Clustering of Cholesterol in DMPC Bilayers as Indicated by Membrane Mechanical Properties

T. HIANIK[1] and M. HABURČÁK[2]

1 *Department of Biophysics and Chemical Physics, Faculty of Mathematics and Physics, Comenius University, Mlynská Dolina F1, 842 15 Bratislava, Slovakia*
2 *Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Sienkiewiczova 1, 813 71 Bratislava, Slovakia*

Abstract. Mechanical characteristics of bilayer lipid membranes (BLM) composed of dimyristoylphosphatidylcholine (DMPC) and cholesterol in the gel and liquid crystalline state were studied by measuring the modulus of elasticity in direction perpendicular to the BLM plane, $E_\perp$. The value of $E_\perp$ varied nonmonotonically with the cholesterol concentration, with a maximum around $c = 50$ mol% cholesterol. $E_\perp$ of BLM in gel state was about 2 times higher than that measured for the liquid crystalline state but the shape of $E_\perp(c)$ curves was similar for both states of the membrane. This may be due to the formation of cholesterol clusters at $c > 50$ mol% in both phase states of BLM.

Key words: Bilayer lipid membranes — Cholesterol — Phase transitions — Elasticity modulus — Clusters

Introduction

The study of the effects of cholesterol on the physical and structural properties of lipid bilayers is important for the understanding of the mechanism of interaction between cholesterol and phospholipids in biomembranes. It is particularly important from the biological standpoint since cholesterol is frequently present in biomembranes and influences their ion permeability, enzymatic activity, elasticity etc. (Yeagle 1985; Needham and Nunn 1990). So far, cholesterol-phospholipid interactions have been studied mainly by the use of differential scanning calorimetry (DSC), by spectroscopic methods (e.g., ESR, NMR and fluorescence spectroscopy) and by X-ray diffraction. Using these methods consistent results were obtained with pure hydrated samples of phospholipids (Finean 1990). However, apparently conflicting results emerged when the above methods were applied to mixtures of

phospholipids with cholesterol. A detailed study of different phosphatidylcholines with various cholesterol content using DSC (Davis and Keough 1983) suggested that cholesterol-phospholipid interactions could be explained by assuming that cholesterol may interact with phospholipids in certain fixed proportions to form intermediate complexes containing 15–30 mol% cholesterol, dependending on the structure of hydrocarbon chains. At concentrations exceeding 30–50 mol% cholesterol there are probably complexes with low and high cholesterol concentrations. A similar interpretation of the results obtained on bilayer lipid membranes (BLM) from egg phosphatidylcholine with different cholesterol contents using measurements of mechanical membrane parameters was presented in our earlier work (Hianik et al. 1984). These experiments were done at $T = 22$ °C, i.e. in lipids in liquid crystalline state. In contrast to these results spectroscopic experiments (Kintanar et al. 1986) as well as X-ray diffraction studies (Finean 1990; Needham et al. 1988) indicated that a nonuniform lateral distribution of cholestrol in phospholipid bilayers exists only in the gel state, while in the liquid crystalline state the membranes behave like a homogeneous phase. Inconsistent results were obtained especially for bilayers from dimyristoylphosphatidylcholine (DMPC) (see Finean 1990 for a review). Therefore, we focused our attention on the possibility of inhomogeneities in the lateral distribution of cholesterol in BLM from DMPC with different cholesterol contents. Both the gel and the liquid crystalline state were studied. We measured the elasticity modulus perpendicular to the BLM plane, $E_\perp$. The value of $E_\perp = -p/(\Delta h/h)$ characterizes the capacity of a membrane to change its thickness, h. The transversal elasticity modulus $E_\perp$ is very sensitive to the structural state of the inner-hydrophobic region of the membrane. It should be noted that so far, the mechanical properties of BLM at the phase transition region of lipids have been studied by Glazunov et al. (1985) only. These authors used the potentiodynamic method to study the dependence of the electrostriction coefficient, α, on temperature for BLM from dipalmitoylphosphatidylcholine (DPPC) in n-decane. However, as shown by Bagaveev et al. (1981), BLM containing hydrocarbon solvent does not represent a suitable model for the study of phase transitions of lipids since to a considerable extent, the observed changes of physical parameters reflect redistribution of the solvent in the lipid bilayer rather than structural changes of the lipids themselves.

