Alcohol Induces Interdigitated Domains in Unilamellar Phosphatidylcholine Bilayers†

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ABSTRACT: Using the atomic force microscope (AFM) *in situ*, we have demonstrated that acyl chain interdigitation can be induced reversibly by alcohol in supported unilamellar phospholipid bilayers. At alcohol concentrations considerably lower than the critical values determined by other experimental techniques, it was found that interdigitated domains can be induced and these domains were stable over a long period of time. The mechanism of such domain formation remains to be elucidated. This work also serves as an example to illustrate the uniqueness of the AFM as a powerful tool in the study of membrane structure and conformation under physiological conditions at high spatial resolution.

Phospholipids are the major components in cell and organelle membranes which play a very important role in the function of a cell, by providing not only necessary compartmentalization but also a proper matrix for membrane-associated proteins such as channels, receptors, and pumps to perform their physiological functions (Yeagle, 1993; Kotyk et al., 1988; Glaser, 1993). The physical states of the self-assembled phospholipids are characterized by their different phases at various temperatures (Ladbrooke & Chapman, 1969; Chen et al.; 1980). A main phase transition of the first order occurs reversibly when lipid changes from the liquid-crystalline phase to the gel phase or vice versa. At temperatures above the main transition temperature (T_m), the bilayer is in the liquid-crystalline phase in which the lipid molecules can diffuse laterally. The gel phase becomes favorable as the temperature is lowered below T_m, in which the lipid molecules are essentially immobile. In addition to temperature, many external perturbants were also found to cause changes in the structure of the lipid bilayer. For example, the commonly used buffer compound tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) was found to reversibly induce the so-called ripple phase in various phosphatidylcholine (PC) bilayers at room temperature (Mou et al., 1994). In addition, many chemicals and drugs were thought to exert their physiological/pharmacological effects by directly dissolving into the lipid membrane, as demonstrated by their effects on the alternation of lipid packing in membranes (Rowe, 1983, 1987; Simon & McIntosh, 1984; Nambi et al., 1988; Ueda et al., 1986). It has been well established that, by X-ray diffraction of stacked-up PC bilayers, alcohol can induce an interdigitated state in the lipid bilayers. Similar research was also reported recently with vesicles using fluorescence spectroscopy (Komatsu et al., 1993; Boni et al., 1993). Specifically, the acyl chains of saturated diacylphosphatidylcholines packed in the two leaflets of the bilayer extend across the entire hydrocarbon width, resulting in the disappearance of the bilayer midplane, commonly occupied by the chain terminal methyl groups in normal bilayers. This acyl chain interdigitation occurs as the concentration of the chemical in the solution is higher than a threshold value (Rowe, 1992). One characteristic of the interdigitated state is drastic reduction in the thickness of the lipid bilayer. For example, the hydrocarbon thickness of fully interdigitated bilayers is significantly reduced (Simon & McIntosh, 1984). Such a thickness change may have a strong effect on the function of membrane-associated proteins due to the mismatch of the hydrophobic region (Slater & Huang, 1988). The interdigitated state in a single bilayer of phospholipid has not been directly demonstrated, and information on interdigitated domains is virtually nonexistent.

In the present work, AFM was used to study the morphology of the unilamellar phosphatidylcholine bilayer on a mica surface in solution at room temperature. We found that both ethanol and 2-propanol can reversibly induce interdigitation in 1,2-dipalmitoyl-*sn*-glycero-3- and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DPPC and DSPC) bilayers. In particular, stable interdigitated domains were directly observed over a period of several days with alcohol concentrations far below the threshold values determined by thermodynamic and X-ray diffraction studies on multilayer systems prepared from these two lipids (Rowe, 1985, 1987; Simon & McIntosh, 1984; Nambi et al., 1988).

MATERIALS AND METHODS

DPPC, DSPC, and dilauroylphosphatidylethanolamine (DLPE) powders were purchased from Sigma Chemicals (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL), and used directly without further purification. According to the suppliers, the purity is better than 99%. 2-Propanol (Sigma Chemicals) and ethanol (AAPER Alcohol and Chemical Co.) were of reagent grade. Deionized water (18 MΩ) from a Continental water system (Model LBMPP 1002) was used in the experiment.

