Ripples and the Formation of Anisotropic Lipid Domains: Imaging Two-Component Supported Double Bilayers by Atomic Force Microscopy

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ABSTRACT Direct visualization of the fluid-phase/ordered-phase domain structure in mica-supported bilayers composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/1,2-distearoyl-*sn*-glycero-3-phosphocholine mixtures is performed with atomic force microscopy. The system studied is a double bilayer supported on a mica surface in which the top bilayer (which is not in direct contact with the mica) is visualized as a function of temperature. Because the top bilayer is not as restricted by the interactions with the surface as single supported bilayers, its behavior is more similar to a free-standing bilayer. Intriguing straight-edged anisotropic fluid-phase domains were observed in the fluid-phase/ordered-phase coexistence temperature range, which resemble the fluid-phase/ordered-phase domain patterns observed in giant unilamellar vesicles composed of such phospholipid mixtures. With the high resolution provided by atomic force microscopy, we investigated the origin of these anisotropic lipid domain patterns, and found that ripple phase formation is directly responsible for the anisotropic nature of these domains. The nucleation and growth of fluid-phase domains are found to be directed by the presence of ripples. In particular, the fluid-phase domains elongate parallel to the ripples. The results show that ripple phase formation may have implications for domain formation in biological systems.

INTRODUCTION

Multicomponent lipid bilayer membranes can have several lipid phases coexisting at a given temperature (Mabrey and Sturtevant, 1976; Jørgensen et al., 1993; Jørgensen and Mouritsen, 1995; Silvius et al., 1996; Leidy et al., 2001). As a result, a lipid phase domain pattern can emerge, which varies in structure and dimensions depending on the mixing properties of the lipid components (Jørgensen and Mouritsen, 1995). For example, if a bilayer has two lipid components that differ in their fluid-phase/ordered-phase transition temperatures by several degrees, fluid-phase and ordered-phase domains can emerge in the temperature range between the two transition temperatures (Bagatolli and Gratton, 2000b). In-plane lipid phase separation and domain formation has now been shown to occur in biological membranes (Brown and London, 1997; Pralle et al., 2000; Schutz et al., 2000), leading to the organization of components such as protein receptors and signaling molecules (Varma and Mayor, 1998; Viola et al., 1999). In addition, the interface between coexisting fluid-phase and ordered-phase domains has been shown to act as a site for increased phospholipase activity (Hønger et al., 1996). Clearly, understanding the domain structures that emerge from phase separation in lipid bilayer membranes provides insights into the general organization and functional properties of biological membranes.

Recently, several fluorescence microscopy studies on giant unilamellar vesicles composed of binary lipid mixtures have shown coexisting fluid-phase and ordered-phase domains that vary in shape and size, depending on composition (Korlach et al., 1999; Bagatolli and Gratton, 2000a,b; Feigenson and Buboltz, 2001). In particular, these studies revealed quite remarkable straight edged ordered-phase domains for certain phosphatidylcholine mixtures (Korlach et al., 1999; Bagatolli and Gratton, 2000a), also observed in earlier studies by diffraction-contrast electron microscopy (Hui, 1981). The presence of straight-edged domains in the fluid-phase/ordered-phase coexistence regime suggests that the ordered-phase has a two-dimensional crystal arrangement that influences the domain shapes. Straight interfacial edges will form when the anisotropy in the energy of the crystal lattice dominates the line tension between the two phases. For example, the interfaces that arise from reorganization of cadmium arachidate Langmuir-Blodgett films show straight edges that are a result of the molecular crystalline packing of the films (Schwartz et al., 1992). In contrast, circular domains would be expected to form when the line tension energy is large and the coexisting phases do not show any long-range molecular ordering, such as in the case of fluid-phase/fluid-phase coexistence (Dietrich et al., 2001). In the case of fluid-phase/ordered-phase coexistence, solid phase is characterized by a short range molecular ordering and would not be expected to induce the straight-edged micron size domains that are observed in the giant vesicles. However, saturated phosphatidylcholines form a P_{β}' phase or ripple phase below the main phase transition that shows hexagonal lattice packing with long-range orientational correlation (Janiak et al., 1979; Zasadzinski and Schneider, 1987), which is likely to influence domain shapes.

