Cholesterol Induced Suppression of Large Swelling of Water Layer in Phosphocholine Floating Bilayers

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The effect of the addition of 10 mol % cholesterol to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) floating bilayers has been investigated by means of neutron reflectivity measurements. The large swelling of the water layer between the two bilayers found in pure phosphocholine systems around the lipid main phase transition[1] is greatly reduced in the 9:1 DPPC:cholesterol mixture. The analysis of the structure of the bilayer reveals that in the gel phase cholesterol induces the presence of a high rms roughness that disappears in the fluid phase.

1. Introduction

Cholesterol is the main sterol of animal organisms. It is an amphiphilic molecule important in the regulation of fluidity and order−disorder behavior of the lipid part of membranes.[2] Its amount varies from cell to cell. It is equimolar with phospholipids in membranes of the liver cells, erythrocytes and myelin while in the outermost layer of human epidermis it represents about 30 mol % of the lipid fraction. The ability of cholesterol to modulate the fluidity of membranes seems to be important in many biological processes as for example cell fusion.[3] Cholesterol is oriented in the membrane such that the long axis lies parallel to the lipid chains, increasing order in their upper part while decreasing packing constraints at the terminal methyl groups. The structure of cholesterol containing membranes has been probed by neutron and X-ray diffraction and small angle scattering[4−6] from either stacked bilayers or multilamellar systems. The accurate vertical location in the membrane is still controversial.[7]

Much work is still needed to define fully the organization of cholesterol in membranes and the effect of structure on function. The modification to the lipid phase behavior induced by cholesterol has been widely studied as this may help understanding its biological role. Lipid phases commonly occurring with phosphocholines are the gel (Lα'), ripple (Pβ') and fluid (Lβ) phases. While in the gel and ripple phases phospholipid acyl chains are conformationally ordered, in the fluid phase they are disordered. The incorporation of cholesterol into the phospholipid membrane usually broadens or eliminates the gel to liquid-crystalline (Lα) phase transition; it increases the orientational ordering of the hydrocarbons in the Lα phase of bilayers while decreasing it in the gel phase. At high concentrations it stabilizes a "liquid ordered" (*lo*) phase that presents high lateral diffusion and high degree of conformation order.[8] At low concentrations the molecule becomes interfacially active and promotes the formation of lipid domains.[2] The *lo* phase is characterized by increased bilayer thickness and area compressibility modulus.[9,10] Model membranes have proved to be powerful tools for studying lipid phase behavior. Studies have been classically carried out on either multilamellar phases or stacked bilayers.

Recently, Charitat et al.[11] have succeeded in the preparation of stable and reproducible double bilayers, in which a bilayer floats at 20−30 Å above an adsorbed one. This model makes it possible to do reflectivity studies on a highly hydrated, accessible and fluctuating bilayer, where the composition of each leaflet can be chosen separately. The system proved useful to probe bilayer−bilayer entropic interactions. Neutron reflectivity measurements on floating bilayers of phosphocholines with saturated acyl chains of different length (number of carbon atoms per chain varying from 16 to 20) allowed the determination of the average and root-mean-square

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(1) Fragneto, G.; Charitat, T.; Bellet-Amalric, E.; Cubitt, R.; Graner, F. Swelling of phospholipid bilayers: the effect of chain length. *Langmuir* **2003**, *19*, 7695−7702.

(2) Mouritsen, O. G.; Jorgensen, K. Dynamical order and disorder in lipid bilayers, *Chem. Phys. Lipids* **1994**, 73 3−25.

(3) Nakanishi, M.; Hirayama, E.; Kim, J. Characterisation of myogenic cell membrane: II. Dynamic changes in membrane lipids during the differentiation of mouse C2 myoblast cells. *Cell Biol. Int.* **2000**, *25*, 971−979.

(4) Wiener, M. C.; White, S. H. Fluid bilayer structure determination by the combined use of X-ray and neutron-diffraction. 2. Composition-space refinement method. *Biophys. J.* **1991**, *59*, 162−173.

(5) Knoll, W.; Schmidt, G.; Ibel, K.; Sackmann, E. Small-Angle Neutron Scattering Study of Lateral Phase Separation in Dimyristoylphosphatidylcholine-Cholesterol Mixed Membranes. *Biochemistry* **1985**, *24*, 5240−5246.

(6) Mortensen, K.; Pfeiffer, W.; Sackmann, E.; Knoll, W. Structural properties of a phosphatidylcholine-cholesterol system as studied by small-angle neutron scattering: ripple structure and phase diagram. *Biochim. Biophys. Acta* **1988**, *945*, 221−245.

(7) Leonard, A.; Escrive, C.; Languerre, M.; Pebay-Peyroula, E.; Néri, W.; Pott, T.; Katsaras, J.; Dufourc, E. J. Location of cholesterol in DMPC membranes. A comparative study by neutron diffraction and molecular mechanics simulation. *Langmuir* **2001**, *17*, 2019−2030.

(8) Vist, M. R.; Davis, J. H. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^{2}H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry* **1990**, *29*, 451−464.

(9) Needham, D.; McIntosh, T. J.; Evans, E. Thermomechanical and Transition Properties of Dimyristoylphosphatidylcholine/Cholesterol Bilayers. *Biochemistry* **1988**, *27*, 4668−4673.

(10) Trouard, T. P.; Nevzorov, A. A.; Alam, T. M.; Job, C.; Zajicek, J.; Brown, M. F. Influence of cholesterol on dynamics of dimyristoylphosphatidylcholine bilayers as studied by deuterium NMR relaxation. *J. Chem. Phys.* **1999**, *110*, 8802−8818.

(11) Charitat, T.; Bellet-Amalric, E.; Fragneto, G.; Graner, F. Absorbed and free bilayers at the solid−liquid interface. *Eur. Phys. J. B* **1999**, *8*, 583−593.

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position of the floating bilayer.[1] Data were interpreted in terms of the competition between the interbilayer potential and membrane fluctuations and used to estimate the bending rigidity of the bilayer. A big swelling of the floating bilayer around the main phase transition temperature corresponded to a minimum of the bending modulus in that region.

