Cholesterol Effects on the Phosphatidylcholine Bilayer Polar Region: A Molecular Simulation Study

Marta Pasenkiewicz-Gierula,* Tomasz Róg,* Kunihiro Kitamura,† and Akihiro Kusumi‡§

*Department of Biophysics, Institute of Molecular Biology, Jagiellonian University, Kraków, Poland; †Department of Molecular Science, Research Center, Taisho Pharmaceutical Co., Saitama 330, Japan; ‡Department of Biological Science, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan; and §Kusumi Membrane Organizer Project, ERATO, Japan Science and Technology Corporation, Nagoya 460-0012, Japan

ABSTRACT A molecular dynamics (MD) simulation of a fully hydrated, liquid-crystalline dimyristoylphosphatidylcholine (DMPC)-Chol bilayer membrane containing ~22 mol% Chol was carried out for 4.3 ns. The bilayer reached thermal equilibrium after 2.3 ns of MD simulation. A 2.0-ns trajectory generated during 2.3–4.3 ns of MD simulation was used for analyses to determine the effects of Chol on the membrane/water interfacial region. In this region, 70% of Chol molecules are linked to DMPC molecules via short-distance interactions, where the Chol hydroxyl group (OH-Chol) is 1) charge paired to methyl groups of the DMPC choline moiety (~34%), via the hydroxyl oxygen atom (Och); 2) water bridged to carbonyl (~19%) and nonester phosphate (~14%) oxygen atoms, via both Och and the hydroxyl hydrogen atom (Hch); and 3) directly hydrogen (H) bonded to carbonyl (~11%) and nonester phosphate (~5%) oxygen atoms, via Hch (~17% of DMPC-Chol links are multiple). DMPC's γ-chain carbonyl oxygen atom is involved in 44% of water bridges and 51% of direct H bonds formed between DMPC and Chol. On average, a Chol molecule forms 0.9 links with DMPC molecules, while a DMPC molecule forms 2.2 and 0.3 links with DMPC and Chol molecules, respectively. OH-Chol makes hydrogen bonds with 1.1 water molecules, preferentially via Hch. The average number of water molecules H bonded to the DMPC headgroup is increased by 7% in the presence of Chol. These results indicate that inclusion of Chol decreases interlipid links and increases hydration in the polar region of the membrane.

INTRODUCTION

Cholesterol (Chol) is an important constituent of eukaryotic cell membranes. It is located mainly in the plasma membrane and often amounts to 50 mol% of the membrane lipids (e.g., Sackmann, 1995). The biological roles of Chol involve maintenance of proper fluidity (e.g., Kusumi et al., 1983; Mouritsen and Jørgensen, 1994), formation of glycosphingolipid-Chol-enriched raft domains (Simons and Ikonen, 1997), reduction of passive permeability (e.g., Bittman et al., 1984; Subczynski et al., 1989, 1994), and increasing the mechanical strength (e.g., El-Sayed et al., 1986; Bloom et al., 1991; Bloom and Mouritsen, 1995) of the membrane.

Because of the prevalence and such important roles of Chol, phospholipid-Chol interactions in the membrane have been studied extensively (for a recent review, see McMullen and McElhaney, 1996). Langmuir film balance studies indicated that Chol causes a reduction in the average cross-sectional area of phosphatidylcholines (PCs) in the liquid-crystalline membranes—the so-called condensing effect (e.g., Hyslop et al., 1990; Smaby et al., 1994, 1997). The magnitude of the effect depends on the degree of unsaturation of the PC acyl chains and whether the unsaturated chain is in the β or γ position (Davis and Keough, 1983, 1984; Keough et al., 1989; Smaby et al., 1994, 1997). In dioleoylphosphatidylcholine-Chol membranes, because of steric nonconformability between the *cis*-unsaturated double bond in the alkyl chain and the rigid tetracyclic ring of Chol, small Chol domains with a lifetime of 1–100 ns are formed (Subczynski et al., 1990; Pasenkiewicz-Gierula et al., 1990, 1991). Meanwhile, the role of the polar part of the PC molecule in the PC-Chol interaction is not certain (e.g., de Kruyff et al., 1973; Bicknell-Brown and Brown, 1980; Bush et al., 1980). A comparison of the effects of 3β-OH- and 3α-OH-Chol (epiChol) indicates that the β-configuration of the hydroxyl group is important for interaction between phospholipids and Chol (Demel et al., 1972; de Kruyff et al., 1973; Murari et al., 1986; Cheetham et al., 1989). However, how the hydroxyl group of Chol interacts with the polar groups of PC has yet to be clarified.