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The P_β' phase is one of the more intriguing ordered lamellar phases for phosphatidylcholines (Nagle and Tristram-Nagle, 2000; Meyer and Richter, 2001). The P_β' phase is involved in the formation of periodic ripples in the membrane surface (Ververgaert et al., 1972; Sun et al., 1996). Since its discovery (Ververgaert et al., 1972; Verkleij et al., 1972; Tardieu et al., 1973), evidence has accumulated over several decades showing that the structure of the P_β' phase is in fact rippled. The bulk of these studies have been done using freeze fracture electron microscopy (Copeland and McConnel, 1980; Hicks et al., 1987; Zasadzinski, 1988; Meyer et al., 1996; Meyer, 1996) and x-ray diffraction (Janiak et al., 1976, 1979; Stamatoff et al., 1982; Alecio et al., 1985). Several structural models have been proposed for describing ripple formation. Some models are based on bilayer thickness modulation (Marder et al., 1984), whereas others assume a nearly constant thickness with undulations arising from packing frustrations (Doniach, 1979; Carlson and Sethna, 1987). The undulations have been assumed to have either a sinusoidal (Doniach, 1979) or a sawtooth profile (Chen et al., 1995). X-ray diffraction (Sun et al., 1996), scanning tunneling microscopy (Woodward and Zasadzinski, 1997), and freeze-fracture electron microscopy (Meyer, 1996) now have provided evidence with general agreement that the ripple profile shows an asymmetric sawtooth shape. A metastable ripple phase with approximately double the ripple spacing distance can form depending on the thermal history of the sample (Tenchov et al., 1989; Koynova et al., 1996; Vladkova et al., 2000). Another distinction from the stable ripple phase is that the metastable phase appears to have a symmetric sawtooth profile (Katsaras et al., 2000).

Ripples appear in a temperature range below the main phase transition temperature and above a low enthalpy transition called the pretransition (Luna and McConnell, 1977). Outside of the ripple-phase range the membrane is locally planar, whereas within the ripple-phase temperature range, the membrane adopts the characteristic corrugated structure with defined periodicity ranging from 100 to 300 Å, depending on the lipid (Ververgaert et al., 1973; Janiak et al., 1979). A recent model attributes the formation of ripples to the melting of a small fraction of lipids at the pretransition temperature, arranging to form linear arrays of fluid state molecules (Heimburg, 2000). This model is corroborated by evidence of the formation of a small fraction of fluid-phase lipids at the pretransition temperature (Rappolt et al., 2000), the presence of two distinct membrane thicknesses in the ripple profile (Sun et al., 1996; Katsaras et al., 2000), and the superposition of ordered- and disordered-type electron spin resonance spectra in the ripple regime (Tsuchida and Hatta, 1988).

Ripples have been shown to dissipate at the main phase transition in single component membranes (Tenchov et al., 1989), mainly due to the very narrow temperature range of melting for one-lipid systems. However, for certain multi-component mixtures, the melting temperature range is very broad (Mabrey and Sturtevant, 1976), giving rise to the formation of coexisting fluid-phase and ordered-phase domain structures (Jørgensen and Mouritsen, 1995). Freeze fracture evidence (Ververgaert et al., 1973; Lentz et al., 1981; Peters et al., 1984) points to the possible coexistence of ripple phase regions and fluid-phase regions within the melting temperature range for certain lipid mixtures. However, a detailed study on ripple phase formation in mixtures that include ripple-forming components has not been done. We have investigated in situ domain formation in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/1,2-distearoyl-*sn*-glycero-3-phosphocholine (DMPC/DSPC) bilayers by atomic force microscopy (AFM). This system is composed of two ripple-forming lipids and presents a broad melting profile.

Most AFM studies on domain formation in bilayers have been performed on supported single bilayers (Hui et al., 1995; Hollars and Dunn, 1998). Interactions with the support are believed to strongly influence bilayer behavior. Recently, differential scanning calorimetry (DSC) of supported bilayers on microsocopic mica chips showed a splitting and a shift to higher temperature of the main phase transition compared with lipid suspensions (Yang and Appleyard, 2000). Although single bilayer studies are useful in understanding membrane behavior, several key membrane properties are altered due to the interaction with the support. One marked effect is the lack of ripple formation on supported single bilayers. Recently, through a combination of vertical and horizontal depositions of monolayers, a mica supported DPPC and DSPC double bilayer system was obtained using a Langmuir trough (Fragneto et al., 2001). The top bilayer in this double bilayer system was shown by neutron diffraction to have a more free-standing behavior, which is attributed to the fact that the second bilayer (floating bilayer) is not in direct contact with the mica support. We have formed DMPC/DSPC double bilayers supported on mica through vesicle fusion and visualized by AFM. The high resolution of this technique reveals the presence of ripple phase in the top bilayer, which is indication of the free-standing behavior. We investigated the ripple structure in the DMPC/DSPC system as the temperature was raised into the fluid-phase/ordered-phase coexistence regime. The ripple phase is found to persist within the coexistence temperature regime, and the emerging fluid-phase/ordered-phase domain structure is found to be highly influenced by the presence of ripples.