The same method as described in Mou et al. (1994) was used to prepare supported unilamellar bilayers via direct vesicle fusion. Lipid powder was first suspended in 170 mM NaCl, at a concentration of about 0.5 mg/mL. The suspension was then subjected to sonication under nitrogen for several times at ~30 min in an ultrasonic bath (Fisher FS-28). The temperature never exceeded 50 °C. When the suspension

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became transparent, devoid of any aggregates, it was considered to contain unilamellar vesicles 30–50 nm in diameter. About 30 μl of the vesicle suspension was directly applied to a freshly cleaved mica surface ($\sim 3 \times 4$ mm^2) at room temperature. After incubation for several hours at room temperature or 4 °C, the specimen was washed with buffer at room temperature to remove excess vesicles, followed by heating the specimen to above T_m. By this procedure, clean unilamellar bilayers are normally formed on the mica surface with excellent coverage.

For the study of the alcohol effect, alcohol (ethanol or 2-propanol) was first mixed with deionized water. This alcohol–water mixture was then added to a 35 mm petri dish containing the specimen to replace the original buffer. The amount of original buffer was about 200 μL, in the form of a droplet covering the bilayer. Repeating this operation for several times, the original buffer should be essentially eliminated. During this procedure, the bilayer was never allowed to be exposed to air. The petri dish was then sealed off by parafilm to prevent evaporation of the alcohol. It was heated to above the T_m (41 °C for DPPC, 55 °C for DSPC, and 30 °C for DLPE) for 1 h and then cooled down to room temperature prior to AFM-imaging in the same solution.

The AFM has been described in great detail elsewhere, and its application to biological samples has been well documented (Engel, 1991; Hoh & Hansma, 1992; Bustamante et al., 1993; Lyubchenko et al., 1993; Yang et al., 1993a,b; Hansma & Hoh, 1993). All images presented in this work were obtained with a NanoScope III AFM from Digital Instruments (Santa Barbara, CA). Commercially available Si_3N_4 tips, with a triangular cantilever 200 μm long, were used (Digital Instruments). According to the manufacturer, the nominal spring constant is 0.06 N/m. Since the spring constant varies over a wide range, the probe force used was in the range of 0.5 nN to perhaps 2 nN. Scanning speed was at a line frequency of 5–8 Hz with 512 pixels per line. A homemade fluid cell retrofitted to the NanoScope III AFM (Yang et al., 1993b) was used which was a self-sealed type to ensure that the alcohol concentration remained constant during AFM imaging.

RESULTS AND DISCUSSION

The unilamellar bilayer of DPPC prepared by direct vesicle fusion on a mica surface was very stable under repeated scans as long as the probe force was kept low (a few nanonewtons). Figure 1 shows a typical AFM image of a DPPC bilayer on a mica surface, obtained at room temperature (22 ± 1 °C) in deionized water. Since the T_m for DPPC is 41 °C, the bilayer under study is thus in the gel phase at room temperature. The thickness measured from natural defects was 5.7 ± 0.3 nm, indicating a single bilayer on the mica surface with a thin layer of water between the mica surface and the bilayer, consistent with other reports (Tamm & McConnell, 1985; Mou et al., 1994). The same thickness was also measured at those defects created by high-speed scanning of the AFM probe at high force (>30 nN), showing the self-consistency of the method (see Figure 1). It is noticed that the natural bilayer in deionized water is basically flat without any feature, except a few defects, which should be expected. The head groups of the lipid molecules were never resolved.

