J. Membrane Biol. 161, 227–233 (1998)

The Journal of
Membrane
Biology

Manipulation and Molecular Resolution of a Phosphatidylcholine-Supported Planar Bilayer by Atomic Force Microscopy

M. Beckmann,[1] **P. Nollert,**[2,*] **H.-A. Kolb**[3]

[1]Becton Dickinson, Tullastr. 8-12, D-69126 Heidelberg
[2]Department of Microbiology, Biozentrum, University of Basel, Klingelbergstr. 70, CH-4056 Basel
[3]Institute of Biophysics, University of Hannover, Herrenhäuserstr. 2, D-30419 Hannover

Received: 12 March 1997/Revised: 3 October 1997

Abstract. The morphology of supported planar bilayers has been investigated below phase transition temperature by atomic force microscopy in contact and tapping mode. The bilayers were formed by the vesicle-spreading technique. In contact mode at low scanning forces of about 1 nN true molecular resolution could be achieved for supported phosphatidylcholine bilayers. The resolution was confirmed by experiments that captured the location, average area of individual lipid headgroups and the manipulation of the bilayer surface. Repeated scanning in contact mode shifted the random topology of the surface consecutively to a striped pattern. Height profiles of defect-containing bilayers were analyzed. The shape of the defects became smooth by repeated scanning. The height profiles allowed the estimation of the indentation of the tip into the surface-adsorbed membrane. In tapping mode a disordered pattern of headgroups became visible. Our morphological data at molecular resolution suggest that the native arrangement of the choline headgroups is disordered, free of large packing defects and becomes ordered in Schallamach waves by scanning in contact mode.

Key words: Lipid head groups — Packing order

**Present address:* Max-Plaack-Institut für Biologie, Abteilung Membranbiochemie, Correnstrasse 38, D-72076 Tübingen, Germany

Correspondence to: H.-A. Kolb

Abbreviations: diC15-PC, 1,2-Dipentadecanoyl-*sn*-Glycero-3-Phosphocholine; SPB, supported planar bilayer; AFM, atomic force microscopy, contact mode, tapping mode

Introduction

Recently, atomic force microscopy (AFM) has been applied to the study of the topography of biological membranes (for reviews *see* Hansma & Hoh, 1994; Lal & John, 1994; Shao & Yang, 1995). However, due to their softness biological membranes of living cells become significantly indented upon contact with the scanning tip, even at low forces of about 1 nN (Hoh & Schoenenberger, 1994; Beckmann et al., 1995). Therefore, molecular resolution could not be achieved to date. Yet, the indentation can be reduced drastically by an underlying rigid substrate, as already shown for a physiological intact membrane of a cell that contained latex beads (Beckmann et al., 1994) and for artificial supported planar bilayers (SPBs) under wet incubation conditions (Raedler, Radmacher & Gaub, 1994). Hence, SPBs appear to be a useful tool for deriving information about the topography of lipid bilayers, especially in the gel phase in which the lipid molecules are essentially immobile (Egger et al., 1990; Engel, 1991; Zasadzinski et al., 1991; Hoh & Hansma, 1992; Brandow et al., 1993; Yang et al., 1993*a,b;* Hansma & Hoh, 1993; Mou, Yang & Shao, 1994; Sackmann, 1996). With respect to molecular resolution, this approach has been successfully employed for Langmuir-Blodgett films of phosphatidyl-ethanolamine (Singh & Keller, 1990), phosphatidylcholine (Hui et al., 1995) and phosphatidylglycerol (Egger et al., 1990).