MATERIALS AND METHODS

DMPC and DSPC were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Ruby muscovite mica was obtained from Plano W. Plannet GmbH, (Wetzlar, Germany). Appropriate amounts of DSPC and DMPC were dissolved and mixed in chloroform. The samples were then dried under nitrogen gas and placed under vacuum

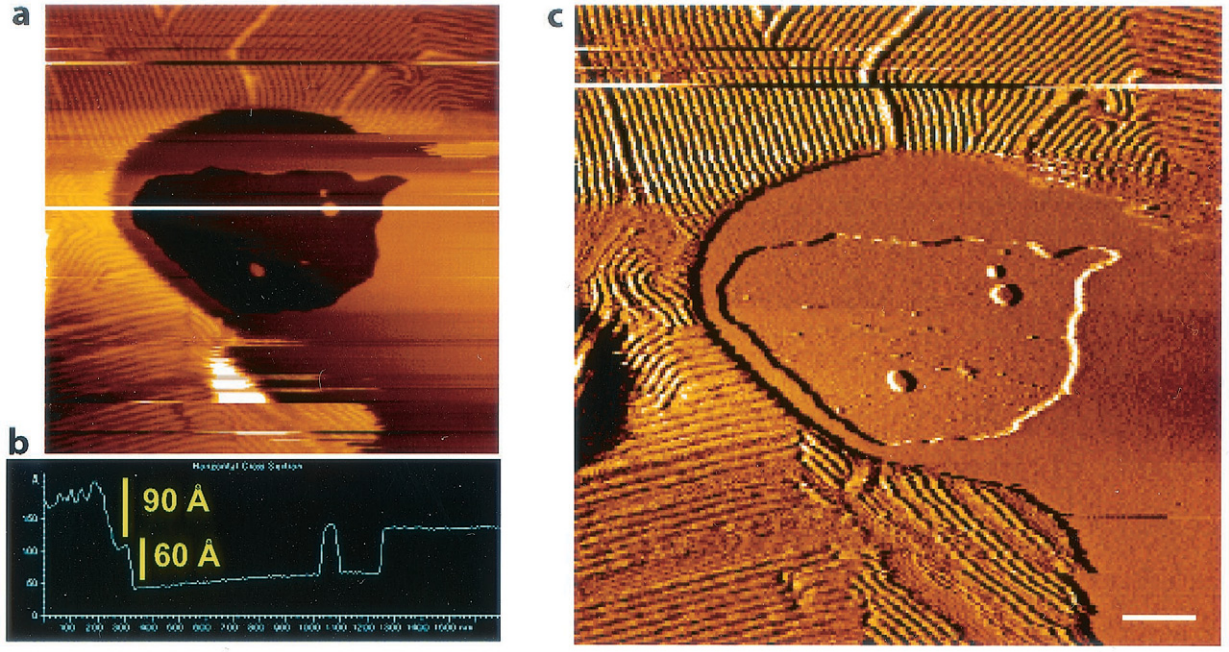

FIGURE 1 AFM image of a DPPC double bilayer supported on a mica substrate. (*a*) In this height mode image, the second bilayer does not fully cover the imaged surface, therefore exposing the first bilayer. A hole was scratched in the first bilayer using a high force setting, exposing the mica substrate. (*b*) Height profile of a cross-section represented by a white line in *a* shows the mica, first bilayer, and second bilayer surfaces. The bars correspond to the height differences between the mica surface and the first bilayer and between the first bilayer and the second bilayer. (*c*) Deflection mode image showing that ripple formation only occurs in the second bilayer. Scale bar = 200 nm.

overnight to remove the residual solvent. The dried lipids were dispersed in Milli-Q water to a final concentration of 3 mM. Aqueous multilamellar lipid dispersions were prepared by heating the sample to 65°C, followed by vortexing. Small unilamellar vesicles (SUVs) were prepared by sonication using a Labsonic U tip sonicator (B. Braun Biotech International, Melsungen, Germany) at 65°C for two periods of 7 min. Residual titanium was removed from the vesicle solution by centrifugation for 5 min at 2750 × *g*.

The SUVs were immediately rewarmed to 65°C, which is above the main phase transition for the mixture, and 1 mL was added to a small home built cell for the atomic force microscope containing a piece of freshly cleaved mica at 24°C. We allowed the sample to incubate before rinsing for 1 h at 24°C, which is ~5°C below the solidus phase line for the mixture. The sample was then rinsed by exchanging 10 times the incubation solution with 20 mM NaCl solution never allowing the supported bilayer to dry. Adding the warmed SUV solution, and allowing the sample to cool down during incubation to a temperature below the solidus phase line transition was generally a successful procedure for obtaining double bilayers. The pure DPPC double bilayers were prepared by warming the solution to 65°C and incubating the DPPC SUVs at 37°C, which is 4°C below the DPPC main phase transition, for 1 h.