Materials and Methods

In our study we used BLM without solvent, formed according to the technique of Montal and Mueller (1972) on a circular hole (0.1–0.2 mm in diameter) in a hydrophobic copolymer foil (~ 5 μm thick) separating two aqueous solutions in a teflon cup. The teflon cup was placed in a metallic thermostat. DMPC with and without cholesterol (Fluka) dissolved in n-hexane (0.1 mg/ml) was applied to the surface of the electrolyte (0.25 mol/l KCl in redistilled water, pH 6) from both sides of the hole at a concentration of ~ 2 μl

lipid solution per cm^2. The electrolyte level was first kept under the lower rim of the hole and after $\sim$ 30 min, when n-hexane had evaporated, the water level was increased to reach over the upper hole rim on which the self assembling formation process of BLM started. BLM formation and the stabilization of parameters were very quick, and the latter practically did not change in time. The temperature was measured to an accuracy of $\sim$ 0.2 °C using a Hg-thermometer placed near the BLM. Membranes were formed at $T \sim 35 - 40$ °C ($T > T_c$, where $T_c \sim 24$ °C is the phase transition temperature of DMPC), following which the temperature was slowly reduced by $\sim$ 0.5 °C/min.

$E_\perp$ was determined using the electrostriction method developed by Passechnik and Hianik (1977). According to this method, an alternating voltage with amplitude V_0 and frequency $f(V = V_0 \sin 2\pi ft)$, applied to a membrane, will compress the membrane with pressure $p = C_s V^2/2h$, where C_s is the specific membrane capacity and h the thickness. Due to the nonlinear relationship of capacity and voltage ($C = C_0(1 + \alpha V^2)$, where α is the coefficient of electrostriction), the membrane current $i = \mathrm{d}(CV)/\mathrm{d}t$ will contain components with frequencies f and $3f$ and amplitudes A_1 and A_3. Parameter $E_\perp$ can be obtained from

$$E_\perp = 3C_s V_0^2 A_1/4hA_3. \tag{1}$$

Thus to determine parameter $E_\perp$ it is sufficient to measure amplitudes A_1 and A_3 of the membrane current harmonics, e.g. by using resonance amplifiers (see Passechnik and Hianik 1977; 1991). To calculate $E_\perp$ the following values of h and C_s were used: $h = 2.04$ nm (Benz et al. 1975) corresponding to the hydrophobic thickness of the lipid bilayer in the liquid crystalline state. Specific capacity of BLM was calculated using $C_s = \epsilon\epsilon_0/h$ (Kruglyakov and Rovin 1978), where $\epsilon_0 = 8.85 \times 10^{-12}$ F/m is the dielectric permittivity of vacuum, and $\epsilon \cong 2.1$ is the relative dielectric permittivity of the hydrophobic part of the membrane (Ivkov and Berestovsky 1981). For DMPC BLM in the liquid crystalline state $C_s = 9.12 \times 10^{-3}$ F/m^2. The changes in membrane capacity and thickness with changes in temperature and cholesterol concentration were calculated using the expression: $C = A_1/2\pi f V_0$. Calomel electrodes were used to apply an alternating voltage with $V_0 =$ 60–80 mV and $f = 1$ kHz.

Results and Discussion

The temperature dependence of $E_\perp(a)$ and its first derivative $E'_\perp(b)$ are shown in Fig. 1. It is evident that considerable changes of $E_\perp$ occur within a narrow interval of temperatures. To determine the width of this interval (ΔT_c) we approximated the curve $E_\perp(T)$ by three straight lines (see Fig. 1*a*). ΔT_c was taken to be the difference in temperature between the two points of intersection. For DMPC BLM, ΔT_c averaged from 4 measurements was 5.1±0.5 °C. The temperature corresponding to the middle of this interval was $T_c = 23.0 \pm 0.4$ °C. Practically identical values of T_c were obtained from the position of the peak of the first derivative of $E_\perp(T)$ (Fig. 1*b*). This temperature characterizing the strong change of $E_\perp(T)$ corresponds to the phase transition temperature of DMPC determined by other methods (see, e.g. Lee 1977). Fig. 1*a* shows that $E_\perp$ increases during cooling, and in the gel state it is about 2 times higher than in the liquid crystalline state. It is obvious that these changes in compressibility are closely associated with conformational