Although most of the physical properties of SPBs are in perfect agreement with those of nonsupported bilayers, a previous study of SPBs on glass revealed that they are more permeable to ions than the membrane of lipid vesicles (Nollert, Kiefer & Jähnig, 1995). The leakiness of SPBs was attributed to membrane defects of

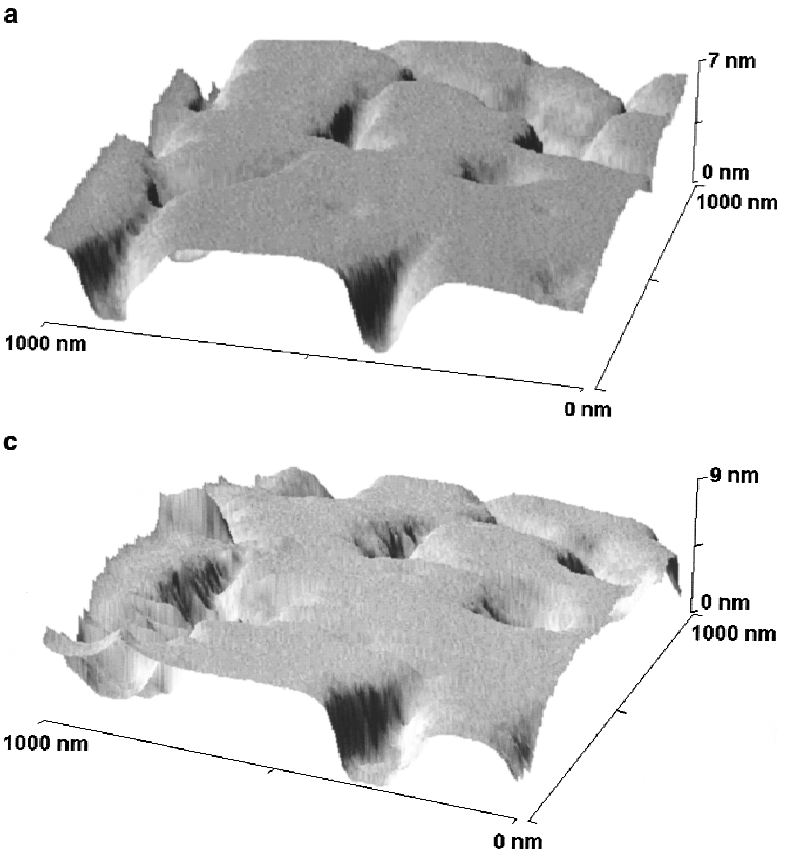
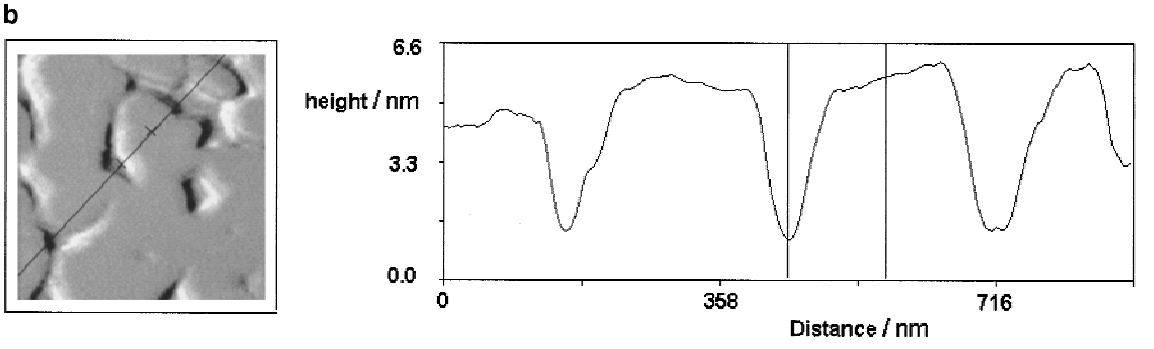
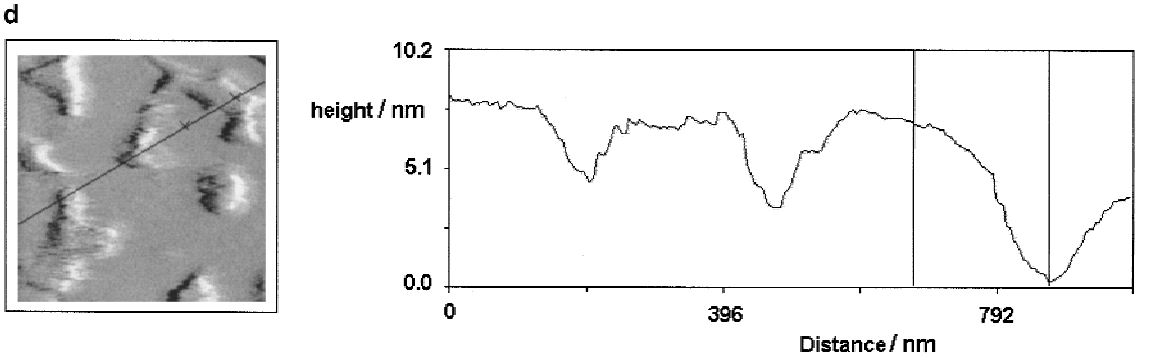