Model lipid bilayers containing cholesterol can be used for studies of specific interactions of proteins with rafts. It is important to have a good working model membrane. For this reason, we tested the possibility of including cholesterol in double bilayer systems and investigated its effect on bilayer fluctuations, We succeeded in including different amounts of cholesterol, varying from 0.5 to 30 mol %, in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers. DPPC was chosen because long chain phosphocholines are major components of cell membranes, because of the abundance of structural data and molecular dynamics simulations existing in the literature, useful to validate our results and because our sample preparation procedure is well established with this phospholipid species. In cell membranes, one of the long lipid chains is usually unsaturated and this ensures fluidity of the membrane. Our sample preparation procedure works well only if lipids are in the gel phase during deposition. For this reason, saturated chains are normally used, and the fluidity is ensured by raising the temperature in order to overcome the gel/fluid lipid phase transition. We measured the specular reflectivity of neutrons from these systems in the gel and fluid phases and recorded the behavior around the main phase transition. We observed that while at low cholesterol concentrations the swelling around the phase transition is maintained and even enhanced up to 2 mol %, from 6 mol % onward it is greatly reduced.[12] Cholesterol leads to structural modification of the bilayer as well. In the following sections the analysis of the system containing 10 mol % cholesterol in the gel, transition and fluid phases will be described. Data from a single adsorbed bilayer containing 10% mol cholesterol are presented as well.

2. Neutron Reflectivity Principles and Measurements

When a neutron beam arrives at a planar surface, at a grazing angle, the atoms of the surface act as a mirror and, above a critical wavelength, neutrons are totally reflected. At values of the wave vector transfer $q = 4\pi/(\lambda \sin\theta)$ (where λ is the wavelength and θ the angle of the incoming beam to the surface) higher than the value for total reflection, reflectivity is related to the scattering length density across the interface by the approximate relation:

$$R \approx \frac{16\pi^2}{q^4} SLD^2 \qquad (1)$$

which is the reflectivity in the Born approximation.[13] The scattering length density, SLD, is given by

$$\mathrm{SLD} = \sum_j b_j n_j \qquad (2)$$

where n_j is the number of nuclei per unit volume and b_j is the scattering length of nucleus j. When a thin layer is deposited on the surface, there is interference between the beam reflected by the surface and the interface. The technique is very sensitive to the thickness of the layer and the composition as well as to the roughness of the interfaces. It is a nondestructive technique allowing in situ studies and giving structural information perpendicular to the surface with a fraction of nm precision.[14] The technique is therefore well suited for the study of thin layers as lipid monolayers or single bilayers.

Specular neutron reflectivity, $R(q)$, defined as the ratio between the reflected and incoming intensities of a neutron beam, is measured as a function of the wave vector transfer. Measurements can be done by either changing θ at a fixed value of λ (monochromatic mode) or inversely by using a spread of wavelengths at fixed angles (time-of-flight mode). The sample and the detector move in order to keep the angle of incidence equal to the angle of reflection.

Neutron reflectivity is widely used for the investigation of buried interfaces. For biological systems it presents the advantage that light elements as H, C, O, and N are strong scatterers and different isotopes of the same element have different scattering lengths so that isotopic substitution may be used to highlight different parts of the interface. Another advantage is that very small amounts of material are required. High resolution can be obtained for aqueous systems when it is possible to measure the reflectivity profiles of the same sample with different H_2O/D_2O contents or different isotopic composition of the layers at the interface (i.e., the same physical samples with different contrasts). Data from all profiles are fitted simultaneously, and this reduces the uncertainty in the structural parameters to the fraction of nanometer scale.

Measurements described here were performed on the D17 reflectometer[15] at the high flux reactor of the Institut Laue-Langevin (ILL, Grenoble, France) in time-of-flight mode using a spread of wavelengths between 2 and 20 Å with two incoming angles of 0.7 and 4°. Background from the solvent in the sample usually limits the useful q range to 0.25 $Å^{-1}$. Data collection per measurement usually took around 1 h for good statistics. Temperature scans were done, warming and cooling through the gel and fluid phases. All samples were allowed to equilibrate for about 15 min at each temperature before measuring the reflectivity profile.

3. Materials and Methods

3.1. Lipids and Substrates. Protonated DPPC and deuterated d75-DPPC (purity >99%) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma Chemicals. All chemicals were used without further purification. Silicon substrates (8 cm × 5 cm × 2 cm) were polished on one side to an average root-mean-square roughness of 3 Å by the ESRF optics laboratory (Grenoble, France). All solvents used were of analytical grade. D_2O (99% purity) was supplied by the ILL and ultrapure water was of Millipore grade (18 MΩ cm). Samples were measured in D_2O, H_2O, and silicon-matched water (SMW). SMW (SLD = 2.07×10^{-6} $Å^{-2}$) consists of 38 vol % D_2O (SLD = 6.35×10^{-6} $Å^{-2}$) and 62 vol % H_2O (SLD = -0.56×10^{-6} $Å^{-2}$). Prior to deposition the silicon blocks were cleaned in chloroform, ethanol, and ultrapure water in an ultrasound bath for 10 min per solvent. They were then made highly hydrophilic

(12) Stidder, B.; Fragneto, G.; Roser, S. J. The effect of low amounts of cholesterol on the phase behaviour of floating bilayers. *Langmuir*, accepted.

(13) Born, M.; Wolfe, E. *Principles of Optics*; Pergamon Press: Oxford, U.K., 1989.

(14) Penfold, J.; Thomas, R. K. The application of specular reflection of neutrons to the study of surfaces and interfaces. *J. Phys. Condens. Mater.* **1990**, *2*, 1369−1412.

(15) Cubitt, R.; Fragneto, G. D17: the new reflectometer at the ILL. *Appl. Phys. A* **2002**, *74*, S329−S331.

with the UV/ozone treatment for 30 min[16] and deposited on immediately.