After the addition of an ethanol/water mixture to the AFM fluid cell at room temperature, the bilayer remained featureless at the beginning. At high concentrations of ethanol ($\sim$200 mg/mL = 19.6% v/v) and long incubation time, some thinner domains started to appear at a very slow rate. However, in the presence of ethanol, heating the specimen to above T_m and cooling it down to room temperature greatly facilitated this

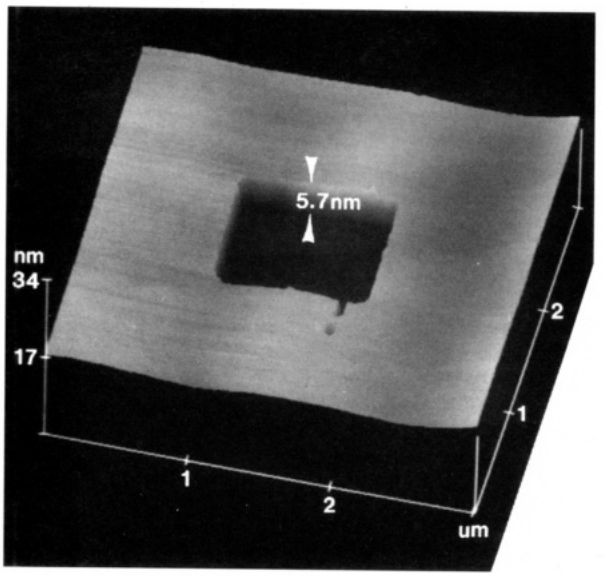

FIGURE 1: DPPC bilayer on the mica surface imaged by AFM at room temperature and in deionized water. This surface plot shows the 3-D perspective of the bilayer with a square defect created by the AFM tip with high-speed ($\sim$200 Hz), high-force ($\sim$30 nN) scanning. The gray level corresponds to the height of the features, the same as in a top projection view (Figure 2–4). Apparently, the bilayer in this area was scraped away by the tip. The thickness measured at the edge of the defect is 5.7 ± 0.3 nm, consistent with the thickness of a single bilayer with a water layer between the mica surface and the lower leaflet of the bilayer (Tamm & McConnell, 1984; Mou et al., 1994).

transition. Figure 2 shows a series of AFM micrographs of the DPPC bilayer at various ethanol concentrations after such a heating/cooling treatment. From Figure 2a–c, the domain structure is more than apparent, which is typical for specimens treated with this procedure. Upon heating, ethanol molecules may be facilitated to intercalate into the head group region of the lipid assembly and to displace H_2O molecules there; hence, the transition from the noninterdigitated to the interdigitated state is promoted by the heating/cooling cycle. Alternatively, it is possible that more ethanol can partition into the fluid phase bilayer at $T > T_m$, so that, upon temperature decrease, the majority of them were retained at the bilayer/water interface due to the strong interaction between the ethanol and the lipid. The thickness difference between the two different domains is 1.9 ± 0.2 nm, while the thicker domain has the same thickness as the one before ethanol was added, as measured from the edges of the defects (which can be seen in Figure 2f in the surface plot). Since the bilayer thickness of DPPC in the gel state is known to be 4–5 nm (Janiak et al., 1976; Simon & McIntosh, 1984), the thinner domains should, therefore, correspond to the interdigitated state of the DPPC bilayer at room temperature. The values measured here are consistent with those obtained by X-ray diffraction of stacked-up DPPC bilayers, where, upon interdigitation, the lamellar repeat period of the gel-state bilayer was reduced by 1.6 nm (Simon & McIntosh, 1984). The slight difference could be due to the increased flexibility of the head groups in the interdigitated bilayer where the interdistance between the head groups is increased significantly. At very high ethanol concentrations (>300 mg/mL = 29.4% v/v), the bilayer structure could no longer be maintained, and small globular structures appeared, presumably formed by the DPPC molecules (as shown in Figure 2d). The packing of the lipid molecules in these globular structures cannot be determined from these measurements alone, due to the limited resolution achieved on these structures by the AFM.