Fig. 1. Sequence of subsequent AFM images in the presence of a mica-supported, defect-containing diPC15-PC bilayer at low resolution (1000 × 1000 nm) in Na-phosphate buffer. *(a)* First scan. The topographical image is given as 3D-presentation. *(b)* Left panel: Image of *(a)* in 2D-presentation. A height profile is given which follows the drawn line shown in the left panel. For the two marked points on the line, the profile shows a height difference of 4.6 nm. *(c)* Second scan. The topographic image is given as 3D-presentation. *(d)* Left panel: Image of *(c)* in 2D-presentation. A height profile along the drawn line is presented. At two marked points a height difference of 6.2 nm is calculated. *(e)* Image in 2D-presentation after solubilization of the lipid bilayer by DMSO and washout with Na-phosphate buffer. All images were recorded at a scan rate of 5 μm/sec and consist of 200 lines. The procedure for generation of defects in the SPB is described in Material and Methods.





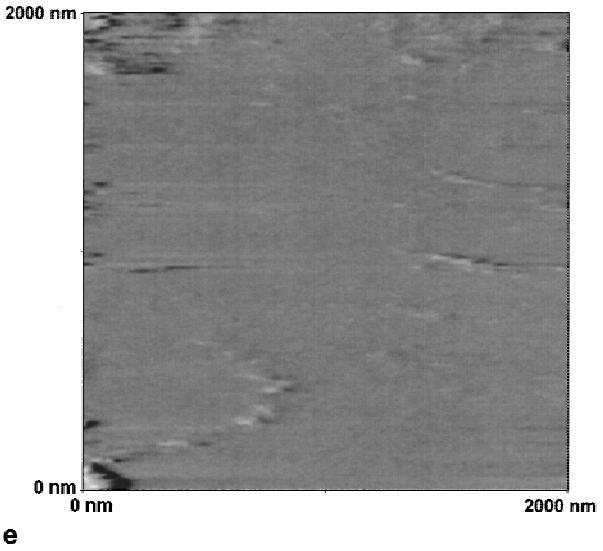


Fig. 1. Continued.

molecular size which might have been induced by the rough glass surface. The size of the defects was too small to be detectable with fluorescence microscopy. Accordingly, a perfectly smooth surface like mica should harbor an SPB without membrane defects. The motivation for the work reported here is to attempt to analyze the known property of the AFM to act in contact mode as a molecular ''comb'' (Zasadzinski et al., 1991) at loading forces of about 1 nN for support lipid bilayers under liquid conditions in the highest spatial resolution obtainable and to compare the images with those obtained in tapping mode.

The planar lipid membrane was fabricated by the vesicle fusion technique with lipid vesicles consisting of phosphatidylcholine (McConnell et al., 1986), a common phospholipid in biological membranes. The lipid bilayer was formed with and without defects and thus permitted us to measure height profiles of the defect-containing bilayer which allowed an estimation of the thickness of the layer and the detection of the influence of repeated scanning on the shape of the defects. Our experiments strongly indicate that detection at a scale of individual lipid molecules in a phosphatidylcholine SPB is possible. Since the experiments were performed in contact mode the interaction between tip and bilayer surface could be studied by monitoring the effect of repeated scanning on the molecular arrangement. By high-resolution scans headgroups of individual lipid molecules were imaged and their molecular arrangement was shown to be disordered and free of large packing defects.