The mica supported lipid bilayers were imaged in contact mode in the home built fluid cell using a PicoSPM atomic force microscope (Molecular Imaging, Phoenix, AZ). The cantilevers were oxide sharpened silicon nitride cantilevers (ThermoMicroscopes, Sunnyvale, CA) with a nominal spring constant of 0.02 N/m. To ensure that the force was kept minimal during scanning, the force was frequently decreased until the tip left the surface and subsequently slightly increased until just regaining contact. In general, ripples could only be resolved when the force was at an absolute minimum. Scanner hysteresis and small variations in temperature during scanning makes precise statements about the scanning force difficult to make. Even temperature fluctuations of the order of 0.01°C cause noticeable thermal bending of the gold coated cantilevers. However, a conservative estimate of the force range would be 20 to 300 pN based on the nominal spring constant.

Single bilayers, double bilayers or multiple bilayers are formed on the mica support depending on the incubation conditions. AFM was used to distinguish between these possibilities and to select for the conditions that produced double bilayers. Single bilayers were identified by similarities in domain patterns when compared with single bilayers of the same mixture formed by Langmuir Blodgett techniques (μTrough, Kibron, Inc., Helsinki, Finland). For multiple bilayer samples, holes on the top bilayer allowed us to visualize the domain pattern in the bilayer immediately below the top bilayer. In the case of double bilayers, the bilayer immediately below presents the domain pattern observed in single bilayers, which is noticeably different from the domain pattern observed on the top bilayer. Imaging was more difficult for preparations that produced more than two bilayers. The surface became very soft, and the resolution was poor. However, it was possible to resolve that, starting with the second bilayer, the subsequent bilayers present similar domain patterns, which are drastically different from the domain patterns found in the first bilayer in close contact with the mica support. We chose sample preparations that produced double bilayer systems with approximately 75% to 90% surface coverage of the top bilayer.

RESULTS AND DISCUSSION

Supported double bilayer

Fig. 1 shows an atomic force microscopy scan of a DPPC double bilayer supported on a mica surface. The double bilayers are formed through vesicle deposition as described in Materials and Methods. In Fig. 1 *a*, the second bilayer does not fully cover the imaged surface, resulting in a large

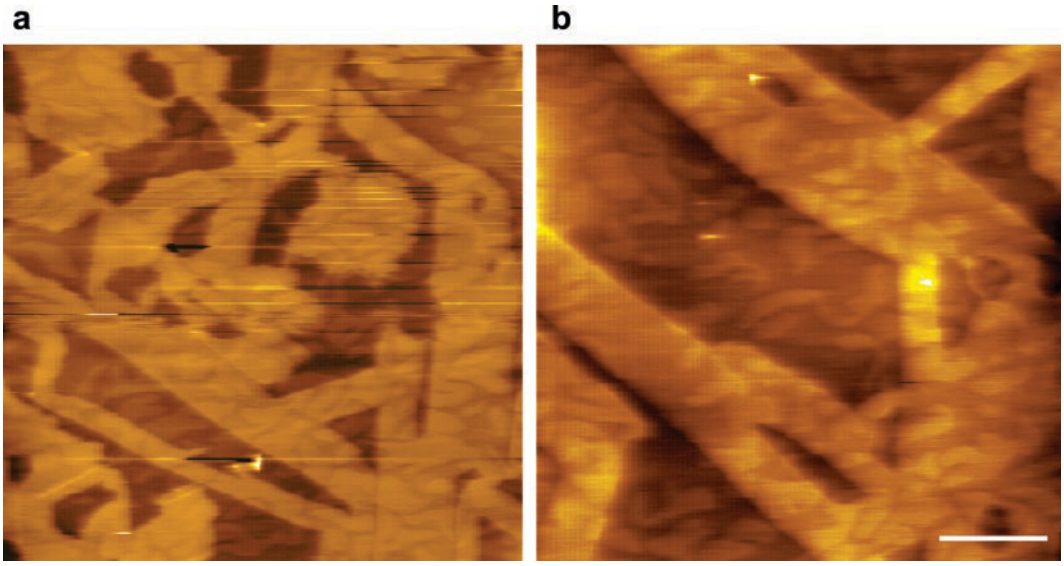

FIGURE 2 AFM images of 1:1 DMPC/DSPC (molar ratio) double bilayers supported on a mica substrate. Brightness variations correspond to height differences in the membrane. The brighter angular shapes correspond to the domain structure in the top bilayer. The small faint uneven domains are imprints of the domain structure in the bottom bilayer. (*a*) Domain structure at 30.5°C. (*b*) Domain structure at 32.5°C. Scale bar = 1 μm.