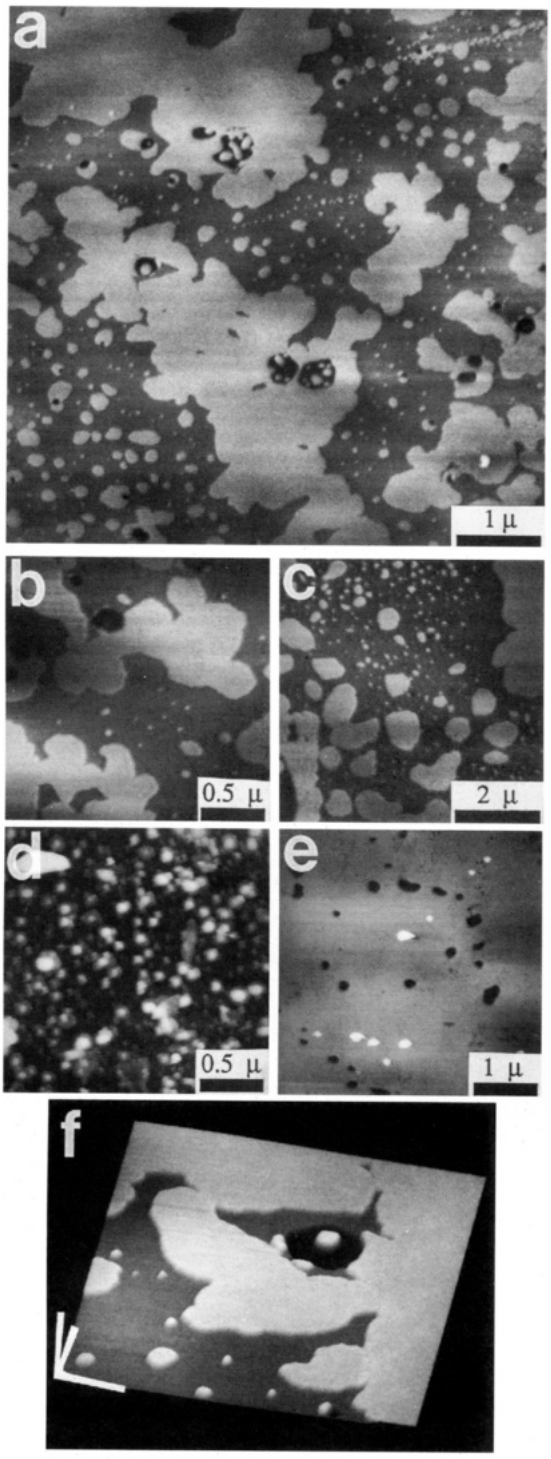

FIGURE 2: Surface morphology of the DPPC bilayer at various ethanol concentrations. (a) is a large-scale view of the DPPC bilayer with 50 mg/mL (4.9% v/v) ethanol. (b) and (c) are smaller scale views of the DPPC bilayer with 50 (4.9% v/v) and 100 mg/mL (9.8% v/v) ethanol, respectively. From (a) to (c), two domains with different thicknesses are seen. Measured from the edge of the defects of a large number of specimens, the thicker domain has the same thickness as the normal DPPC bilayer, and the thinner domain has a 1.9 ± 0.2 nm thickness reduction. This indicates that the thinner domains are in the interdigitated state. At an ethanol concentration of 200 mg/mL (19.6% v/v), the interdigitation is almost complete. At very high ethanol concentrations (>300 mg/mL = 29.4% v/v), the planar structure can no longer be maintained (d), and small globular structures appeared. (e) shows that upon the removal of ethanol (50 mg/mL = 4.9% v/v), the bilayer structure was restored, but many holes appeared, due to the area reduction for the transition from the interdigitated state to the bilayer state. (f) is a surface plot of interdigitated domains with natural defects, showing the thickness difference among the domains explicitly. Horizontal scale bars, 0.2 μm; vertical scale bar, 40 nm.

Upon the removal of ethanol at room temperature, the interdigitated domains remained stable for a long period of time, up to several days. Only by heating the specimen to a temperature above the T_m can the noninterdigitated bilayer structure be fully restored (see Figure 2e). It is interesting to note from Figure 2e that many structural defects are present in the two-dimensional plane of the noninterdigitated bilayer. Such defects are most likely caused by the reduced area at the lipid/H_2O interface when the bilayer is transformed from the interdigitated state to the noninterdigitated state. The persistence of the interdigitated domains after removal of ethanol from the solution seems to suggest that ethanol molecules prefer to remain at the interface between the interdigitated bilayer and the aqueous medium. The interaction between the lipid and ethanol is perhaps strong enough to retard somewhat the diffusion of ethanol away from the interface.