PC-Chol membranes have also been studied by theoretical (Scott, 1991; Scott and McCullough, 1993) and computer simulation (Scott and Kalaskar 1989; Scott, 1991; Edholm and Nyberg, 1992; Robinson et al., 1995; Tu et al., 1998) methods. Monte Carlo (MC) studies concentrated mainly on the ordering effect of Chol on the hydrocarbon chains (Scott and Kalaskar 1989; Scott, 1991). They showed that Chol significantly decreased the *trans-gauche* isomerization of neighboring chains. The effect was stronger for chains that were near neighbors to more than one Chol molecule. However, even those chains did not assume all-*trans* conformations. Results of MC calculations were subsequently used to define a theoretical model for phase equilibria in a PC bilayer containing Chol (Scott, 1991) and

Received for publication 20 August 1999 and in final form 22 November 1999.

Address reprint requests to Dr. Marta Pasenkiewicz-Gierula, Jagiellonian University, Institute of Molecular Biology, al. Mickiewicza 3, 31-120 Kraków, Poland. Tel.: 48-12-634-20-08; Fax: 48-12-633-69-07; E-mail: mpg@mol.uj.edu.pl.

0006-3495/00/03/1376/14 $2.00

to predict the effect of Chol on the ripple phase (Scott and McCullough, 1993). Increased ordering of hydrocarbon chains close to the Chol molecules was also observed in molecular dynamics (MD) simulation studies of Edholm and Nyberg (1992) and Robinson et al. (1995). However, Tu et al. (1998) observed only moderate effects of Chol. Interactions of Chol with PCs and water at the membrane/water interface were analyzed by MD simulation by Robinson et al. (1995) and Tu et al. (1998). In both studies, formations of hydrogen bonds between the Chol hydroxyl group (OH-Chol) and the phosphate and carbonyl oxygen atoms, as well as between OH-Chol and water, were observed. Furthermore, Tu et al. (1998) showed that interaction of Chol with the γ-chain carbonyl oxygen atom was twice as common as that with the β-chain carbonyl oxygen atom, and that in the presence of Chol the choline group moved toward the bilayer center.

In this paper, results of a 4.3-ns, constant temperature and pressure MD simulation of the fully hydrated, liquid-crystalline dimyristoylphosphatidylcholine (DMPC)-Chol bilayer membrane containing ~22 mol% Chol are described. The bilayer reached thermal equilibrium after 2.3 ns; thus a 2.0-ns trajectory was used for analyses. The primary aim of these analyses was to investigate short-distance interactions among polar groups of PC, Chol, and water at the interfacial region of the membrane. Like Robinson et al. (1995) and Tu et al. (1998), we observed the formation of H bonds between OH-Chol and oxygen atoms of PC, particularly the γ-chain carbonyl oxygen atom, and between OH-Chol and water. However, in the present research, we found that interactions between Chol and PC via water bridges and charge pairs are much more prevalent than direct hydrogen bonding. In a series of MD simulation studies of pure DMPC bilayer membranes in the liquid-crystalline phase (Pasenkiewicz-Gierula et al., 1997; 1999), we showed the occurrence of two types of important interactions between polar groups of DMPC molecules—indirect, via H bonded water molecules (water bridging), and direct, via Coulombic attraction between the positively charged choline moiety of one DMPC molecule and the negatively charged nonester phosphate (Op) or carbonyl (Oc) oxygen atoms of another (charge pairing). In the bilayer, 76% of DMPC molecules were linked by water bridges and 93% by charge pairs. Water bridges and charge pairs formed an extended network of interactions among PC headgroups. These interactions linked 98% of all PC molecules in the membrane; on average, only one PC molecule in the bilayer built of 72 DMPC molecules was not linked to the remaining ones in the polar region. Thus major issues of the present study were to determine how interactions between OH-Chol and PC headgroups, and between OH-Chol and water, contribute to the organization of the membrane/water interface and the extent to which they interfere with the formation of DMPC-DMPC water bridges and charge pairs observed in the pure DMPC bilayer. The key finding of this study is that in the polar region of the PC-Chol bilayer membrane, PC-Chol-water links form an extended network. This network, however, is less branched than a PC-PC-water network in the pure DMPC bilayer. A similar conclusion was drawn from experimental studies (Slater at al., 1993; Ho et al., 1995). In accordance with experimental data (Kusumi et al., 1986; Ho et al., 1995), increased headgroup hydration is observed in the presence of Chol. Interactions between DMPC and Chol in the nonpolar, hydrocarbon chain region of the membrane will be analyzed and published elsewhere.