3.2. Bilayer Deposition. Ratios of DPPC and cholesterol were dissolved in chloroform and co-spread to an initial surface pressure of 2 mN/m on a Nima LB trough (Nima technology, Coventry, U.K.) allowing 20 min for solvent evaporation. Depositions were performed at temperatures of about 17 °C with the monolayer held throughout at surface pressure of 40 mN/m. Deposition onto the silicon substrate was by a combination of the three vertical Langmuir−Blodgett depositions and one horizontal Langmuir−Schaefer deposition as described previously.[11]

Transfer ratios, defined as the monolayer reduction over the dipped surface area were recorded throughout each monolayer deposition. They were typically 1.1 for the first layer, 0.95 for the second layer and 1.08 for the third. It was found that to obtain a successful Schaefer deposition the degree of horizontality and lowest speed possible of immersion (20 μm/s) was crucial. This was achieved by the use of an in-house built microtable controlled manual dipper with precision adjusted verticality. The pressure jump at contact was around 5 mN/m. The samples were then sealed in a neutron reflectivity cell under water ready for measurement. Single bilayer samples were fabricated by one vertical and one horizontal deposition.[17]

3.3. Reflectivity Data Analysis. The method of analysis often used for specular reflection data involves the construction of a model of the interface that may be represented by a series of parallel layers of homogeneous material. Each layer is characterized by a SLD and a thickness, which are used to calculate a model reflectivity profile by means of the optical matrix method.[13] The interfacial roughness between any two consecutive layers may also be included in the model by the Abeles method. The calculated profile is compared to the measured profile and the quality of the fit is assessed by using χ^2 in the least-squares method. This box model technique is successful for simple homogeneous systems, but deteriorates for more complex systems as shown by X-ray measurements at high momentum transfer values.[18] Recently a different method, the quasi-molecular approach, has been employed to model reflectivity data of monolayers[19] and hybrid double bilayers.[20] This method describes the bilayer fragments as distribution functions rather than slabs and was first developed by Wiener and White for the analysis of diffraction from multilamellar vesicles.[21] A detailed account of the method applied to neutron reflectivity of lipid bilayers can be found in Hughes et al.[20] An overview will be given below.

The SLD of the headgroups are described in terms of Gaussian distribution functions, where each Gaussian represents the contribution of a particular molecular fragment to the overall SLD profile. The headgroup is divided into four fragments; the choline, phospho, glycero, and carbonyl fragments, with each assigned a separate Gaussian. The center of the Gaussian corresponds to the center of mass of the fragment along the bilayer normal and the width accounts for fluctuations around the mean position. Its size (height) is the calculated scattering length contribution of the fragment. The Gaussian functions may vary within molecular considerations their center position and half-widths along the bilayer normal representing the disorder present in bilayers. The alkyl chain region is assumed to be one homogeneous region and is thus treated as a single alkyl layer. In other words cholesterol is treated simply as though it were

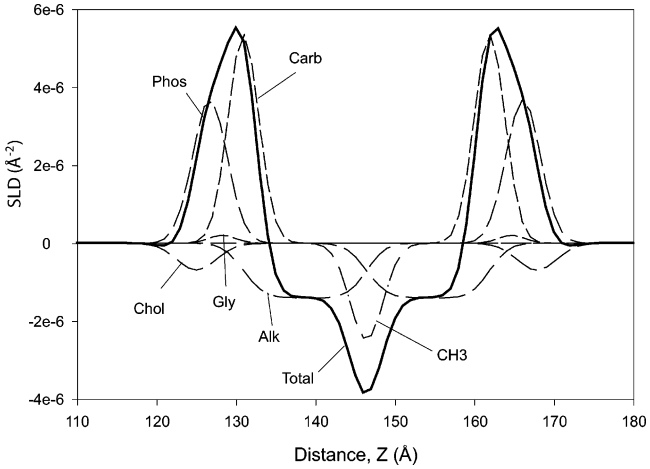

Figure 1. Model breakdown of the bilayer fragments. It represents the raw bilayer in a vacuum, the water is added by a simple volume fraction filled method. For details see text and ref 20.

part of the alkyl chain region (we do not attempt to define its vertical position in the layer). Since only measurements from hydrogenated lipids could be analyzed, in the model it is assumed that the scattering length density of cholesterol is the same as that of the alkyl chains. This seemed a reasonable assumption given the relative molecular volumes, chemical formulas and small amount of cholesterol. Moreover, results were validated by using a program based on the classical optical matrix method. The scattering length density of cholesterol is 0.22×10^{-6} Å^{-2};[22] therefore inclusion of cholesterol in the layer changes the scattering length density in the chain region and in the gel from -0.4×10^{-6} to -0.36×10^{-6} Å^{-2}. With the available resolution, this difference is negligible. Figure 1 shows an example of the model breakdown of the bilayer fragments. It represents the raw bilayer in a vacuum, the water is added by a simple volume fraction filled method (i.e., the percentage of space not filled by molecules is filled with water).[20]

The silicon oxide layer is described by a single slab. The coverage of each bilayer is determined by scaling between a calculated 0% and 100% coverage until it fits the measured reflectivity profile.[20] All unoccupied space in the headgroups, between the bilayers, and between the lower bilayer and silicon surface is assumed to be occupied by water molecules. The total scattering length density of the sample is simply the sum of the Gaussians, alkyl slab, and silicon oxide slab.

The distribution function method is a more realistic representation of the bilayer constitution rather than large slab regions. It has successfully been used to fit synchrotron reflectivity of lipid monolayers to momentum transfer out to 0.8 Å^{-1}, while box models were found to be rather inadequate to such momentum transfer.[18]

By defining the relative position of fragments relative to the center of the bilayer and the reliance of certain parameters on others,[19] the total number of variable parameters for a double bilayer system can be reduced down to nine. Each bilayer is described by an area per molecule (APM), position along the bilayer normal, roughness and coverage. The overall roughness of the bilayer is an independent parameter which acts upon the net structure of each bilayer. The bilayer thickness, D_B, is inversely proportional to APM. The scattering length density of each fragment is calculated from atomic scattering length tables and fragment volumes from molecular dynamics simulations and experimental results.[23] It is assumed not to vary significantly within the temperature range studied and is therefore kept constant. For the silicon oxide only one parameter was allowed to vary, the thickness, because the roughness was characterized

(16) Vig, J. R. UV ozone cleaning of surfaces. *J. Vac. Sci. Technol.* **1985**, *3*, 1027−1034.

(17) Fragneto, G.; Graner, F.; Charitat, T.; Dubos, P.; Bellet-Amalric, E. Interaction of the third helix of antennapedia homeodomain with a deposited phospholipid bilayer: a neutron reflectivity structural study. *Langmuir* **2000**, *16*, 4581−4588.

(18) Schalke, M.; Kruger, P.; Weygand, M.; Losche, M. Submolecular organisation of DMPA in surface monolayers: beyond the two-layer model. *Biochim. Biophys. Acta* **2000**, *1464*, 113−126.