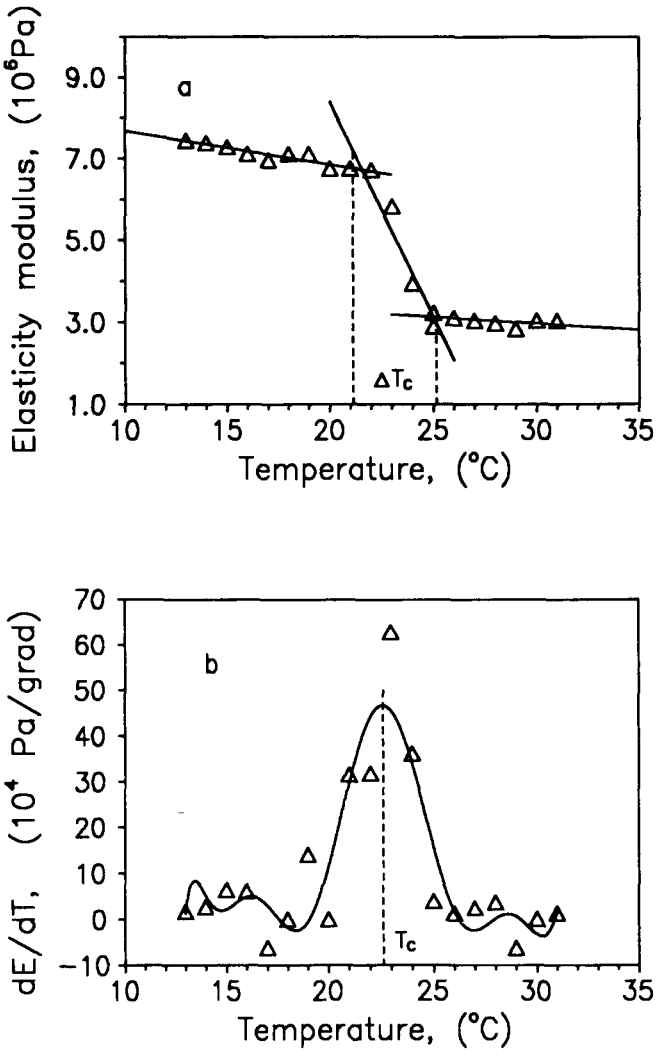

Figure 1. Temperature dependences of elasticity modulus $E_\perp(a)$ and its first derivative $E'_\perp(b)$ for BLM prepared of DMPC. The curves represent the best fits by the least square method (a) or spline (b).

transitions of hydrocarbon chains that are passing from a condition with a higher mobility (rotation around C–C bonds) in the liquid crystalline state to the full trans conformation in the gel state with a considerably lower mobility (Janiak et al. 1976).

The temperature dependence of the elasticity modulus $E_\perp$ for BLM of DMPC with different cholesterol contents (0–66 mol%) and for BLM from pure cholesterol are shown in Fig. 2. It should be noted that stable membranes from pure cholesterol can be formed only by the method of Montal and Mueller (1972). As a rule, by