Materials and Methods

Materials

diC15-PC (1,2-Dipentadecanoyl-*sn*-Glycero-3-Phosphocholine) was purchased from Avanti (Alabaster, AL). Non-ruby V-1 muscovite mica was a gift from S & J Trading (NY, NY). Dimethylsulfoxide (DMSO) was obtained from SIGMA (St. Louis, MO). Silicon wafers were a gift of Wacker Chemie (Burghausen, Germany). For cleaning purpose silicon wafers were treated with 2% Hellmanex II (Hellma, Müllheim, Germany) and thereafter rinsed extensively with deionized water.

Lipid Vesicle Preparation and SPB Formation

Required amounts of a diC15-PC stock solution in chloroform were filled in a glass tube and the solvent was removed by evaporation at room temperature. The resulting lipid film was further dried at reduced pressure for more than 30 min and agitated in 20 mM NaCl, 10 mM sodium phosphate buffer, pH = 7.4, yielding a 1 mg/ml solution. The large multilamellar vesicles were then sonicated with a Branson sonifier 250 (Danbury, CT) for 15 min at approx. 40°C, cooled to room temperature and then centrifuged on a benchtop centrifuge in order to remove titanium particles. The size of the resulting lipid vesicle suspension was measured by photon correlation spectroscopy with a Coulter N4/SD sub-micron particle analyzer (Hialeah, FL). The mean diameter obtained was approx. 50 nm.

SPBs were prepared by the vesicle fusion method adopted from Brian and McConnell (1984). Care was taken to avoid air contact of the lipid covered surface during the entire preparation procedure. SPBs of discontinuous spread were formed on freshly cleaved mica by incubating the substrate with the vesicle suspension above the main transition temperature of 33°C for about 30 min and thereafter rinsing with buffer at that temperature. Discontinuities in the membrane were produced by cooling to room temperature (approx. 23°C). This temperature shift induces a membrane shrinkage which introduces holes into the membrane bilayer (Tamm & McConnell, 1985). These holes cannot be filled by membrane because bulk vesicles were removed from the bathing solution. SPBs of continuous spread were formed by incubating the substrate with the vesicle suspension above the main transition temperature of 33°C. After incubation for 15 min the surface and the liquid were cooled to room temperature and surplus lipid vesicles were removed by extensive rinsing with buffer at room temperature.

Both substrates, freshly cleaved mica or silicon wafers (SiO) of about 3 × 4 mm were mounted in a fluid chamber which allowed the exchange of various solutions at room temperature.

Atomic Force Microscopy

AFM images were obtained as previously described (Beckmann et al., 1994) with a commercial TMX 2010 (TopoMetrix, Santa Barbara, CA). Microfabricated silicon nitride tips on a cantilever were used with a spring constant of 0.032 N/m according to the manufacturer. An uncertainty of a factor of two cannot be excluded. In contact mode, images of supported bilayers were scanned in a closed fluid chamber (TopoMetrix, Santa Barbara, CA) under electrolyte solution at a constant image force of about 1 nN at room temperature. Thus the bilayer can be considered to be in the gel phase. The force was not independently calibrated, but taken from the corresponding force-distance curve obtained with the TMX 2010. Following this procedure a loading force of about 1 nN was applied in the different experiments. Simultaneously in forward and reverse scan direction, the error signal (Putman et al., 1992) and the topography were recorded. The x,y and z scale was not independently calibrated. To reduce the penetration of the tip into the bilayer, scanning velocities of about 10 μm/sec were used (Raedler et al., 1994). Images in tapping mode were recorded with the commercial option of TopoMetrix. In this mode the feedback parameter is the damping of the amplitude of the piezo driven oscillating cantilever (Zhong et al., 1993). Since the tapping mode was not appropriate for the closed liquid chamber, an open chamber was used. The tip had an I-shape (Model 1620, Topometrix) with a radius of

about 20 nm. The cantilever had a length of 450 μm, a width of 30 μm. The driven frequency of the cantilever was derived from the corresponding frequency spectrum and set to 299.3 kHz. Images were captured with a scanner for a maximal area of 7 μm × 7 μm. All images of supported bilayers were scanned under aqueous solution.

