Scanning Tunneling Microscopic (STM) Analysis of the Phospholipid Bilayer Surface Characteristics in the $P\beta$ Phase. Secondary Ripples

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Summary. Previous investigations have elaborated on the necessity for dimyristoylphosphatidylcholine (DMPC) molecules to conform in a ripple phase at the temperature intermediate between the transition and pretransition in order to protect the hydrophilic-hydrophobic polarity of the bilayer. Present observations, in addition to the asymmetric $P\beta$ phase of 11.1 ± 1.3 nm wave-length, demonstrate orthogonal corrugations perpendicular to the main wave direction. Whether this is a prerequisite for maintaining the molecular order-disorder balance at such specific temperature, or it results from yet unknown source is unclear at present.

Physicochemical properties of polar fatty acids and/or phospholipid molecules were extensively investigated by optical microscopy, transmission electron microscopy (TEM) of replicated freeze-fractured surfaces, STM of Langmuir-Blodgett films and replicated surfaces, electron densitometry, nuclear magnetic resonance, X-ray diffraction, and other techniques. Dimyristoylphosphatidylcholine (DMPC), due to a strict dependence on the level of saturation and thermal fluctuation, was the substrate of choice in most of previous investigations. The polar head of this molecule supports three methyl groups and is, for this reason, probably too large in proportion to the hydrocarbon chains at a subtransitional temperatures (HAUSER et al., 1981). It was, furthermore, suggested by ZASADZINSKI and SCHNEIDER (1987) that the DMPC bilayer assumes a ripple configuration ($P\beta$) as an alternate way of satisfying the area incompatibility at the mid-range temperature between the main transition and the pretransition temperatures. The same, however, is not true of the ethanolamine molecule, whose head methyl groups are substituted with hydrogen atoms, thus resulting in a stronger intermolecular binding. Temperature-dependent and

reversible interlayer order-disorder transition of phospholipids is based on a simultaneous presence of solid and fluid-like membrane properties, as it was ascertained by the nuclear magnetic resonance investigation of WITTERBORT et al. (1981).

FALKOVITZ et al. (1982) further postulated that the solid molecules are coupled with the fluid-like molecules across the bilayer in the $P\beta$ phase, resulting in a rippled surface configuration. The latter, according to TARDIEU et al. (1973), may also be due to the orientational tilt of hydrocarbon chains, which is typical of chemically homogeneous phospholipids, and the interlocking of methyl groups. Because of a hexagonal packing of DMPC hydrocarbon chains in the $P\beta$ phase the directions of ripples assume a three-axial pattern (ZASADZINSKI and SCHNEIDER, 1987; VIDIĆ and OBCEMEA, 1991) with a separation of 120° between each axis.

Replicated ripple phase of DMPC bilayers were simultaneously analyzed by the TEM and STM by VIDIĆ and OBCEMEA (1992, in press) and OBCEMEA and VIDIĆ (1992, under consideration). Based on the quantitative assessment of replicas and the wave-length of ripples by the two techniques, which were in good correlation, it was concluded that the STM data were derived from the veritable images of investigated samples in real time. The DMPC molecules, due to the utilized concentration of water in the phospholipid solution, were predominantly arranged in multiple concentric bilayers, liposomes, with an average thickness of the bilayer of 5.8 nm. This was estimated by the TEM on the perpendicularly fractures liposomes showing a clear bilayer profile. The STM findings established an asymmetry of the wave revolution with respect to the topography of the left and right wave inclinations to the bilayer normal. On cross section, a wave consisted of a left and a right