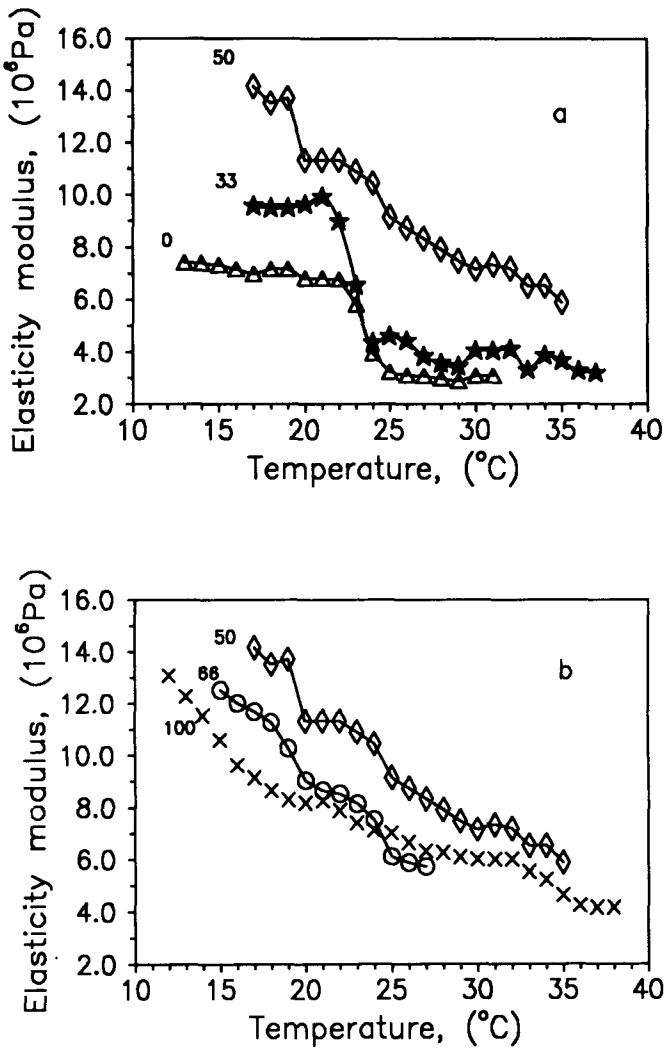

Figure 2. Temperature dependence of $E_\perp$ for BLM of DMPC with different cholesterol contents. The cholesterol content (in mol%) is shown.

the method of Mueller et al. (1962) it is not possible to prepare stable solvent-containing BLM with cholesterol concentrations higher than 80 mol%. As seen in Fig. 2*a*, a clear phase transition occurs only at a cholesterol concentration of 33 mol%. At 50 mol% ΔT_c is so broad that a phase transition is practically not detectable, a trend that continues at even higher concentrations. $E_\perp(T)$ for cholesterol concentrations of 50, 66 and 100 mol% are shown in Fig. 2*b*. Significant changes of $E_\perp$ with the increasing cholesterol concentration are also evident. Fig. 3 represents the dependence of $E_\perp$ on cholesterol concentration in the membrane in

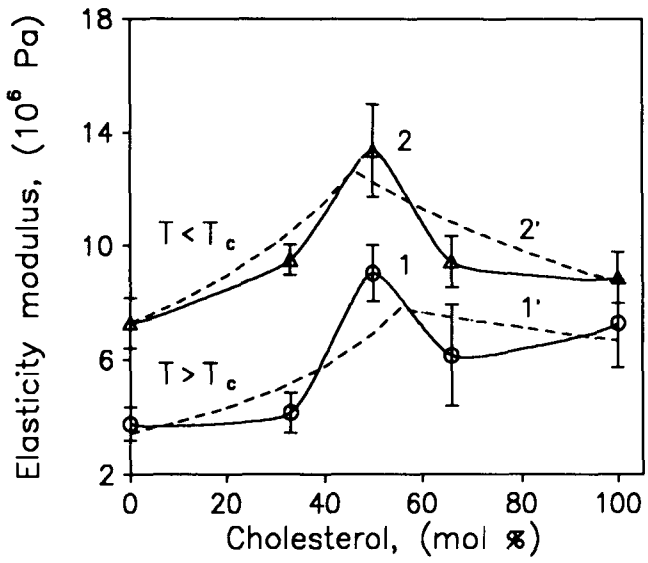

Figure 3. Dependence of $E_\perp$ on cholesterol concentration in BLM of DMPC at 17 °C (1) and 25 °C (2). The corresponding theoretical dependences calculated using equations (2) and (3) are represented by curves 1′ and 2′.