Results

Supported Planar Bilayer of Discontinuous Spread

A planar lipid bilayer of discontinuous spread was formed on mica and the surface was scanned with the AFM. The discontinuities within the surface of the lipid bilayer allowed the analysis of the thickness of the bilayer as well as the influence of repeated scanning in contact mode on the surface topography under identical selection of the parameters for the adjustment of the feedback circuit and image processing. Two representative images, scanned consecutively at the same location, are shown in Fig. 1 *a* and *c*. The result of the first scan is given in Fig. 1*a* which shows the area of 1000 × 1000 nm. Cleft- and troughlike structures are clearly visible. A representative height profile along a straight line is given in Fig. 1*b* (right panel). The height profile indicates two levels. We assign the upper level to the lipid surface and the lower level to the underlying mica surface. From the difference of the corresponding plateau values in Fig. 1*b* a height of 4.9 ± 0.2 nm was determined for the total thickness of the supported layer using six different height profiles of random orientation.

After the second scan of the same area (*see* Fig. 1*c*) the clefts and troughs became smeared out (compare with Fig. 1*a*) which is also indicated in the corresponding height profiles (*see* Fig. 1*d*). Here, the height difference between the two levels was 4.7 ± 0.6 nm as derived from six different profiles. One representative height profile is drawn in Fig. 1*d* (right panel).

To test whether the image actually represented a lipid bilayer the aqueous buffer was exchanged with pure DMSO. After solubilization of the bilayer within about 5 min and washout of DMSO the same area was scanned as used for Fig. 1*a* and *c*. The mica surface with a few remaining flatspots became visible (*see* Fig. 1*e*).

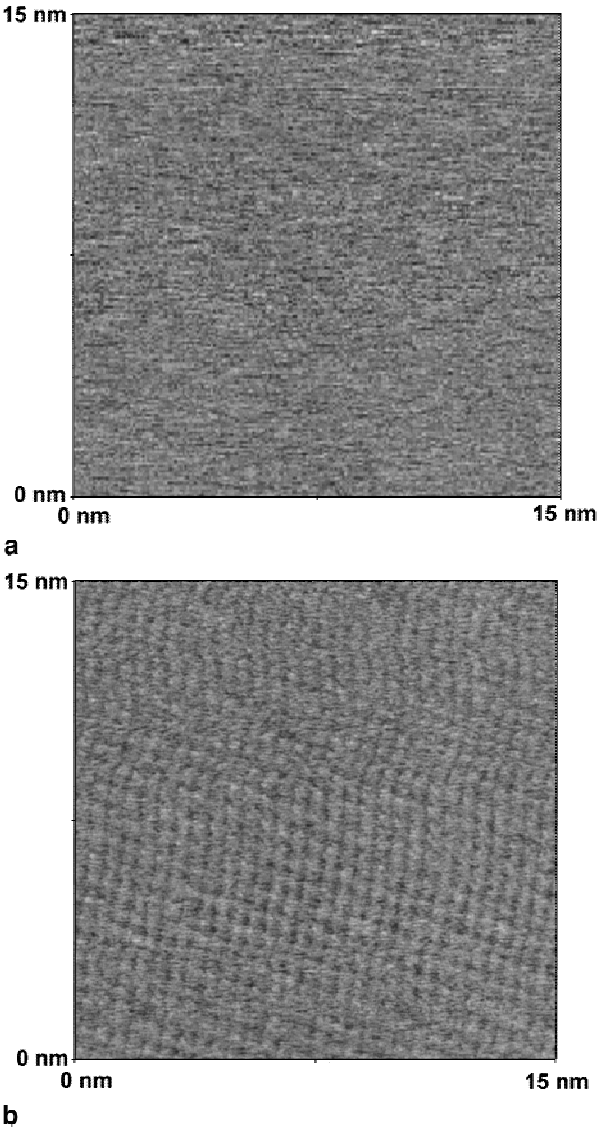


Fig. 2. Sequence of subsequent AFM images of a mica supported continuous bilayer patch in Na-phosphate buffer at high resolution (15 × 15 nm). *(a)* High resolution image of the first scan of a diC15-PC bilayer on mica in Na-phosphate buffer. It consists of 200 lines. *(b)* Image of the 7th scan consisting of 400 lines. All images were processed in error mode and scanned at 10 μm/sec.

