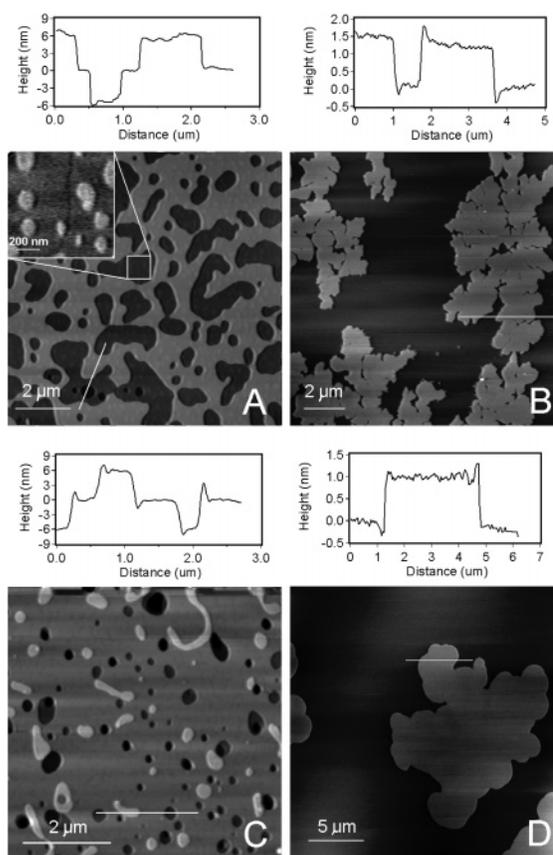


Figure 6. Domain formation in supported membranes following hydration of spin-coated films of two binary lipid mixtures. Dry and hydrated samples of POPC–DPPC (1:1) are shown in parts A and B, and the analogous dry and hydrate samples of POPC + 16% BC are shown in parts C and D. The inset in part A shows an enlargement of the indicated square area.

A simplified model system for this phase separation would be membranes of the binary lipid mixture POPC–BC. Recently Hsueh et al.⁵⁰ have constructed a partial phase diagram for pure palmitoyl ceramide and POPC based on NMR data and showed that a gel–fluid coexistence region covers a wide range of temperatures and compositions. So far, no microscopic imaging has been reported of domains in ceramide/phospholipid mixtures.

Figure 6C,D shows the AFM images obtained with the mixture POPC + 16% BC. The sample was prepared, hydrated, and annealed by the same procedure as for the POPC–DPPC film. The dry film again exhibits a dewetting pattern with a step height between bilayers of around 60 Å while ceramide-enriched membrane domains of 5–20 μm in size are observed in the supported membrane at 20 °C. The domains are more regular and rounded in shape than the DPPC/POPC gel domains and have a height difference relative to the fluid phase of 10 Å. The relatively large differences in bilayer heights found in both part B and part D of Figure 6 support the notion that the coexisting phases are of the gel and fluid type.

Fluorescence imaging of the supported DPPC–POPC (1:1, DiIC₁₈-labeled) membrane is shown in Figure 7. This image gives further support to the conclusion that a single bilayer is present on the support after hydration. The dark domain regions correspond to the DPPC-rich gel phase which excludes the fluorescence probe and which is imaged

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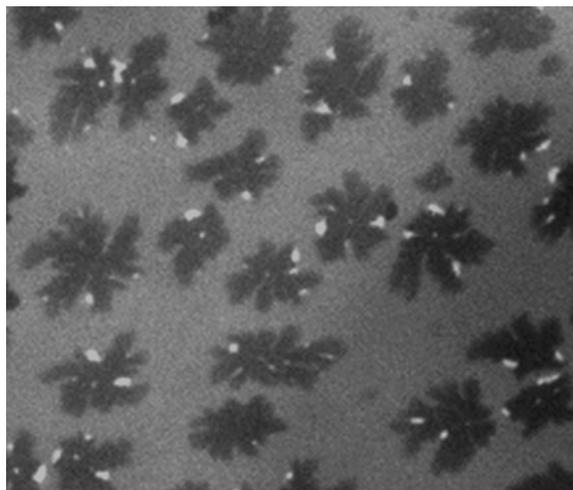


Figure 7. Fluorescence image of a supported membrane composed of DPPC–POPC (1:1, 0.5% DiI_{C18}). The image covers approximately $50 \times 40 \mu\text{m}^2$. The dark DDPC-rich domains are in the gel state and correspond to the bright (tallest) domains in Figure 6B.

as taller and brighter in the AFM topography image of Figure 6B. If multiple membranes were present, we would have expected to see the projection of overlapping domains as a contribution from several membranes, but this is not the case. Therefore, it is concluded that only a single membrane is present on the support. In addition, the image shows that the membrane domains are coupled between the bilayer leaflets as normally observed in free-standing membranes (i.e., in GUVs).