region where the first bilayer is exposed. The exposed area allowed us to corroborate that we were truly visualizing a double bilayer. This was done by scanning at a high force setting in the center of the image and forming a hole that reached the mica surface. The resulting image is shown in Fig. 1 *a*. Fig. 1 *b* shows the height profile of a cross-section of the image, which is indicated by a white line in Fig. 1 *a*. The height profile shows three distinct levels, indicating the mica, the first bilayer, and the second bilayer. The height difference between the first bilayer and the mica is 60 to 70 Å, whereas the height difference between the second bilayer and the first bilayer is 90 to 100 Å. In any event, these thicknesses lie within the expected range for supported bilayers. Fig. 1 *c* shows a deflection mode image of Fig. 1 *a*, which clearly shows the presence of ripples in the top bilayer, whereas the first bilayer shows no indication of ripple formation, corroborating the fact that ripple formation only occurs in the second bilayer (Fang and Yang, 1996; Fragneto et al., 2001). By choosing an appropriate protocol for vesicle deposition, a double bilayer system can be formed and imaged through AFM.

Fluid-phase/ordered-phase domain structure

Fig. 2 shows atomic force microscopy scans of a 1:1 DMPC/DSPC double bilayer supported on mica in the fluid-phase/ordered-phase coexistence temperature range. Although we did not obtain images in the case of the DMPC/DSPC mixture that were good enough to present a height profile showing that we indeed have a double bilayer, we are confident that the images we present are from double bilayers for the DMPC/DSPC mixture. Variations in the protocol, such as changes in vesicle concentration, produced either single bilayers with no ripple formation or multiple layers with ripples forming at multiple levels (data not shown). Due to an increased softness, the multilayers (more than two layers) did not generate images that had as high of a resolution as the double bilayers, therefore we chose to focus on the double bilayers.

The domain structure of fluid-phase and ordered-phase domains is clearly resolved in Fig. 2, which at that temperature corresponds to fluid-phase DMPC-rich domains and ordered-phase DSPC-rich domains (Leidy et al., 2001). We measured a uniform height difference between the ordered-phase and fluid-phase domains of ~16 Å. Growth and fusion of the fluid-phase domains occurs as the temperature is raised from 30.5°C (Fig. 2 *a*) to 32.5°C (Fig. 2 *b*).

Domain shapes in the second bilayer are clearly angular and show straight edges (Fig. 2, *a* and *b*). Similar, straight-edged domains with sharp angles are also seen in 1,2-dilauroyl-*sn*-glycero-3-phosphocholine/1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DLPC/DPPC) giant unilamellar vesicles by fluorescence microscopy (Korlach et al., 1999; Bagatolli and Gratton, 2000a). In contrast, binary mixtures that include phosphoethanolamines, which do not form ripples, show rounded domains (Bagatolli and Gratton, 2000a). The observation that straight-edged domains are only seen in mixtures that include ripple forming components hints at an intimate relationship between ripple phase formation and domain shape. For one-component systems, within the temperature range immediately below the melting temperature, the hexagonal packing of the ripple phase presents long-range positional order and unidirectional long-range modulated order (Janiak et al., 1979). This long-range order is

likely to play a role in inducing the straight-edged domain shapes.

The angular domain shapes appear to be characteristic of phosphatidylcholine mixtures with specific chain-length differences. Phosphatidylcholine mixtures with chain length differences of six or eight carbons do not show sharp edged domains (Bagatolli and Gratton, 2000a). The high disparity in chain length implies that these lipid mixtures show almost complete immiscibility and the ordered-phase domains therefore consist almost entirely of the high melting lipids. Consequently, the phase behavior of the ordered-phase domains is completely dominated by the high melting lipid component, and ripple formation is therefore only expected in a narrow temperature range just below the fluidus phase line. In contrast, although the range for ripple formation in partially miscible binary phosphatidylcholine systems (such as DMPC/DSPC) has not previously been defined, this range would be expected to span a broad temperature region that includes the fluid-phase/ordered-phase coexistence regime.

The formation of ripples in the DMPC/DSPC binary mixture and the influence of the ripple long-range molecular order on domain formation is addressed in Figs. 3 and 4. The data support the proposal that the ripple-phase persists in the coexistence temperature regime and that the straight-edged domain shapes in the DMPC/DSPC bilayers are induced by the anisotropic nature of the ripple phase structure.

Ripple formation

The AFM images in Fig. 3 (*a–c*) show ripple phase formation in the top layer of a DMPC/DSPC double bilayer membrane. The scans were performed at 24°C, which is approximately 5°C below the solidus phase line for this system. The DSC scan of 1:1 DMPC/DSPC multilamellar vesicles in Fig. 4 *a* shows a pretransition 10°C below the onset of the main phase transition, indicating the formation of the ripple phase. Ripple phase has previously been visualized in single lipid systems by AFM (Fang and Yang, 1996). However, to our knowledge AFM imaging of ripples has not been reported for lipid mixtures. Fig. 3 *a* is a height mode image, which shows the characteristic modulated ripple structures with ripple regions angled at approximately 60° and 120° from each other. Two predominant ridge spacings of ~12.5 and 25 nm are clearly observed in Fig. 3 *b*. The 12.5-nm spacing has been previously characterized for one-component lipid bilayers as the stable ripple phase, whereas the 25-nm spacing has been reported to be meta-stable (Tenchov et al., 1989; Koynova et al., 1996; Katsaras et al., 2000; Vladkova et al., 2000).