(19) Schalke, M.; Losche, M. Structural models of lipid monolayers from X-ray and neutron reflectivity measurements. *Adv. Colloid Interface Sci.* **2000**, *88*, 243−274.

(20) Hughes, A. V.; Roser, S. J.; Gerstenberg, M.; Golder, A.; Stidder, B.; Feidenhans, R.; Bradshaw, J. Phase behavior of DMPC free supported bilayers studied by neutron reflectivity. *Langmuir* **2002**, *18*, 8161−8171.

(21) Wiener, M. C.; Suter, R. M.; Nagle, J. F. Structure of the fully hydrated gel phase of dipalmitoylphosphatidylcholine. *Biophys. J.* **1989**, *55*, 315−325.

(22) Deme, B.; Lee, L.-T. Adsorption of a hydrophobically modified polysaccharide at the air−water interface: kinetics and structure. *J. Phys. Chem. B* **1997**, *101*, 8250−8258.

(23) Armen, R. S.; Uitto, O. D.; Fellar, S. E. Phospholipid component volumes: determination and application to bilayer structure calculations. *Biophys. J.* **1998**, *75*, 734−744.

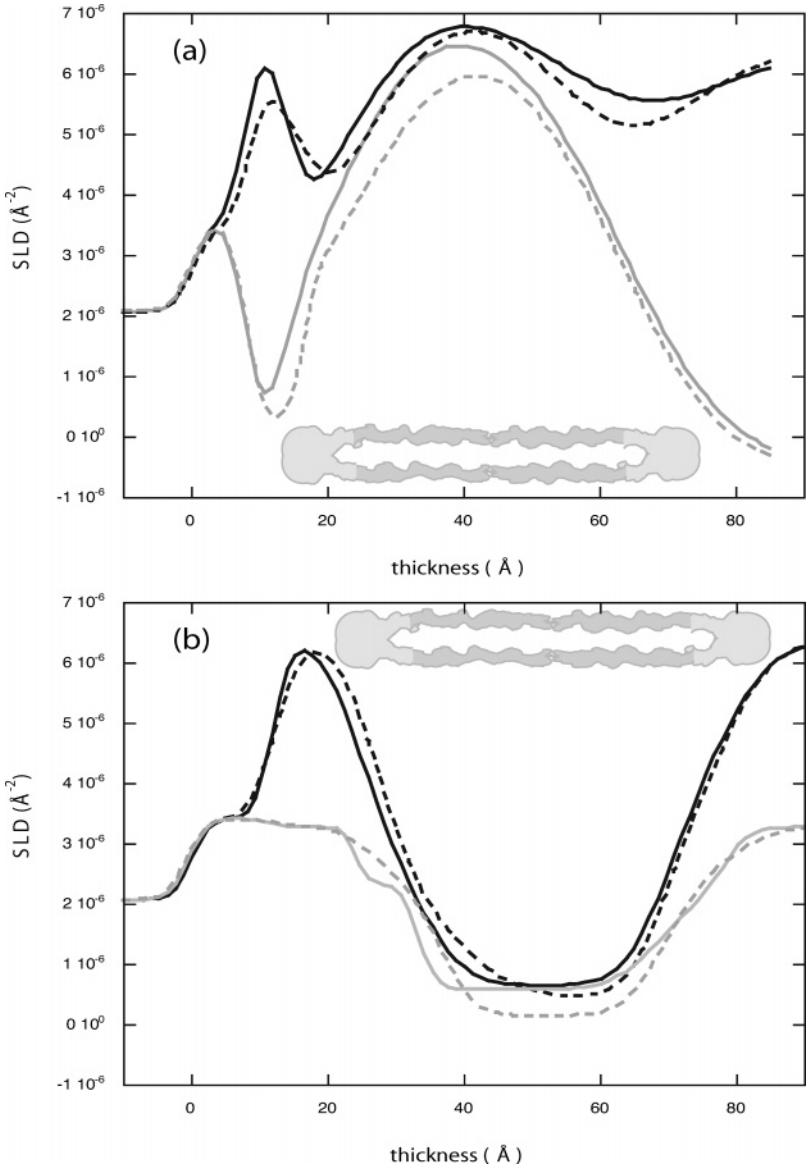

Figure 2. Scattering length density profile of 10% cholesterol 90% deuterated (a) and hydrogenated (b) DPPC single bilayers. The black lines are the best fits to the data in the D_2O contrast with the complete line at 25 °C and broken line at 47 °C. Grey lines correspond to the H_2O contrast (a) and the SMW contrast (b) with the complete line at 25 °C and broken line at 47 °C. Other contrasts are not included for reasons of clarity. The cartoon of the lipid bilayer is for helping the eye, cholesterol is not included in the cartoon as it is.

by AFM measurements (ESRF optics laboratory, Grenoble) on all bare silicon blocks and the value found for the rms of 3 ± 1 Å was confirmed by reflectivity measurements on other silicon blocks from the same series. The complete model is implemented in a "Matlab" environment, with the reflectivity profile calculated by use of the recursive Parratt algorithm.[24] The program calculates a range of acceptable fits from randomly starting points within the realistic parameter boundary limits to minimize the weighted χ^2 of the model to measured profile. The parameters are varied until χ^2 is unable to decrease by at least 10^{-6}. The SLD profiles were sliced into a histogram of 200 slabs, each 1 Å thick, which means that the resolution is not affected by the slicing.

The area per molecule, APM, parameter obtained from the reflectivity data analysis is the average value for the DPPC and cholesterol values combined. Molecular areas for cholesterol in phosphocholine-cholesterol systems vary widely depending on the technique to the extent of a spread of 22−39 Å^2 and are generally assumed to remain constant regardless of the cholesterol mole fraction.[25−29] An estimation of the cholesterol APM by molecular dynamic simulations is 33 Å^2.[30,31]

(24) Parratt, L. G. Surface studies of solids by total reflection of X-rays. *Phys. Rev.* **1954**, *95*, 359−369.

4. Results and Discussion

4.1. 90 mol % DPPC 10 mol % Cholesterol Single Bilayers. Single bilayers from both hydrogenated and

(25) Chiu, S. W.; Jakobsson, E.; Mashl, R. J.; Scott, H. J. Cholesterol induced modifications in lipid bilayers: a simulation study. *Biophys. J.* **2002**, *83*, 1842−1853.