slope (inclination) from the wave peak to the respective trough. The direct measurements indicated that the left slope made, on average, a steeper inclination to the horizontal plane and a greater angle than the right slope, resulting in a shorter left than right side of a wave revolution. Because a similar asymmetry was earlier observed for the $P\beta$ phase of DMPC, also by ZASADZINSKI et al. (1988), an attempt was made, by theoretical means, to test the model for such a molecular arrangement at this specific molar concentration of phospholipids and at this same temperature. Two conditions were considered for this approach: 1) the van der Waals radius of the polar head-group of DMPC and the distance between the adjacent head-groups were ascertained as 0.76 and 1.6 nm, respectively, by LOU et al. (1990); and 2) although the tilt of hydrocarbon chains to the bilayer normal of 30° was suggested for the temperature of 19°C by TARDIEU et al. (1973), it may be even greater for the molar concentration used in this study (JANIAK et al., 1979). In considering the one-half length of the hydrocarbon chain bilayer as 2.5 nm, distance between the adjacent DMPC head-centers as 2.35 nm, the initial tilt minus 30° as 60° , and the angular complement of the latter value as 30° , the left and right angles of a ripple revolution with the horizontal plane were calculated as 38° and 21° respectively. These values, especially the left angle, deviated somewhat from those obtained by the direct image analysis ($21.5 \pm 7.3^\circ$ and $18.1 \pm 4.5^\circ$ for the left and right angles respectively). Such discrepancy between the theoretically and directly obtained values may to some degree be due to the assumption of the theoretically analyzed molecular model that all hydrocarbon chains were in solid state, fully extended and tilted to the bilayer normal. From the previous observations by FALCOVITZ et al. (1982) it is clear, however, that certain areas of the phospholipid bilayer, especially those around the

peaks and troughs of ripples, consist indeed of hydrocarbon chains in a fluid-like configuration. The theoretically derived values were, consequently, magnified in comparison to the results of direct measurements of the STM images. The theoretical approach indicated, in any event, that the tilting pattern of at least some hydrocarbon chains may be the cause of the ripple asymmetry to satisfy the order-disorder balance and to protect the hydrophilic-hydrophobic polarity of DMPC molecules in the bilayer.

MATERIALS AND METHODS

A mixture of crystalline $L\alpha$ DMPC (purity $>99\%$; SIGMA, St. Louis, MO) in doubly-distilled water was made at a molecular concentration of 1 to 23 and at the temperature of 35°C . The mixture was shaken and maintained for several hours to allow an even saturation of the phospholipid compound. The substrate was subsequently cooled to the mid-range point ($18 \pm 1^\circ\text{C}$) between the pretransition and transition states, then spread in several hundred μm thick layers between the planchettes (Balzers BUO-12-056T, Hudson, NH) and frozen to -190°C by liquid propane cooled in liquid nitrogen. The specimens were fractured at -170°C and 10^{-5} torr in a Reichert-Jung Cryofract (freeze-fracture device, Cambridge Instruments, Buffalo, NY) and initially replicated with a 2.5 nm thick platinum-carbon mixture followed by a 30 nm thick carbon film. The deposition of the platinum-carbon mixture, which is believed to result in a superior resolution, and the carbon film were achieved under the normal angle to prevent surface distortion of the replica. The replicated specimens on the planchettes were removed from the vacuum chamber and dissolved in chromic acid (ZASADZINSKI and SCHNEIDER, 1987). The replicas were subsequently washed in

Fig. 1. An almost perpendicular fracture of a liposome reveals either individual bilayers (*arrowheads*) or stacks of bilayers (*arrows*). Fractured profiles similar to this were used to assess the cross diameter of the DMPC bilayer. $\times 40,000$

Fig. 2. The line-mode presentation of the ripple phase shows the primary waves (*arrows*) separated from one another by ca. 11 nm. Shallow secondary (perpendicular) ripples, the ribconfiguration (*arrowheads*), connect the adjacent wave-slopes along the troughs without making noticeable perpendicular indentation on the peak area of ripples. The scale is in nanometers.

Fig. 3. A secondary ripple (*arrowheads*) perpendicularly transects the continuity of primary waves (*arrows*). The indentation by the secondary corrugation is in this instance as deep as the average amplitude of the ripple phase, 1.5 nm. The scale is in nanometers.

Fig. 4. An example of the step-wise right inclination of ripples is demonstrated in this image. The left slope, starting from the peak of a wave (*large arrows*), gradually and steeply descends to the trough area (*small arrows*). The right slope, however, makes a step-wise inclination with the plateau of the step (between *arrowheads*) positioned approximately at the mid-level of the ripple amplitude. The scale is in nanometers.

Figs. 1-4. Legends on the opposite page.

chloroethanol-water mixture, 50:50% by volume, rinsed in doubly-distilled water and placed on 300-mesh gold grids. Electron microscopic observations were made with a JEOL 1200 operated at 60–80 kV. The analysis of the surface features of replicas was achieved by either a prototype STM designed for electrochemistry (Angstrom Technology, Mesa, AZ), or a commercial STM NanoScope II (Digital Instruments, Santa Barbara, CA).