Supported Planar Bilayer of Continuous Spread

An SPB was prepared and scanned at room temperature which is well below phase transition temperature. Figure 2*a* shows a typical image at high magnification (15 × 15 nm). The pattern of the surface topology appears to be disordered. Repeated scanning of the identical area significantly altered the pattern. The degree of order increased by consecutive scanning, but did not change significantly after the third or fourth scan. Figure 2*b* shows the result of the seventh scan. Virtually unordered areas, as obtained in the first scan, and those of well-defined rows running about perpendicular to the scanning direction are visible. Domains of differently oriented rows were always separated by unordered areas. Crossing of rows was not observed. The corresponding two-dimensional Fourier transform of areas of higher order (*data not shown*) indicates an increased order by repeated scanning, but not the typical pattern expected for hexagonal symmetry. To exclude imaging artefacts the scan direction was changed by 90°C after the rows became visible. After changing the imaging direction the

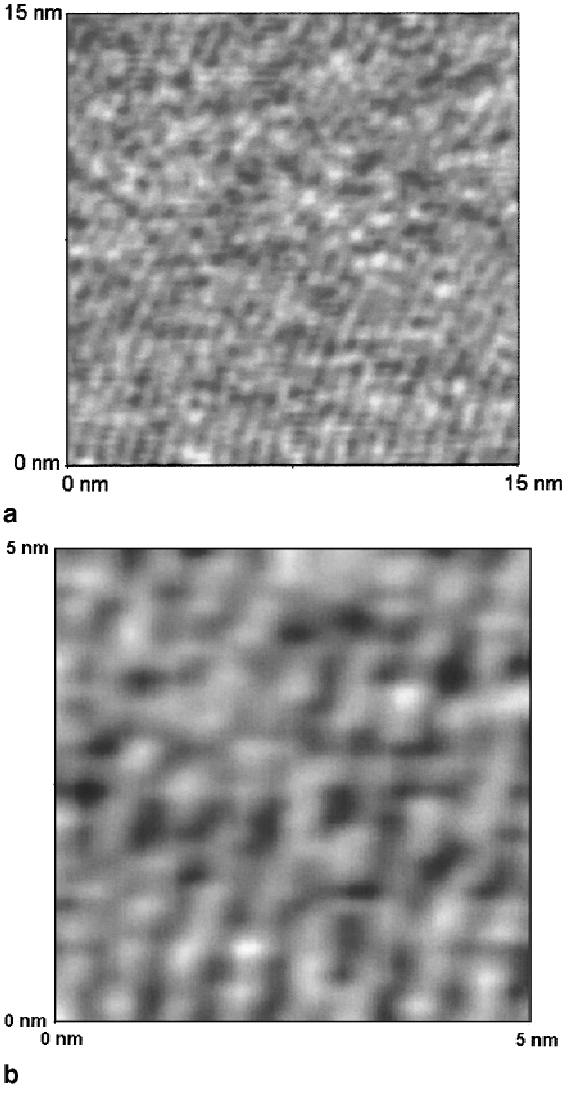


Fig. 3. High resolution image of a mica-supported continuous diC15-PC bilayer in Na-phosphate. *(a)* Image in 2D-reconstruction of an area of 15 × 15 nm with 100 lines in error mode. *(b)* Magnified area of *(a)* in 2D-reconstruction with 34 lines. Scan rate was set to 10 μm/sec.

ordered pattern disappeared and reappeared after repeated scans.