(26) Almeida, P. F. F.; Vaz, W. L. C.; Thompson, T. E. Lateral diffusion in the liquid phases of dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis. *Biochemistry* **1992**, *31*, 6739−6747.

(27) Chong, P. L. G. Evidence for regular distribution of sterols in liquid crystalline phosphatidylcholine bilayers. *PNAS* **1994**, *91*, 10069−10073.

(28) Ipsen, H. I.; Mouritsen, O. G.; Bloom, M. Relationships between lipid membrane area, hydrophobic thickness, and acyl-chain orientational order. The effects of cholesterol. *Biophys. J.* **1990**, *57*, 405−412.

(29) Hyslop, P. A.; Morel, B.; Sauerheber, R. D. Organization and interaction of cholesterol and phosphatidylcholine in model bilayer membranes. *Biochemistry* **1990**, *29*, 1025−1038.

(30) Smondyrev, A. M.; Berkowitz, M. L. Molecular dynamics simulation of the structure of dimyristoylphosphatidylcholine bilayers with cholesterol. *Biophys. J.* **2001**, *80*, 1649−1658.

(31) Tu, K.; Klein, M. L.; Tobias, D. J. Constant-pressure molecular dynamics investigation of cholesterol effects in a dipalmitoylphosphatidylcholine bilayer. *Biophys. J.* **1998**, *75*, 2147−2156.

Table 1. Summary of Fitted Parameters for DPPC Double Bilayers in D_2O Containing 10 mol % Cholesterol[a]

temp (°C)	dw (Å)	uAPM (Å^2)	uDc (Å)	uRou (Å)	Dw (Å)	lAPM (Å^2)	lDc (Å)	lRou (Å)
25.0 ± 0.1	14 ± 1	43 ± 2	37 ± 1	17 ± 1	37 ± 1	45 ± 2	35 ± 1	12 ± 2
39.9 ± 0.1	12 ± 1	48 ± 2	34 ± 1	13 ± 1	38 ± 1	50 ± 2	32 ± 1	9 ± 2
45.0 ± 0.1	11 ± 1	49 ± 2	33 ± 1	3 ± 1	33 ± 1	51 ± 2	31 ± 1	3 ± 2
25.0 ± 0.1	9 ± 1	44 ± 2	37 ± 1	9 ± 1	34 ± 1	47 ± 2	34 ± 1	3 ± 2

[a] The prefix u refers to the upper bilayer and l the lower. APM is the average area per molecule, Dc is the chain region thickness, and Rou is bilayer roughness. dw is the water layer separating the lower bilayer from the solid substrate and Dw is the water layer separating the two bilayers.

fully deuterated DPPC were prepared and reflectivity measurements taken from samples immersed in D_2O, SMW, and H_2O in the gel (25 °C) and fluid (47 °C) phases. Data from all contrasts were fitted simultaneously. Figure 2 shows examples of the SLD profiles obtained from the model that best fitted the experimental data in the case of deuterated lipids in D_2O and H_2O in the two phases. The box model technique described in section 3.3 was used in this case. It was observed that the bilayer is much rougher than in the case of the pure DPPC bilayer[17] with roughness of the order of 8 ± 2 Å. There is no drop in roughness in the fluid phase as compared to the gel phase. As we will see below, this differs from what was observed in the case of the floating bilayer. The structure of the deuterated sample differs slightly from that of the hydrogenated one. This is likely due to different interaction forces between the lipids and the silicon substrate. Below we will see that deuterated lipids behave differently than hydrogenated ones in double bilayer systems.

For both samples there is a large decrease in the thickness of the chain region going from 36 ± 2 Å in the gel phase to 30 ± 2 Å in the fluid phase.

4.2. 90mol % DPPC 10% Cholesterol Double Bilayer. Before investigating the effect of cholesterol on floating bilayers, a control experiment was carried out with double bilayers of protonated DPPC containing no cholesterol and immersed in D_2O. The layers were prepared with the standard procedure described above and neutron reflectivity data collected in the gel and fluid phases and transition region. In agreement with previous data,[32] the water layer between the bilayers showed a big increase in thickness around the transition temperature. The effect on the reflectivity profiles can be seen in the inset of Figure 3 where reflectivity profiles collected at 44.4 (fluid phase) and 37.7 °C (transition region) are shown. Because of the large fluctuations in the transition region, a complete analysis of the data is still under progress, but it is clear from the position of the first minimum of the reflectivity profile, which shifted to lower values of q, that the system is thicker in the transition region than in the gel or fluid phases and we estimated that this is mainly due by a swelling of the water layer from 27 ± 1 Å in the gel phase and 31 ± 1 Å in the fluid phase to 43 ± 3 Å in the transition region.

Samples containing 10 mol % cholesterol were prepared from both deuterated and hydrogenated lipids.

Our past experience is that it is difficult to prepare double bilayers from deuterated DPPC. This time also we detected the presence of a Bragg peak in the reflectivity profile in the gel phase indicating the formation of multilamellar structures. The sample was then investigated no further. The presence of cholesterol helped in the preparation and a reflectivity profile without the presence of the Bragg peak could be recorded in the gel phase, but when the sample was warmed to reach the fluid phase, it became unstable and multilamellar structures formed on the surface. This might be due to the presence of small impurities in the purchased lipids. Therefore, the analysis of the deuterated systems is not reported here. The hydrogenated lipids gave stable, reversible, and reproducible layers. Reflectivity measurements were taken on double bilayers immersed in either D_2O or SMW at various temperatures in the gel, transition, and fluid lipid phases. For clarity, data will be shown at three temperatures where lipids are in the gel and fluid phases and transition region. Examples of fits of the reflectivity data collected at 25, 39.9, and 45 °C and down to 25 °C from DPPC + 10% cholesterol double bilayers in D_2O are given in Figure 3, and the parameters used to calculate the reflectivity are summarized in Table 1. The resulting scattering length density profiles are given in Figure 4. The two minima correspond to the two alkyl regions (hydrogenated lipids), while the higher parts correspond to the water layers (deuterated). The thickness of the water layer between the bilayers in the gel phase is higher than in the pure bilayer system. There is indeed a trend, which is discussed more fully in ref 12, of this thickness that increases with increasing amounts of cholesterol (at least from 1 to 10 mol %). It is thought that at high cholesterol amounts the decrease of thermal fluctuations may keep the two bilayers further apart.