the gel ($T = 17$ °C, curve 1) and in the liquid crystalline state ($T = 25$ °C, curve 2). We can see that in both cases there is a nonmonotonic behavior with a maximum at ~ 50 mol% cholesterol. A cholesterol concentration of 50 mol%, which corresponds to a cholesterol to lipid molar ratio of 1 : 1, obviously represents a special situation in the membrane. Similar observations have been made by other methods, and this ratio is considered the most effective for stabilization of the phospholipid-cholesterol complex. It has been assumed that an interaction occurs between the glycerol oxygen at position 2 on the phospholipid head group and the β-OH group of cholesterol (Finean 1990). The curves in Fig. 3 suggest that BLM are most compactly ordered at 50 mol% cholesterol. We suggest a three phase model for the lipid-cholesterol system: pure lipid, a 1 : 1 association, and pure cholesterol. At $c < 50$ mol%, a pure DMPC phase with an elasticity modulus of E_L is in equilibrium with a DMPC : cholesterol = 1 : 1 phase, with elasticity modulus E_{LC}. A single phase is present at $c = c_k = 50$ mol%, while two phases are again present at $c > 50$ mol%: one with elasticity modulus E_{LC}, and another composed solely of cholesterol, with a modulus E_C.

This model, implying an inhomogeneous distribution of cholesterol in the membrane, allows the modulus of elasticity of the system to be described as follows:

$$E_\perp = E_L/[1 - (c/c_k)(1 - E_L/E_{LC})] \qquad 0 \le c \le c_k \qquad (2)$$

$$E_\perp = E_C/\{1 - [(1 - c)/(1 - c_k)](1 - E_C/E_{LC})\} \qquad c_k \le c \le 100 \text{ mol\%} \qquad (3)$$

where $E_\perp$ is modulus of elasticity of the membrane as a whole. The dependence $E_\perp(c)$ calculated according to (2) and (3) is shown in Fig. 3 (curves 1′ and 2′ for $T = 17\,^\circ\mathrm{C}$ and $25\,^\circ\mathrm{C}$ respectively). The calculated curves agree well with the experimental data. The values of parameters E_L, E_LC, E_C as well as those of critical concentrations c_k were estimated by the least squares method from experimental values of the dependence $1/E_\perp(c)$. The values of critical concentrations c_k determined from the dependences $1/E_\perp(c)$ are 46.0 mol% ($T = 17\,^\circ\mathrm{C}$) and 55.5 mol% ($T = 25\,^\circ\mathrm{C}$). It means that in dependence on the phase condition of the lipid bilayer, the value of c_k can differ from the "optimal" concentration 50 mol%. The decrease of $E_\perp$ at $c_\mathrm{k} \leq c \leq 100$ mol% may partially be due to the boundary regions surrounding clusters of pure cholesterol, but E_C must be really less than E_LC (the value for BLM at the region of maximum bilayer ordering). The apparently unexpected results concerning lower ordering of clusters from pure cholesterol are supported by NMR experiments which indicate that cholesterol rotation is faster than that of the phospholipid (Yeagle 1985). Thus, the results obtained provide evidence for an inhomogeneous lateral distribution in the lipid bilayer in both the gel and the liquid crystalline state.

The question arises concerning a certain disparity of the results obtained by the macroscopic methods (DSC and electrostriction) and the microscopic ones (ESR and fluorescence spectroscopy). In the latter case, as mentioned in Introduction, no irregularities in the lateral distribution of cholesterol in phospholipid vesicles were found in the liquid crystalline state of the membrane. In contrast to the macroscopic methods, both the shape and the parameters of spin and fluorescence label spectra in inhomogeneous membranes depend on the size of the clusters and on the label concentration within them. The measurements of microscopic parameters of labels may therefore yield different results for membranes containing numerous small-sized clusters and those containing few large clusters. In the liquid crystalline state, when as a result of higher mobility of hydrocarbon chains the process of cluster crumbling occurs, the border area between clusters increases. Spin or fluorescence labels are probably localized in less ordered regions, e.g. at the border regions between clusters. X-ray diffraction studies also show broad X-ray reflection characteristics at $T > T_c$ indicating the existence of a homogeneous phase. In this case the X-ray reflection may be influenced by the interaction between neighboring bilayers in a multilayer system (Evans and Kwok 1982). In contrast to microscopic methods, the measurement of $E_\perp$ supplies information about a membrane as a whole, i.e. the same value of $E_\perp$ is obtained regardless of either the number of clusters of one kind (e.g., type C with modulus of elasticity E_C) or their size. The only important condition is that the overall cluster area remain unchanged. It is however obvious that the problem concerning cholesterol-phospholipid interaction requires further studies. The nonmonotonic behavior found for $E_\perp$ need not apply generally to different lipid compositions of membranes but can differ depending on

the length and degree of saturation of hydrocarbon chains. Moreover, mechanical properties of membranes are characterized by considerable anisotropy (Passechnik and Hianik 1991). As a result their full description requires measurement of membrane deformation in different directions. Thus for example, the recently obtained results of Needham and Nunn (1990) showed monotonic growth of volume modulus compressibility of large liposomes from stearoyloleylphosphatidylcholine (SOPC) with increasing cholesterol concentration in lipid bilayers in gel state.