The patterned surface allowed a closer inspection of the molecules at higher magnification. Figure 3*a* presents an image of 15 × 15 nm as 2D-reconstruction which was obtained after the fifth scan. An enlarged area of 5 nm × 5 nm is given in Fig. 3*b*. Structures are visible which are regularly ordered in parallel rows with a spacing of approximately 0.63 nm. These structures were neither observed in the first scan nor prior to SPB formation. The number of individual elevations per patterned 5 × 5 nm area was approximately 60. This corresponds to about 0.42 nm^2 per exalted object, which is close to 0.47–0.54 nm^2, the area required for one phosphatidylcholine molecule in a bilayer membrane (Hauser et al., 1981). Even at this molecular level the bilayer was free of defect structures exceeding a 1.5 × 1.5 nm area, corresponding to the area required by about four lipid head groups. The appearance of structures of higher order did not depend on the support type, as similar pattern of rows were also observed for diC15-PC on a silicon surface (*data not shown*). For comparison, images in tapping mode were recorded at molecular resolution. A representative image is shown in Fig. 4 after the first scan. It indicates a disordered pattern of the bilayer surface which is formed by the choline headgroups. A comparison of height profiles in sub-nanometer resolution with those derived in contact mode is hindered by the fact that the cantilever has to be replaced for the application of the tapping mode technique. Due to the force applied on the membrane, the membrane thickness is underestimated in both cases.

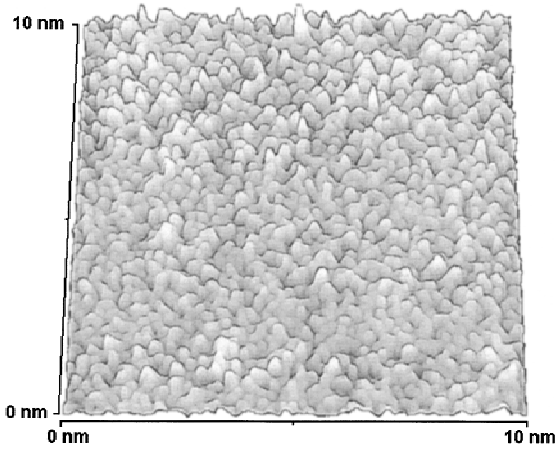


Fig. 4. High resolution image of a mica-supported continuous diC15-PC bilayer in tapping mode. Image in 3D-presentation of an area of 10 × 10 nm with 400 lines. The scan rate was 20 μm/sec and the resonance frequency 299.3 kHz.

Discussion

Our results provide detailed structural information on the influence of repeated scanning by AFM on the defects in supported phosphatidylcholine bilayers in the gel phase and on the pattern of choline headgroups at molecular resolution. The first scan at molecular resolution revealed a disordered arrangement of the molecules. Yet, after consecutive scanning of the lipid surface parallel rows of molecules appeared using either mica or silicon wafer as support. It is known that in the gel phase the hydrocarbon chains are stiff and hexagonally packed with a spacing of about 0.48 nm (Tardieu, Luzzatti & Reman, 1973; Janiak, Small & Shipley, 1976). It is known that during the scan movement, the tip actually

touches several molecules at a time (Weihs et al., 1991), partly penetrates into the surface of the top layer and indents by about 1 nm at a loading force of 1 nN (Raedler et al., 1994). Obviously, this disturbance provides sufficient energy to disturb the interaction of the neutral phosphatidylcholine headgroups and to align them to the observed packing of higher order which appears as rows about perpendicular to the scan direction (Fig. 2). Since there exists a mutual adaptability of the space requirement between head group and hydrocarbon chains, it is tempting to suggest that the change in order reflects a scanning-induced variable arrangement of the headgroups. It appears to be likely that in the undistorted state the headgroups are disordered. Recently, it has been shown that adsorbed lipid membranes may be cut and fused with the scanning tip (Mou et al., 1994; Brandow et al., 1993). Here we have shown that even sub-nm structures are susceptible to manipulation by the scanning tip.

Furthermore, we observed a parallel spacing of 0.68 nm perpendicular to the scanning direction. This spacing is clearly different from the ripple structure with a periodicity of about 18 nm for mica supported diC15-PC bilayers in the gel phase (Mou et al., 1994). These ripples were induced by addition of tris(hydroxymethyl)-aminomethane to the electrolyte and their orientation appeared to be independent of the scanning direction. Corresponding to our data on single lipid molecules a ripple would correspond to a parallel arrangement of about thirty diC15-PC molecules.