All parameters indicate that both bilayers are exhibiting gel, transition, and fluidlike phases.The roughness in the transition phase was not higher than in the other phases, contrarily to what was found with the lipid bilayer without cholesterol.[1,32] Figure 5 shows the variation in chain region thickness vs temperature on heating and cooling. The

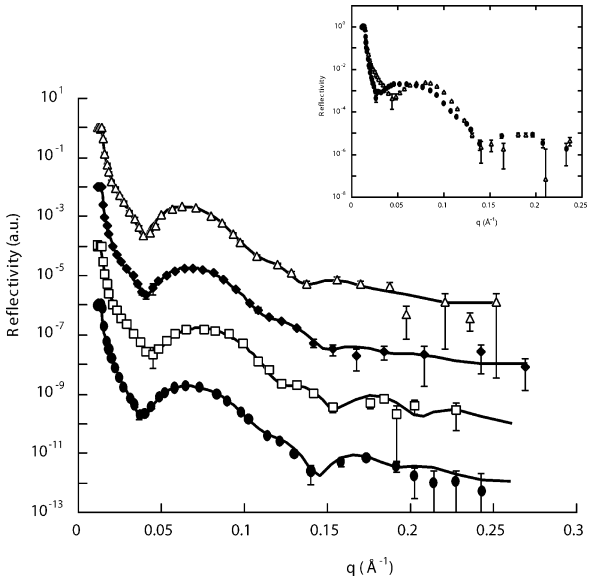

Figure 3. Neutron reflectivity profiles and fits from model described in Table 1: (△) 25.0 °C (as fabricated); (◆) 39.9 °C; (□) 45.0 °C; (●) 25.0 °C (cooled). Black lines are from models that best fit the data. Inset shows data collected from a pure DPPC double bilayer in D_2O at (●) 44.4 (fluid phase) and (△) 37.7 °C (transition region).

(32) Fragneto, G.; Charitat, T.; Graner, F.; Mecke, K.; Perino-Gallice, L.; Bellet-Amalric, E. A fluid floating bilayer. *Europhys. Lett.* **2001**, *53*, 100−106.

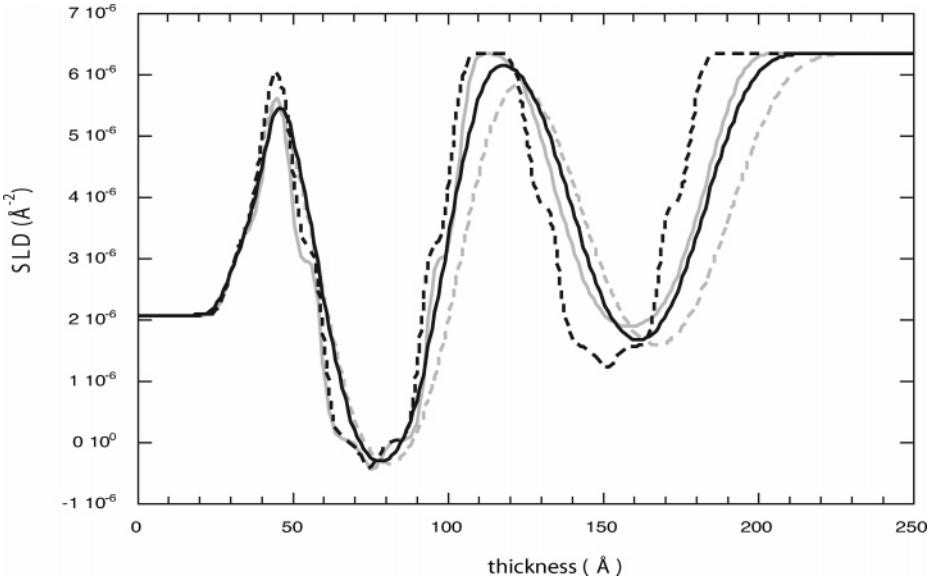

Figure 4. Scattering length density profiles for a DPPC double bilayer in D_2O: dashed gray line, 25.0 °C (as fabricated); black line, 39.9 °C; dashed black line, 45.0 °C; gray line, 25.0 °C (cooled).

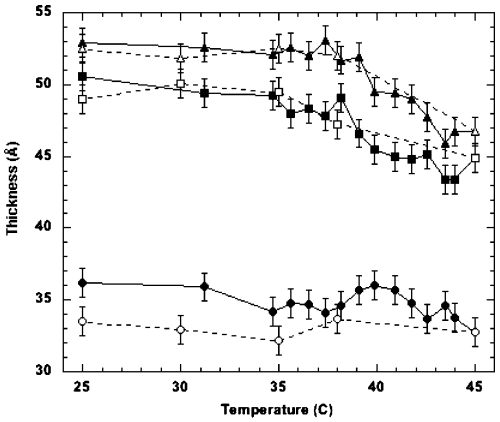

Figure 5. Bilayer and inter bilayer water layer thicknesses from fitted model of reflectivity of DPPC double bilayers in D_2O and SMW: (▲) upper bilayer (D_{Bu}); (■) lower bilayer (D_{Bl}); (●) water layer (D_w). Unfilled symbols and dashed lines indicate the same parameter but for cooling the sample down.

thickness of both chain regions start decreasing at 39 °C, 3 °C lower than the T_m of pure DPPC[33] and continues decreasing until 43.5 °C.