The STM images, consisting of 400×400 pixels, were taken as constant-current topographs in the air. The following conditions were adjusted in the course of the STM analyses of samples: scan size from 20 to 400 V, scan rate from 10 to 20 Hz, set-point current from 1 to 5 nA, applied bias voltage from ± 100 to 200 mV, integral and proportional gains from 20 to 200, 2D gain close to 1. The images of replicas and, in particular of the $P\beta$ ripple phase were doubly filtered before being stored on discs and subsequently analyzed for various surface parameters.

RESULTS

The fluidity of the DMPC mixture was so adjusted as to promote the arrangement of bilayers in the liposomal structure. A typical liposomal profile on the replica consisted of a multitude of phospholipid bilayers fractured either perpendicularly, obliquely, or longitudinally. The former two modes were rather convenient for the assessment of the cross diameter of bilayers and the general stacking pattern of concentric bilayers with respect to the radius of the curvature. The number of bilayers in and the radius of the liposomes (2.5 ± 1.3 nm) varied considerably from one organelle to another; the thickness of a phospholipid bilayer in the liposome, however, was of a constant value of 5.8 nm (Fig. 1). Those liposomes fractured longitudinally, along the hydrophobic gap, displayed a ripple configuration on the phospholipid surface. The hydrocarbon chains, in this phase, were assembled in a three-directional ripples, each two directions separated by 120° . The pattern of ripple topography was found in phase across several phospholipid bilayers, as it was observed in instances where the fractured bilayers only partially overlapped one another. The two distinctly different corrugated phases by the wave-length were ascertained for most samples. One, corresponding to the asymmetric $\Omega/2$ ripples, possessed a wave-length of 11.1 ± 1.3 nm; the other, resembling the symmetric Ω phase (asymmetric $\Omega/2$ and symmetric Ω phases were introduced by RÜPPEL and SACKMANN, 1983), had a wave-length of 22.8 ± 3.6 nm.

Because of its unprecedented resolving power and the precision to manipulate the sample in any desired tilt and/or cross section, the scanning tunneling technology provided additional information about the nanometer-fine substructure of the corrugated phase. The present findings, which are in agreement with an earlier investigation of the DMPC replica by the STM (ZASADZINSKI et al., 1988), identified once again roughly orthogonal and irregular corrugations, extending perpendicular to the main direction of ripples. They most frequently traversed only the troughs of the surface while connecting the adjacent slopes in the form of perpendicular ribs (Fig. 2). The peak areas of the surface, however, were not affected by the topography of secondary ripples and, therefore, remained horizontally flat. In less frequent observations the secondary ripples made a noticeable perpendicular indentation on the primary ripples (Fig. 3) with a depth of indentation corresponding almost to the average value of the wave-amplitude, 1.5 ± 0.3 nm. Such indentations extended perpendicular to the main direction of ripples over the main wave revolutions. Another topographical irregularity of the replicated surface of DMPC bilayers was on occasion confined to the right slope of the wave revolutions only. Instead of a regular and gradual inclination of a wave from the peak to the horizontal plane, a step-wise configuration was encountered (Fig. 4). The plateau of such a step was usually observed at about the mid-level of the wave-amplitude, ca. 0.75 nm from either the trough or the peak of ripples. The horizontal diameter of the plateau was 0.89 ± 0.12 nm. Although it is not clear from the present findings whether these substructures are inherent to the replica or an artifact of another source, they may possibly indicate an irregular arrangement of phospholipid molecules also along the axis perpendicular to the direction of the main ripple phase ($P\beta$).

DISCUSSION

In spite of the uncertainty in interpreting the physicochemical reasons for the occurrence of secondary ripples, the investigation of freeze-fractured DMPC replicas by the STM appears a reliable and reproducible approach. First of all, the metric results of the well established TEM and the novel STM techniques of the DMPC replicas were in good correlation. Secondly, the present observation strongly supports the findings from the same material by an independent laboratory (ZASADZINSKI et al., 1988) in respect to the physical characteristics of the wave revolution and the occurrence of the secondary ripples. The

theoretical model of the molecular arrangement in the ripple configuration, furthermore, integrates well with the previously forwarded assumption that the hydrocarbon chains at the mid-range temperature between the main transition and the pretransition levels: 1) are in a tilted mode to the bilayer normal (TARDIEU et al., 1973); and 2) likely represent a mixture of the solid and the fluid-like states (FALCOVITZ et al., 1982).