Acknowledgements. The authors would like to thank Dr.D.F.Sargent for helpful discussion and for reading the manuscript. This work was supported by Slovak Grant Agency Grant 1/22/92.

References

Bagaveev I. A., Petrov V. V., Zubarev V. S., Rovin Yu. G., Antonov V. F., Nedozorov P. M. (1981): Effect of phase transition on electric capacitance of plane lipid membranes. Biofizika **26,** 464—466 (in Russian)

Benz B., Frölich O., Läuger P., Montal M. (1975): Electrical capacity of black lipid films and of lipid bilayers made from monolayers. Biochim.Biophys.Acta **394,** 323—334

Davis P. J., Keough R. M. W. (1983): Differencial scanning calorimetric studies of mixtures of cholesterol with some mixed-acid and single-acid phosphatidylcholines. Biochemistry USA **22,** 6334—6340

Evans E., Kwok R. (1982): Mechanical calorimetry of large dimyristoylphosphatidylcholine vesicles in the phase transition region. Biochemistry USA, **21,** 4874—4879

Finean J. B. (1990): Interaction between cholesterol and phospholipid in hydrated bilayers. Chem.Phys.Lipids **54,** 147—156

Glazunov I. Yu., Abidor I. G., Fadeev V. G., Tatulyan S. A., Chizmadzhev Yu. A. (1985): Studies on the phase transition in membranes by means of potentiodynamic method. Biol. Membrany. **2,** 1023—1028 (in Russian)

Hianik T., Kvasnička P., Štroffeková K. (1984): Mechanical properties of lipid bilayers with varying cholesterol content. Stud. Biophys. **100,** 23—32

Ivkov V. G., Berestovsky G. N. (1981): Dynamic Structure of Lipid Bilayer, Nauka, Moscow (in Russian)

Janiak M. J., Small D. M., Shipley G. G. (1976): Nature of the thermal pretransition of synthetic phospholipids: dimyristoyl and dipalmitoyllecithin. Biochemistry USA **15,** 4576—4580

Kintanar A., Kunwar A. C., Oldfield E. (1986): Deuterium nuclear magnetic resonance spectroscopic study of the fluorescent probe diphenylhexatriene in model membrane system. Biochemistry USA, **25,** 6517—6524

Kruglyakov P. M., Rovin Yu. G. (1978): Physical Chemistry of Black Hydrocarbon Films. Nauka, Moscow (in Russian)

Lee A. G. (1977): Lipid phase transitions and phase diagrams. I.Lipid phase transitions. Biochim. Biophys. Acta **472,** 237—281

Montal M., Mueller P. (1972): Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. Proc.Nat.Acad.Sci. USA **69,** 3561—3566

Mueller P., Rudin D. O., Tien H. Ti., Wescott W. C. (1962): Reconstruction of cell membrane structure in vitro and its transformation into an excitable system. Nature **194,** 979—980

Needham D., Nunn R. (1990): Elastic deformation and failure of lipid bilayer membranes containing cholesterol. Biophys. J. **58,** 997—1009

Needham D., McIntosh T. J., Evans E. (1988): Thermomechanical and transition properties of DMPC : Cholesterol bilayers. Biochemistry USA **27,** 4668—4673

Passechnik V. I., Hianik T. (1977): Elastic properties of bilayer membranes in the direction perpendicular to the membrane plane. Kolloid. Zh. **39,** 1180—1185 (in Russian)

Passechnik V. I., Hianik T. (1991): Transversal Elasticity of Lipid Membranes. Veda, Bratislava

Yeagle P. L. (1985): Cholesterol and the cell membrane. Biochim. Biophys. Acta **822,** 267—287

Final version accepted April 26, 1993