The lowering of T_m by cholesterol is a known phenomenon.[34] The bilayers were found to be thicker in both the gel and fluid phase than the literature values for pure DPPC.[1,35] This is in agreement with literature data that indicate that the net effect of cholesterol is to increase the lipid bilayer thickness by 3−4 Å independent of temperature (see for example[7] and references therein). This is likely to be caused by cholesterol impeding the PC chains from tilting in the gel phase and increased ordering in the fluid phase. The extended length of the acyl part of the DPPC bilayer is 41 Å (considering 32 carbon atoms), we find a thickness of 37 Å, which corresponds to an average tilt of 25.5° relative to the normal to the plane of the bilayer. This is slightly lower than literature values for pure DPPC bilayers being around 30°.[36,38] It has been shown that for cholesterol/DPPC ratios between 8 and 24 mol % a coexistence of normal gel phase and a gel cholesterol rich phase occurs.[5,8] It is likely that this is the case for the gel phase of the double bilayer, as reflected by the high roughness, and it is discussed below. The lower bilayer is generally slightly thinner throughout than the upper bilayer. This is likely due to a higher restraining influence of substrate on the lower compared to the upper bilayer,[32] although it cannot be excluded that cholesterol transfers from the lower to the upper bilayer so that after equilibrium is established the lower bilayer is thinner. Coverage parameters for the bilayers were also found to be different, with the upper bilayer having a coverage of $70\% \pm 5$ and lower $97\% \pm 3$. Both bilayer coverages slightly increased when going to the fluid phase and decreased on approaching gel phase. The effect of these coverage differences can clearly be seen in the SLD profile (Figure 4), where the upper bilayer has a higher solvent content than the lower one. The difference in coverage is likely to be due to differences in the fabrication procedure of the bilayers, as during the deposition of the third and fourth layers, lipids can fill in holes left on the surface while depositing the previous layers. The voids are assumed to fill with water by calculating their fractional occupancy of space by adding up the occupancy of the subfragments. There is no assumption of coherence between segregated regions, which would be on a much larger scale for this type of experiment than defects seen in monolayers and deposited films by many different Brewster angle microscopy studies. The multidetector shows the presence of Yoneda wings (generated by the enhancement of the transmitted wave amplitude at the inner sample surface; see, for example, ref 39) but it is difficult to distinguish whether it is caused by fluctuations or holes. There is no significant change of this off-specular signal at the

(33) Lipidat (Lipid Thermodynamic Database Project). www.lipidat.chemistry.ohio-state.edu/.

(34) McMullen, T. P. W.; McElhaney, R. N. New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry. *Biochim. Biophys. Acta* **1995**, *1234*, 90−98.

(35) Nagle, J. F.; Tristram-Nagle, S. Structure of lipid bilayers. *Biochim. Biophys. Act.* **2000**, *1469*, 159−195.

(36) McIntosh, T. J. Differences in hydrocarbon chain tilt between hydrated phosphatidylethanolamine and phosphatidylcholine bilayers. A molecular packing model. *Biophys. J.* **1980**, *29*, 237−245.

(37) Smith, G. S.; Sirota, E. B.; Safinya, C. R.; Clark, N. A. Structure of the L-beta phases in a hydrated phosphatidylcholine multimembrane. *Phys. Rev. Lett.* **1988**, *60*, 813−816.

(38) Sun, W. J.; Suter, R. M.; Knewtson, M. A.; Worthington, C. R.; Tristram-Nagle, S.; Zhang, R.; Nagle, J. F. Order and disorder in fully hydrated unoriented bilayers of gel-phase dipalmitoylphosphatidylcholine. *Phys. Rev. E.* **1994**, *49*, 4665−4676.

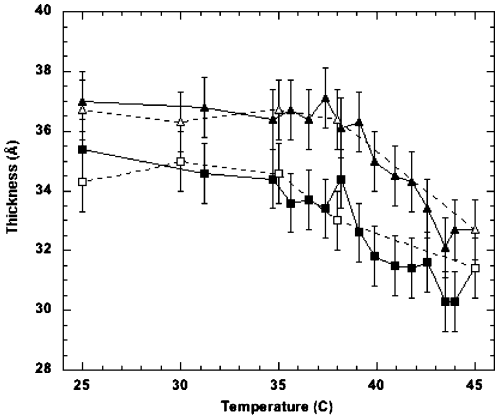

Figure 6. Thickness of chain region of bilayers from fitted model of reflectivity of DPPC double bilayers in D_2O and SMW: (▲) upper bilayer and (■) lower bilayer. Unfilled symbols and dashed lines indicate the same parameter but for cooling the sample down.

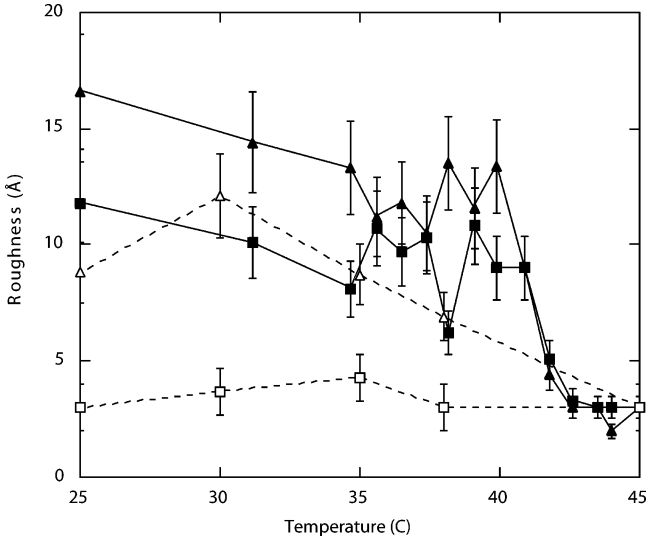

Figure 7. Overall roughness of bilayers from fitted model of reflectivity of DPPC double bilayers in D_2O and SMW: (▲) upper bilayer roughness and (■) lower bilayer roughness. Unfilled symbols and dashed lines indicate the same parameter but for cooling the sample down.

different temperatures which suggests that it is probably not due to fluctuations.

The coherence length of the neutron beam (determined by the incident angular resolution) in the used configuration is of the order of 10 μm. Cholesterol rich domains reported in the literature are of the order of 1 μm or smaller (see for example[40,41]). The measured reflectivity can be correctly considered as from layers with weighted average density.

Because of the low levels and small APM of cholesterol, the calculated area per DPPC lipid was found to be close to the average area per molecule (Table 1). On average the DPPC molecule was ~1.5 Å^2 higher than the average APM. However these were still lower than those of pure DPPC (gel 48 Å^2; fluid 56−73 Å^2 [35]).

4.3. Water Layers. A reduction in the thickness of the thin water layer separating the lower bilayer from the substrate was observed upon heating, after which it remained relatively constant. This can be observed in the SLD profiles, where the lower bilayer has moved closer to the silicon oxide on heating. This is likely due to an equilibrating of this water layer and to interactions between the lower bilayer and the substrate.