We have imaged the interdigitated bilayer under various ethanol concentrations to see whether the interdigitated area can be correlated to the ethanol concentration. Unfortunately, the measurements are too scattered to be conclusive, perhaps due to the nonuniformity of the specimen and the limited scanning range of the AFM. It is also likely that the cooling rate may play some role in the amount of ethanol retained in the bilayer, which could not be well controlled at present. At a concentration of 200 mg/mL (19.6% v/v), almost complete interdigitation can be induced (not shown). At 25 mg/mL (2.5% v/v) ethanol concentration, which is far below the threshold of 55 mg/mL (5.4% v/v) described previously (Rowe, 1992), sizable interdigitated domains were already observed (Figure 3a). The underlying reason for such domain formation is not clear at present. If we assume that a certain minimum amount of ethanol must be present at the interface in order to stabilize the interdigitated domains, this would indicate that the ethanol concentration must be inhomogeneous in the DPPC bilayer. How this inhomogeneity is originated during the heating/cooling cycle remains to be elucidated. Perhaps ethanol molecules dissolved in the bilayer could aggregate to certain regions randomly when the temperature is lowered, like the crystal formation in solution. This would increase the local ethanol concentration in certain areas to above the critical value so that a mosaic pattern of interdigitation can be formed. It will be very interesting to study the shapes and the formation of the interdigitated domains. If the temperature can be well controlled, and the speed of image acquisition can be faster than those available today, such a "real" time observation may shed some light on ethanol aggregation during temperature reduction. However, a theoretical estimate seems to suggest that the lateral resolution could be seriously degraded at high scanning speed due to the limited response of the cantilever (Butt et al., 1993). Therefore, whether such a "real" time imaging can be achieved remains to be demonstrated. It is also interesting to note that, at very low ethanol concentrations, very narrow and long domains could be observed in certain areas (Figure 3b). This seems to indicate that, even at very low ethanol concentration, there may still be some localized structural changes in the membrane due to ethanol aggregation.

The above observation is not confined to DPPC or ethanol. With 2-propanol, which is slightly larger than ethanol in terms of molecular weight with one more methyl group, similar interdigitated domains were also induced in DPPC bilayers (Figure 4a). It turned out that 2-propanol was much more effective in inducing chain interdigitation. At 100 mg/mL (12.8% v/v), complete interdigitation was already induced, compared with 200 mg/mL (19.6% v/v) for ethanol; this