METHOD

Simulation system

The dimyristoylphosphatidylcholine bilayer membrane with intercalated Chol molecules used in this study consisted of 56 DMPC and 16 Chol molecules. It was obtained by replacing 16 DMPC molecules with 16 Chol molecules (eight in each leaflet) in the bilayer built of 72 ($6 \times 6 \times 2$) DMPC molecules and simulated for 1690 ps (Pasenkiewicz-Gierula et al., 1997). The molar content of Chol in the membrane was ~22 mol%. In each leaflet, the Chol molecules were uniformly distributed and well separated from one another by DMPC molecules. The membrane was hydrated with 1622 water molecules and simulated for 4300 ps, using AMBER 4.0 (Pearlman et al., 1991). Details concerning the pure DMPC membrane construction and equilibration have been described by Pasenkiewicz-Gierula et al. (1997, 1999). The initial structure of Chol was the crystal structure of the molecule A determined by Shieh et al. (1981) (the unit cell of the Chol crystal contains eight molecules, labeled A–H; Shieh et al., 1981).

Fig. 1 shows the structure and numbering of atoms in the DMPC and Chol molecules.

Simulation parameters

For DMPC and Chol, optimized potentials for liquid simulations (OPLS) parameters (Jorgensen and Tirado-Rives, 1988) were used. The procedure for supplementing the original OPLS base with the missing parameters for DMPC was described by Pasenkiewicz-Gierula et al. (1999). For Chol, the ring sp3 carbon atoms with four explicit substituents, at the junctions between the rings A and B, and between the rings C and D, i.e., atoms C10 and C15 in Fig. 1 *b*, respectively, were identified as CT atom type. The stretching, bending, and torsion parameters for groups containing CT were set in analogy to OPLS parameters for carbon atoms in hydrocarbons, purines (C5 in Fig. 1 *b*), or pyrimidines (C6 in Fig. 1 *b*). For water, TIP3P parameters (Jorgensen et al., 1983) were used. To reduce computation time, the united-atom approximation was applied to CH, CH_2, and CH_3 groups of DMPC and Chol. The hydroxyl group of Chol was treated with full atomic details. The atomic charges of the DMPC molecule were taken from Charifson et al. (1990) (a detailed explanation is given in Pasenkiewicz-Gierula et al. (1999)). The atomic charges of the Chol molecule were obtained by minimizing the electrostatic energy with respect to the charges. The method is provided with the Extensible Systematic Forcefield (ESFF) from the MSI package (Biosym/MSI, 1995). The only nonzero charges are on the hydroxyl hydrogen (0.42 in units of an electronic charge (*e*)) and oxygen (−0.54 *e*) atoms and on the C3 carbon atom of the A ring to which the hydroxyl group is attached (0.12 *e*) (cf. Fig. 1 *b*).