Figure 5 shows the values of the two bilayers thicknesses and of the water layer between the bilayers, upon heating and subsequent cooling. One of the most interesting phenomena is that the water swelling between the two bilayers observed upon heating through the transition region for pure floating bilayers[1,32] and bilayers containing up to 6 mol % cholesterol,[12] practically disappears with the 10 mol % samples. One could argue that some swelling occurs over the range 37.4−42.6 °C, with a maximum at 39.9 °C and a magnitude of about 1.5 Å. It starts just before the melting of the chains and finishes just before the end of it. Figure 6 shows that the chain melting occurred between 39.1 and 43.5 °C.

The swelling was assumed to be stable and constant over the period of measurement of each temperature (~1 h). This was both based on historical results obtained from similar systems and on the fact that the measurements in the transition region were performed over periods of 12−15 h and profiles were identical within the error bars. Finally, swelling is symmetrical either side of the maximum.

One similar swelling effect previously observed in multilamellar bilayers is that of anomalous swelling of the lattice repeat unit upon cooling in the fluid phase toward the main transition. A small swelling of 2−4 Å is observed for phosphocholine bilayers[42−45] and thought to be due to softening of bilayer around T_m and a lowering of the bending rigidity.[46] It is thought to have a critical point just below the T_m, but this is obscured from observation by the transition events.[43] The swelling is predominately from the water layer, but the bilayer has recently been seen to contribute as well.[44] Enhanced anomalous swelling has also been observed in the presence of low amounts of cholesterol in liposomes,[46] even up to 10 mol %, which gave swelling of 4.2 Å upon cooling.[43]

4.4. Roughness. An unexpected phenomenon is the presence of high roughness in the gel phase (~14 Å) and low roughness in the fluid phase (~3 Å), as summarized in Figure 7. This differs from that previously seen for double bilayers of pure DPPC,[32,47] where the lipid bilayers had a roughness of about 3 Å in the gel phase, rising higher in the transition region and becoming low again in the fluid phase region, except at very high temperatures. It can be rationalized by the cholesterol interfering with the packing of the PC chains. Molecular dynamic simulations

(39) Baumbach, T.; Mikulik, P. X-ray Reflectivity from Rough Multilayers. In *X-ray and Neutron Reflectivity: Principles and Applications*; Daillant, J., Gibaud, A., Eds.; Springer-Verlag: Berlin and Heidelberg, Germany, 1999.

(40) Radhakrishnan, A. McConnell, H. M. Condensed complexes of cholesterol and phospholipids. *Biophys. J.* **1999**, *77*, 1507−1517.

(41) McConnell, H. M.; Radhakrishnan, A. Condensed Complexes of cholesterol and phospholipids. *Biochim. Biophys. Acta* **2003**, *1610*, 159−173.

(42) Hønger, T.; Mortensen, K.; Ipsen, J. H.; Lemmich, J.; Bauer, R.; Mouritsen, O. G. Anomalous swelling of multilamellar lipid bilayers in the transition region by renormalisation of curvature elasticity. *Phys. Rev. Lett.*. **1994**, *72*, 3911−3914.

(43) Richter, F.; Finegold, L.; Rapp, G. Sterols sense swelling in lipid bilayers. *Phys. Rev. E.* **1999**, *59*, 3483−3491.

(44) Mason, P. C.; Gaulin, B. D.; Epand, R. M.; Katsaras, J. Critical swelling in single phospholipid bilayers. *Phys. Rev. E.* **2000**, *61*, 5634−5639.

(45) Pabst, G.; Katsaras, J.; Raghunathan, V. A.; Rappolt, M. Structure and interactions in the anomalous swelling regime of phospholipid bilayers. *Langmuir* **2003**, *19*, 1716−1722.

(46) Lemmich, J.; Mortensen, K.; Ipsen, J.; Hønger, T.; Bauer, R.; Mouritsen, O. G. The effect of cholesterol in small amounts on lipid-bilayer softness in the region of the main phase transition. *Eur. Biophys. J.* **1997**, *25*, 293−304.

(47) Hughes, A. V.; Golder, A.; Gerstenberg, M.; Roser, S. J.; Bradshaw, J. Hybrid SAM phospholipid approach to fabricating a free supported lipid bilayer. *PCCP* **2002**, *4*, 2371−2378.

have shown that very high cholesterol (50 mol %) containing DPPC systems have higher roughness than pure DPPC, due to shorter cholesterol molecules.[48] No information on the roughness of 10 mol % cholesterol bilayers could be found in the literature.

Another explanation for the high roughness in the gel phase is related to miscibility issues. It has been observed in vesicles[5] that for cholesterol ratios between 8 and 24 mol % two coexisting phases are observed. There is a tilted phase resembling that of a pure DPPC bilayer and a nontilted mixture containing 24 mol % of cholesterol. The figure of 24 mol % corresponds to reconciling the concepts of phase separation and complex formation. The same team established that there was complete miscibility in the liquid in vesicle phases up to ratios of 14 mol %, with strong evidence that it was the case up to 45 mol %. Cholesterol is also known to increase the rigidity of the liquid phase,[49] which would be expected to lower the roughness. The different behavior observed with the bilayer adsorbed on the silicon might be related to substrate effects.

5. Conclusions

The addition of 10 mol % cholesterol to DPPC floating bilayers induces not only structural modifications of the bilayer but also differences in the phase behavior. In particular, the large swelling of the water layer between the two bilayers at the transition is practically removed. This indicates that cholesterol induces a modification in the forces holding the upper bilayer to the lower.[50] The result is in agreement with literature data indicating that while very low amounts of cholesterol (less than 4%) soften the bilayer, higher amounts rigidify it.[46] The structure of the bilayer revealed that in the gel phase cholesterol induces the presence of a high rms roughness that disappears in the other phases.

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(48) Smondyrev, A. M.; Berkowitz, M. L. Structure of dipalmitoylphosphatidylcholine/cholesterol bilayer at low and high cholesterol concentrations: molecular dynamics simulation. *Biophys. J.* **1999**, *77*, 2075−2089.

(49) Yeagle, P. L. Cholesterol and the cell membrane. *Biochim. Biophys. Acta Rev. Biomem.* **1985**, *822*, 267−287.

(50) Helfrich, W. Steric interactions of fluid membranes in multilayer systems. *Z. Naturforsch.* **1977**, *33A*, 305−315.