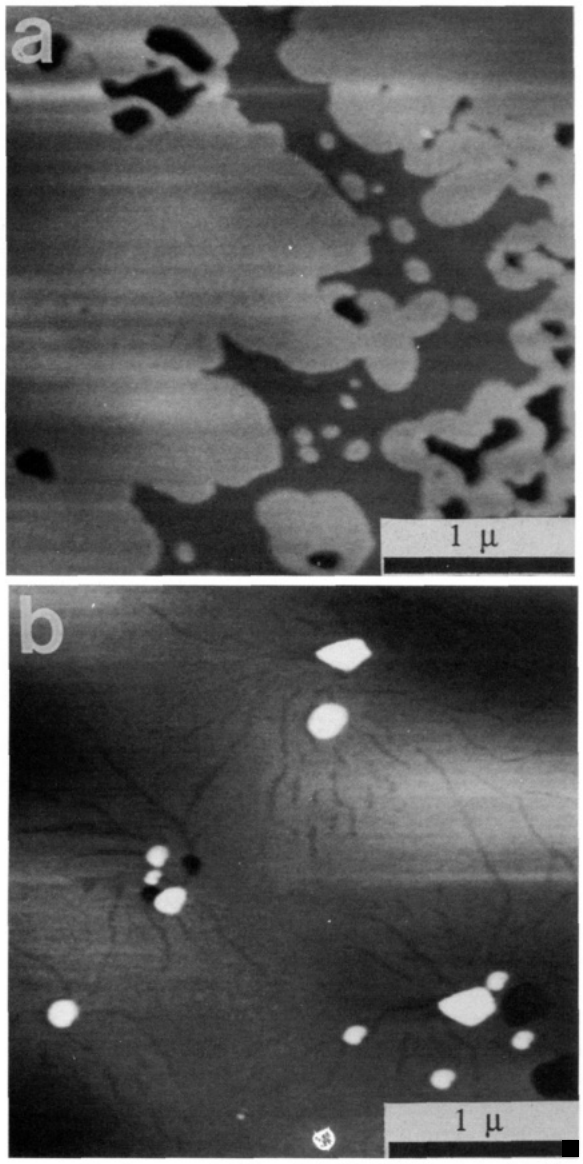

FIGURE 3: Surface morphology of the DPPC bilayer under 25 mg/mL (2.5% v/v) ethanol. (a) shows the interdigitated domains similar to those at higher ethanol concentrations; (b) shows the peculiar line-type domains found on some areas of the specimen. The previously found threshold for interdigitation is 55 mg/mL (5.4% v/v), which is much higher than the one used here. This indicates that the "critical" concentration is actually much lower.

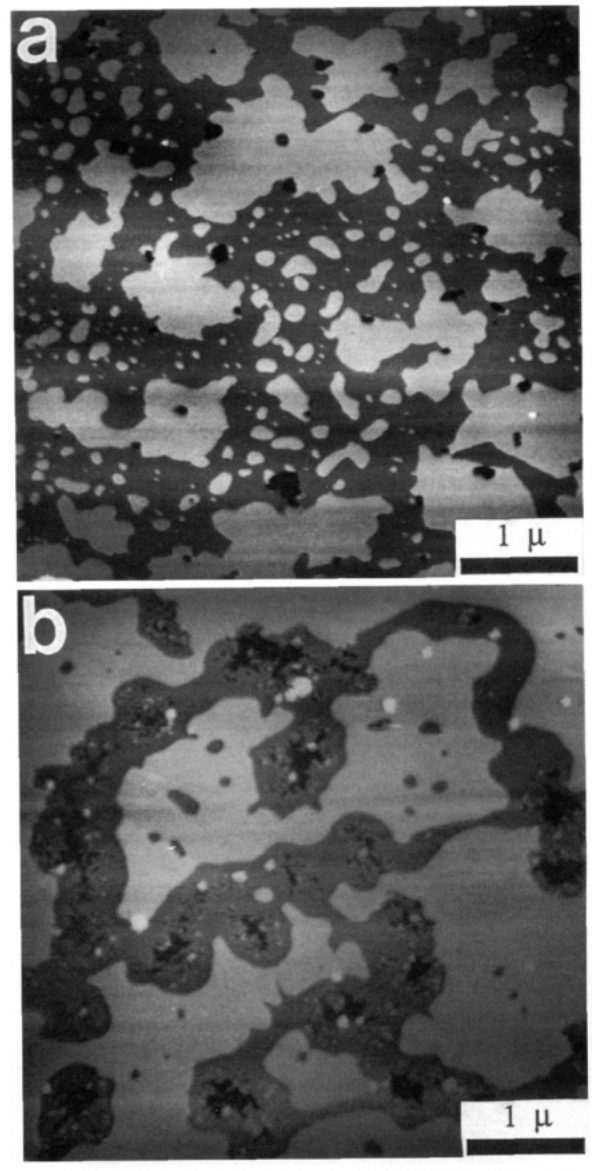

FIGURE 4: (a) Interdigitated domain induced by 2-propanol (25 mg/mL = 3.2% v/v) in DPPC bilayers. (b) Interdigitated domain induced by 2-propanol (100 mg/mL = 12.8% v/v) in DSPC bilayers.

perhaps can be attributed to the larger hydrophobic chain in 2-propanol, resulting in a more favorable partition into the lipid bilayer. With DSPC bilayers prepared by the same method, 2-propanol also induced the interdigitated domains as shown in Figure 4b, where the thickness of the bilayer was 5.9 ± 0.5 nm, and the reduction in thickness for the interdigitated domain is 2.4 ± 0.2 nm, slightly larger than that for the DPPC bilayers, as expected.