Because of the well integrated observations from the temperature dependent behavior of DMPC molecules by various techniques, including the STM, it is assumed that the topography of the freeze-fractured and replicated bilayers was a reliable reflection of the images in real time. Additional findings, the secondary ripples and the step-wise right inclination of the wave, on the other hand, can not be explained on the sole basis of the molecular conduct to the varying temperatures, as evaluated in several previous investigations. It could be only speculated, at present, that some sort of tilt and/or shift between the crystal and fluid states of hydrocarbon chains, which regularly occur for the DMPC bilayer at the mid-range temperature ($18 \pm 1^\circ\text{C}$), along the X axis may also be required in the Y direction. The question, however, remains whether or not the secondary corrugation of the surface occurs as a prerequisite for maintaining the hexagonal lattice of hydrocarbon chains and for protecting the overall hydrophilic-hydrophobic polarity of the entire phospholipid bilayer.

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REFERENCES

- FALCOVITZ, M. S., M. SEUL, H. L. FRISCH and H. M. MCCONNELL: Theory of periodic structures in lipid bilayer membranes. *Nat. Acad. Sci. USA.* 79: 3918-3921 (1982).
- HAUSER, H., I. PASCHER, R. H. PEARSON and S. SUNDELL: Preferred conformation and molecular packing of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta* 650: 21-51 (1981).
- JANIAK, M. J., D. M. SMALL and G. G. SHIPLEY: Temperature and compositional dependence of the structure of hydrated dimyristoyl lecithin. *J. Biol. Chem.* 254: 6068-6078 (1979).
- LOU, C., C. ZHU, L. RUAN, G. HUANG, C. DAI, Z. CHENG, C. BAI, Y. SU, S. XU, K. LIN and J. D. BALDESCHWIELER: Scanning tunneling microscopy of the phosphatidylcholine bilayers. *J. Vac. Sci. Technol. A* 8 (1): 684-686 (1990).
- OBCEMEA, C. H. and B. VIDIĆ: Lipid bilayer molecular organization in $P\beta$ phase studied by scanning tunneling microscopy (STM). *Proc. Xth Int. Conf. STM. Interlaken, Switzerland. August 12-16, 1991*, (p. 165).
- : Bilipid layer molecular organization in $P\beta$ phase studied by scanning tunneling microscopy (STM). *Ultramicro. Under consideration*.
- RÜPPEL, D. and E. SACKMANN: On defects in different phases of two-dimensional lipid bilayers. *J. Phys. (Paris)* 44: 1025-1034 (1983).
- TARDIEU, A., V. LUZZATI and F. C. REMAN: Structure and polymorphism of the hydrocarbon chains of lipids: A study of lecithin-water phases. *J. Mol. Biol.* 75: 711-733 (1973).
- VIDIĆ, B. and C. H. OBCEMEA: Dimyristoylphosphatidylcholine molecular organization studied in $P\beta$ phase by scanning tunneling microscope. *Abs. 73eme Cong. Assoc. Anat. Bruxelles, Belgium, September 8-12, 1991* (p. 141).
- : Assymetry of bimembrane ripples in $P\beta$ phase. Scanning tunneling microscopy of replicas. *Proc. Serbian Acad. Sci. (Belgrade)*. 1992, in press.
- WITTERBORT, R. J., C. F. SCHMIDT and R. G. GRIFFIN: Solid-state carbon-13 nuclear magnetic resonance of the lecithin gel to liquid-crystalline phase transition. *Biochemistry* 20: 4223-4228 (1981).
- ZASADZINSKI, J. A. N. and M. B. SCHNEIDER: Ripple wavelength, amplitude, and configuration in lyotropic liquid crystals as a function of effective headgroup size. *J. Phys. (Paris)* 48: 2001-2011 (1987).
- ZASADZINSKI, J. A. N., J. SCHNEIR, J. GURLEY, V. ELINGS and P. K. HANSMA: Scanning tunneling microscopy of freeze-fracture replicas of biomembranes. *Science* 239: 1013-1014 (1988).

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