Influence of ripples on domain formation

AFM images of DMPC/DSPC mixtures in the fluid-phase/ordered-phase coexistence temperature range reveal the

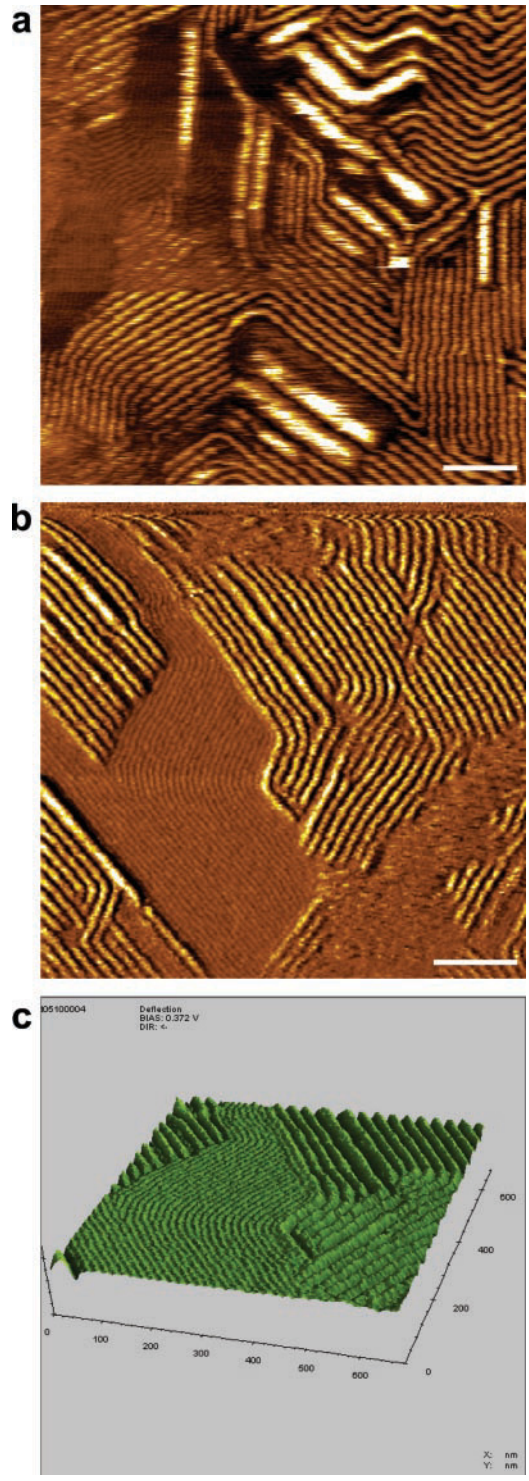

FIGURE 3 AFM images showing ripple phase formation in 1:1 DMPC/DSPC double bilayers at 24.5°C. (*a*) Height mode image showing characteristic ~60° and ~120° ripple orientations and different ripple spacings. (*b*) Deflection mode image showing a region of ~12.5-nm ripples (stable ripple phase) within a ~25-nm ripple region (meta-stable ripple phase). (*c*) Three-dimensional side view of *b* showing the details of the ripple structure. It should be emphasized that ripple formation was not observed in single supported bilayers. Scale bar = 200 nm.