As a control, identical procedures were used on DLPE bilayers for 2-propanol concentrations up to 200 mg/mL (25.6% v/v). Interdigitation in the PE bilayer was never observed, consistent with the previous conclusion that chain interdigitation cannot be induced in PE bilayers by alcohol (Rowe, 1992).

In summary, we have demonstrated by atomic force microscopy that the domains of lipid interdigitation in single PC bilayers in the presence of alcohol can be directly observed. We have further demonstrated that, at alcohol concentrations far below the threshold value as described previously, interdigitated domains can exist in lipid bilayers. However, the mechanism by which such domains are formed remains to be elucidated. These studies also demonstrate that AFM is a powerful tool for investigating the membrane structure and conformation at nanometer resolution under nearly physiological conditions.

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REFERENCES

Boni, L. T., Minchey, S. R., Perkins, W. R., Ahl, P. L., Slater, J. L., Tate, M. W., Gruner, S. M. & Janoff, A. S. (1993) *Biochim. Biophys. Acta 1146*, 247–257.

Bustamante, C., Keller, D., & Yang, G. (1993) *Curr. Opin. Struct. Biol. 3*, 363–372.

Butt, H. J., Siedle, P., Seifert, K., Fendler, K., Seeger, T., Bamberg, E., Weisenhorn, A. L., Goldie, K., & Engel, A. (1993) *J. Microsc. 169*, 75–84.

Chen, S. C., Sturtevart, J. M., & Gaffney, B. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5060–5063.

Engel, A. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 79–108.
Glaser, M. (1993) *Curr. Opin. Struct. Biol. 3*, 475–481.
Hansma, H. G., & Hoh, J. (1994) *Annu. Rev. Biophys. Biomol. Struct.* (in press).
Hoh, J. H., & Hansma, P. K. (1992) *Trends Cell Biol. 2*, 208–213.
Janiak, M. J., Small, D. M., & Shipley, G. G. (1976) *Biochemistry 15*, 4575–4580.
Komatsu, H., Guy, P. T., & Rowe, E. S. (1993) *Chem. Phys. Lipids 65*, 11–21.
Kotyk, A., Janacek, K., & Koryta, J. (1988) *Biophysical Chemistry of Membrane Functions*, John Wiley & Sons, New York.
Ladbrooke, B. D., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 304–367.
Lyubchenko, Y. L., Olden, P. I., Lampner, D., Lindsay, S. M., & Dunker, K. A. (1993) *Nucleic Acids Res. 21*, 1117–1123.
Mou, J., Yang, J., & Shao, Z. (1994) *Biochemistry 33*, 4439–4443.
Nambi, P., Rowe, E. S., & McIntosh, T. J. (1988) *Biochemistry 27*, 9175–9182.
Rowe, E. S. (1983) *Biochemistry 22*, 3299–3305.
Rowe, E. S. (1987) *Biochemistry 26*, 46–51.
Rowe, E. S. (1992) in *Alcohol and Nerobiology: Receptors, Membranes, and Channels* (Watson, R. R., Ed.) pp 239–267, CRC Press, Boca Raton, FL.
Simon, S. A., & McIntosh, T. J. (1984) *Biochim. Biophys. Acta 773*, 169–172.
Slater, J. L., & Huang, C. (1988) *Prog. Lipid Res. 27*, 325–359.
Tamm, L. K., & McConnell, H. M. (1985) *Biophys. J. 47*, 105–113.
Ueda, I., Tseng, H. S., Kaminoh, Y., Ma, S.-M., Kamaya, H., & Lin, S. H. (1986) *Mol. Pharmacol. 29*, 582–588.
Yang, J., Tamm, L. K., Somlyo, A. P., & Shao, Z. (1993a) *J. Micros. 171*, 183–198.
Yang, J., Tamm, L. K., Tillack, T. W., & Shao, Z. (1993b) *J. Mol. Biol. 229*, 286–290.
Yeagle, P. (1993) *The Membranes of Cells*, 2nd ed., Academic Press, San Diego, CA.