4. Discussion

In relation to the results on binary mixtures, it is of particular significance that membrane artifacts and defects are essentially absent when using the spin-coating procedure as evident from Figures 2 and 6. Artifacts that are otherwise typically observed in supported membranes include membrane holes of nanometer to micrometer size and regions of the membrane where lipid domains are out of registry in the two bilayer leaflets.¹⁴ As an example, difficulties were encountered when attempting to prepare supported membranes of the POPC–BC mixture by vesicle spreading (not shown). In this case, several preparation conditions were tested such as variation of sample temperature (20–80 °C) both before and after vesicle spreading and the use of vesicles prepared by extrusion as compared to sonicated vesicles. These attempts resulted in samples with highly different and often nonreproducible structures including the following: the supported membrane was not created, the supported membrane contained many holes, the ceramide-rich domains had several different characteristic heights, and large variations in domain shapes and sizes between different preparations were present. From our data it appears that the lateral lipid density (molecules per area) in dry spin-coated films is sufficiently high to allow for the hydrated membrane to equilibrate without the formation of holes. The presence of a water layer between the hydrated supported membrane and the mica substrate cannot be confirmed directly from our data. However, the ability of the membrane to rearrange laterally into rather large domain structures indicates that the lipid lateral diffusion in the lowest leaflet is not severely hindered and, therefore, suggests the presence of a water layer of some thickness. Regarding domain formation, it is also relevant to what extent the lipid species in binary mixtures are homogeneously

distributed in the dry film or if some degree of lateral phase separation has already occurred at this stage. As shown in the inset of Figure 6A the dry POPC–DDPC film does in fact exhibit domain structure within each bilayer with a height difference between domains of 8–10 Å. These “dry” domains in the inverted bilayer structures are rather small (100–200 nm), and at this point we can infer very little about their origin and composition from the AFM images. In comparison, the dry POPC–BC bilayers in Figure 6C are completely uniform and do not exhibit lateral separation. Overall we conclude that some degree of lateral lipid organization may or may not take place in a dry spin-coated film of a binary lipid mixture for which phase separation exists in the hydrated membrane. However, the important point is that in our samples domain formation in the hydrated membrane takes place on the substrate upon hydration and annealing/re-cooling. In addition there is no reason to assume that the domains in the hydrated membrane will have the same composition as domains that may have formed in the dry lipid film. From these considerations there is a fundamental difference between the mechanisms leading to domain formation in supported membranes made by hydration of a spin-coated film as compared to samples prepared by LB transfer. For this method, equilibrated domains are preexisting in a monolayer before transfer and these domains may or may not end up in a new (nonequilibrium) state after transfer to the substrate. For membranes prepared by vesicle spreading, the vesicles are usually at a temperature above the fluid phase line when the fusion takes place. However, both of these techniques are in contrast to membranes made from a spin-coated film where domains are formed as a result of the lateral rearrangement of lipids upon hydration and heating and after a dry lipid film is already placed on the substrate.

5. Conclusions

The purpose of the present study has been to obtain a detailed morphological characterization of dry and fully hydrated lipid films prepared by spin-coating and to explore the possibility of using this technique as an alternative and advantageous route to the preparation of solid supported membranes. Using POPC and binary mixtures of POPC–DPPC or POPC–BC we find that dry spin-coated films are topologically highly uniform as compared to lipids deposited by the simple drying of a droplet. The spin-coated dry lipid film has a lamellar structure and exhibits a classical dewetting pattern similar to what has typically been observed in polymer thin film studies. The top layers of the film are broken up by formation of holes in each layer in addition to decay of the film into isolated round patches. By investigating the topography of the lamellar film at varying lipid coverages, it is found that a lipid monolayer is formed on the substrate followed by inverted lipid bilayers located successively on top of the monolayer. This structure is explained by the fact that in the dry film the lipid headgroups will tend to orient toward the hydrophilic mica substrate while the acyl chains are oriented toward the hydrophobic air/vacuum space. In accordance with previous studies, we find that full hydration of the lipid film in fluid water leads to detachment of complex bilayer structures from the substrate while one single membrane remains on the substrate. This supported membrane is highly uniform and free from commonly observed defects such as holes, and it can be prepared with binary lipid mixtures. It is demonstrated that a POPC–DPPC membrane exhibits gel–fluid domain formation when at a temperature and composition for which the phase diagram predicts mem-

brane phase coexistence. A gel–fluid domain coexistence pattern is also observed for POPC–BC as would be expected from previous work on POPC–palmitoyl ceramide.

Our study indicates that supported membranes prepared by the procedure described above has at least two main advantages. First, the lateral lipid density is sufficiently high to practically eliminate the formation of holes in the supported membrane. Second, lateral reorganization of lipids into micrometer-sized membrane domains takes place when the membrane is located on the substrate, thereby avoiding artifacts in the domain morphology related to transfer of the membrane to the substrate. Our preliminary studies also show that supported membranes can be prepared analogously with more

complex ternary mixtures containing, for example, cholesterol. The method is relatively easy and fast to apply, and it appears as a useful alternative method for preparing supported membranes.

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