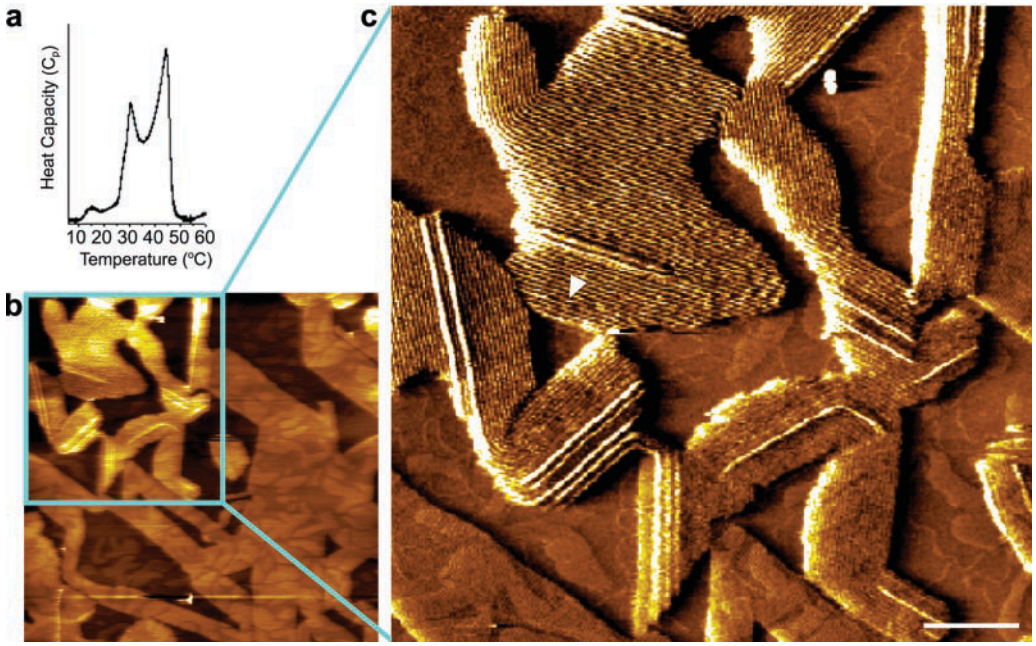

FIGURE 4 Ripples in the fluid-phase/ordered-phase coexistence temperature range in 1:1 DMPC/DSPC double bilayers at 30.5°C. (*a*) DSC scan of 1:1 DMPC/DSPC multilamellar vesicles showing a pretransition at 15°C indicating ripple formation. (*b*) Height mode AFM image showing coexisting ordered-phase and fluid-phase domains at 32.5°C. (*c*) Deflection mode magnification of *b* showing ripples in the ordered-phase domains. Arrow indicates a fluid-phase domain defined by the surrounding ripple structure. Scale bar = 500 nm.

presence of ripple phase in the ordered-phase domains (Fig. 4), showing that ripples persist even after the onset of the main phase transition for the mixture. In Fig. 4, *b* and *c* are height mode and deflection mode scans of coexisting ordered-phase and fluid-phase domains in 1:1 DMPC/DSPC bilayers at 30.5°C. The DSC scan in Fig. 4 *a* indicates that at 30.5°C, the system is well within the fluid-phase/ordered phase coexistence temperature range. In the height mode image in Fig. 4 *b*, the fluid-phase and ordered-phase domains appear as lower and higher regions, due to the differences in thickness of the fluid-phase and ordered-phase regions of the bilayer. The deflection mode image shown in Fig. 4 *c* resolves more clearly the presence of ripples within the ordered-phase domain regions.

Ripple phase formation induces straight-edged fluid-phase domains. This is evident in Fig. 4 *c*, where ripples run parallel to the straight edges that outline the emerging fluid-phase domains. As pointed out by the arrow in Fig. 4 *c*, ripples clearly define the shape of the triangular fluid-phase domain in the center of the AFM image. In addition, the similarity between domain angles (Fig. 2, *a* and *b*) and ripple orientations (Fig. 3 *a*) suggests that ripple angles also play a role in determining the 60° and 120° angles that are commonly observed in domain shapes for this mixture (Fig. 2, *a* and *b*). Since the ripple phase is responsible for inducing long-range positional and orientational order in the bilayer membrane (Janiak et al., 1979), the ripple structure must be directly involved in determining the sharp domain shapes at the micron scale.

Fig. 5 (*a* and *b*) shows AFM height-mode and deflection-mode images of a fluid-phase domain emerging within a region of 125-Å ripples in a 7:3 DMPC/DSPC sample. The fluid-phase domain shows preferential growth in the direction of the ripples, resulting in the anisotropic shape of the domain. Straight edges form on the sides of the fluid-phase domain that run parallel to the ripple direction, whereas the sides that are perpendicular to the domain show uneven edges. The domain shapes suggest that the long-range molecular ordering of the ripple phase dominates the two-dimensional line tension between fluid and ordered phases. The preferential growth along the ripple direction reveals a marked anisotropy in the energy of the lipid-packing. This anisotropy most likely arises from tighter molecular packing along the ripple direction, resulting in a lower energy crystalline face. This is a general mechanism for the formation of two-dimensional crystals in coexistence with a fluid medium and is observed in other two-dimensional crystalline arrays (Schwartz et al., 1992). The higher molecular packing energy along the direction perpendicular to the ripples is likely due to either packing frustrations along the ripple cross-section (Chen et al., 1995) or the presence of fluid-phase lipids above the pretransition temperature (Rappolt et al., 2000). Although some studies suggest that fluid-phase lipids localize at defects in the ripple structure (Kapitza et al., 1984), other studies propose that fluid-phase