Interestingly, the rows never crossed each other. This phenomenon has been attributed to soft material when rubbed by a harder material and is referred to as Schallamach waves (Schallamach, 1971). In AFM studies it has been observed on the nanometer scale also for a polystyrene film on mica (Leung & Goh, 1992). It also appeared after repeated imaging of a bilayer made by the Langmuir-Blodgett technique especially from saturated lipids. In that case a periodicity of 0.49 nm was reported for DPPE at scanning forces ten times larger than we used (Hui et al., 1995). The reported periodicity is smaller but comparable with the presented values. The difference might be caused by the fact that the diameter of the head group of DPPE fits to the cross section of the lipid backbone whereas the diameter of the choline head group of the used diC15-PC molecules exceeds the diameter of the lipid backbone. Due to the different size a different packing order of the head groups has been proposed (Tardieu et al., 1973) which might lead to a different spacing of the corresponding Schallamach waves.

From the high resolution image (Fig. 3*b*) the average area for an individual lipid molecule of about 0.42 nm^2 can be estimated. This value closely agrees with the experimentally derived value of 0.47–0.54 nm^2 for the space requirement of an individual lipid headgroup of phosphatidylcholine (Hauser et al., 1981). A comparable value of 0.38 nm^2 has been obtained for a mica-supported distearoylphosphatidylcholine bilayer in the solid phase (Hui et al., 1995). For mica-supported dimyristoyl-phosphatidylethanolamine a value of 0.4 nm^2 was found (Zadsadzinski et al., 1991) which agrees with the expected value of 0.35–0.42 nm^2 for tightly packed phosphatidylethanolamine headgroups (Hauser et al., 1981). The close agreement indicates in both cases that two dimensional measurements are not significantly biased by the scanning procedure of an AFM. However, the situation is less favorable for the height parameter which was studied on defect containing bilayers.

The defects within a diC15-PC bilayer were induced by the specific procedure of vesicle fusion on the substrate. A mean value of 4.9 nm for the total thickness of the mica-supported layer was estimated. But it is known that there exists a thin hydration layer between the hydrophilic mica surface and the bilayer. Variable values of 1–3 nm have been reported for the thin water layer for various lipids and supports by use of different techniques (Bayerl & Bloom, 1990; Johnson et al., 1991; Adachi et al., 1995; Koenig et al., 1995). Furthermore, the derived height value is biased by the mentioned indentation of the layer by the applied force of about 1 nN. The indentation can be estimated by linear extrapolation of the thickness reported for supported bilayers of diC16-PC and diC18-PC in the gel phase (Tardieu et al., 1972). A value close to 5 nm should be expected for diC15-PC in the gel phase. Assuming a hydration layer of 1 nm between mica and the lower lipid leaflet, a thickness of about 6 nm should be observed for the height of the mica supported layer. Comparison with the measured thickness of 4.9 nm indicates even at low scanning forces of about 1 nN an indentation of at least 1 nm which is in agreement with the estimate of Raedler et al., 1994. Further experiments are necessary to understand the interaction of the scanning tip with the outer leaflet which would yield a quantitative value not only for the indentation but also for the number of head groups which become simultaneously affected.

For mica-supported phospholipid bilayers of distearoylphosphatidylcholine (Hui et al., 1995) which were formed by the Langmuir-Blodgett technique and studied by AFM in the gel phase, a hexagonal symmetry of the surface of lipid molecules has been found after the first scan in contact mode. We could not observe this high packing order at the surface of mica-supported diC15-PC bilayers. Assuming that the Langmuir-Blodgett technique and the vesicle fusion method yield a comparable bilayer structure, then the different observations in contact-mode might be related to the loading force which in our studies was smaller by about an order of magnitude. Our data obtained in tapping mode support this observation and indicate that the pattern of lipid headgroups are

disordered independently of the hexagonal packed hydrocarbon chains. This observation might be caused by the circumstance that the chains must tilt to accommodate the more spacious surface-aligned parallel arrangement of the phosphorylcholine head group lattice (Büldt et al., 1979; Hauser et al., 1981). Further investigations with bilayers formed by lipids of variable headgroups are necessary to support this suggestion.

The authors gratefully acknowledge the support by Dr. F. Lang and Dr. F. Jähnig.

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