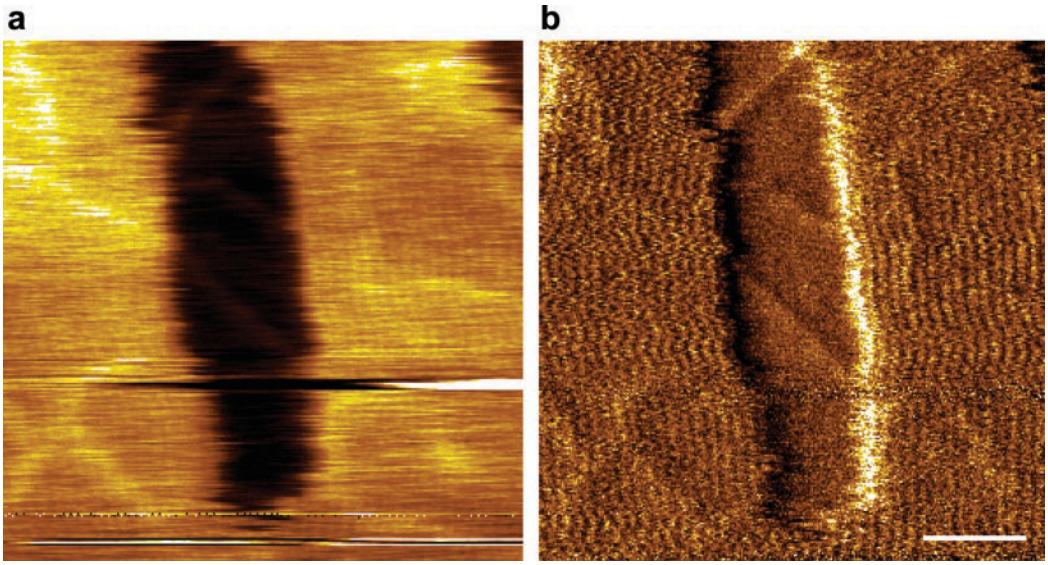

FIGURE 5 AFM scan showing a height mode (*a*), and deflection mode (*b*) image of a fluid-phase domain nucleating within a ripple phase region in a 7:3 DMPC/DSPC double bilayer at 29.5°C. The fluid-phase domain shows preferential growth along the ripples. Scale bar = 100 nm.

lipids form part of the ripple structure and actually play a role in inducing the formation of the ripples (Trandum et al., 1999; Heimburg, 2000). The AFM images clearly show that the formation of straight-edged domains is controlled by the anisotropy of the ripples, however the precise origin of this anisotropy is still to be determined.

CONCLUSION

The results show that the ripple phase persists in the fluid-phase/ordered-phase coexistence temperature range in the DMPC/DSPC binary lipid mixture. In addition, the presence of ripples in the coexistence regime affects the formation and growth of fluid-phase domains, inducing highly anisotropic domain shapes. The formation of straight-edged interfaces and anisotropic domains in a two-dimensional system of coexisting phases is not uncommon. There are examples of this phenomenon in monolayers at the air-water interface (Mohwald et al., 1995) and in Langmuir-Blodgett films (Schwartz et al., 1992). The particular relevance of the current observations lays in the fact that the formation of anisotropic domains occurs in a multicomponent bilayer, therefore showing that the ripple phase has a high enough lattice energy to influence the fluid-phase/ordered-phase domain structure in a multicomponent bilayer environment. This can have direct implications for domain formation in biological membranes. The fact that a ripple phase is present in coexistence with a fluid-phase seems consistent with the possibility that ripples may form in the complex mixtures seen in biological membranes and that the ripples can play a role in influencing the domain structure. We suggest that ripple formation is also plausible in biologically relevant mixtures that contain high melting-temperature components that have ripple-forming characteristics. We find it reassuring in this regard that sphingomyelin, a well-known component of ordered-phase domains in native membranes (Brown and London, 1997) forms ripples at physiologically relevant temperatures (Meyer et al., 1999).

The results also demonstrate that, for the top bilayer, the double bilayer system presents an environment that resembles more closely the conditions found in a free-standing bilayer. This provides a clear advantage over supported single bilayers for studying membrane properties such as domain formation. AFM studies on DMPC/DSPC single supported bilayers (Giocondi et al., 2001; Muresan et al., 2001) show very different domain morphology than the straight-edged angular domains seen in free-standing bilayers (Korlach et al., 1999; Bagatolli and Gratton, 2000a). In addition, ripple formation, an important morphological property of phsophatidylcholines, is inhibited by the surface interactions in supported single bilayers (Fang and Yang, 1996; Fragneto et al., 2001). With the use of a double bilayer system, membrane properties that are sensitive to surface interactions can be studied in a free-standing environment at the high resolution provided by atomic force microscopy.

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