Simulation conditions

Three-dimensional periodic boundary conditions with the usual minimum image convention were used. The SHAKE algorithm (Ryckaert et al.,

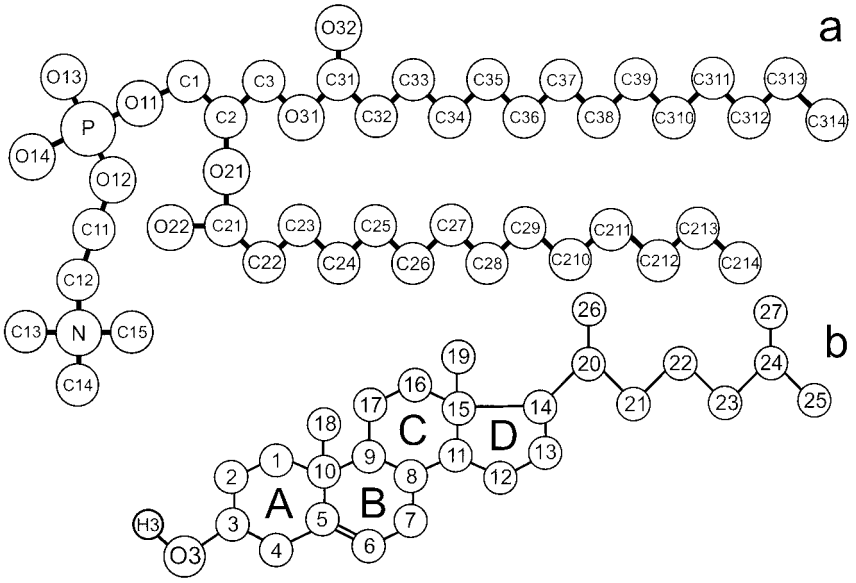

FIGURE 1 Molecular structure of (*a*) DMPC and (*b*) Chol molecules with numbering of atoms (the chemical symbol for carbon atoms, C, is omitted). The Chol rings are labeled A, B, C, and D.

1977) was used to preserve the bond lengths of the water molecule and the hydroxyl group of Chol, and the time step was set at 2 fs (Egberts et al., 1994). For nonbonded interactions, a residue-based cutoff was used with a cutoff distance of 12 Å. To reduce the calculation time of nonbonded interactions, each DMPC molecule was divided into six residues (Pasenkiewicz-Gierula et al., 1997), and each Chol molecule was divided into three residues (residues 1, 2, and 3 consisted of the following atoms, respectively: 1) C1-C5, O3, H3, C10, C18; 2) C6-C9, C11-C17, C19; 3) C20-C27 (cf. Fig. 1 *b*). Each residue was chosen in such a way that the total electrostatic charge on the residue was close to or equal to zero and the integrity of its chemical groups was preserved. The list of nonbonded pairs was updated every 25 steps.

To speed up equilibration of the DMPC-Chol membrane, at the beginning of simulation the temperature of the system was raised to 500 K for 20 ps. Then temperature was gradually lowered to 310 K (cf. Fig. 2 *a*). After this temperature was reached, simulation was carried out at constant temperature (310 K = 37°C), which is above the main phase transition temperature for a pure DMPC bilayer (~23°C), and constant pressure (1 atm). Temperatures of the solute and solvent were controlled independently. Both the temperature and pressure of the system were controlled by the Berendsen method (Berendsen et al., 1984). The relaxation times for temperatures and pressure were set at 0.4 and 0.6 ps, respectively. Applied pressure was controlled anisotropically, where each direction was treated independently and the trace of the pressure tensor was kept constant (1 atm).

RESULTS

Characterization of the membrane systems and comparison with experimental data

The approach to the thermally equilibrated state of the DMPC-Chol bilayer in the liquid-crystalline phase was observed from the onset of simulation until 4300 ps by monitoring the following parameters of the system: the temperature (Fig. 2 *a*), surface area/DMPC (Fig. 2 *b*), number of *gauche* conformations/myristoyl chain (Fig. 2 *c*), and potential energy (Fig. 2 *d*). (While this paper was reviewed and revised, the calculation was continued for 700 ps, up to 5000 ps. Because the system equilibration is more clearly seen with the addition of the latest simulation, Fig. 2 shows the data for the time range between 0 and 5000 ps.) The surface area/DMPC in the DMPC-Chol membrane was obtained by subtracting the cross-sectional area of eight Chol molecules (8×39 Å^2) from the total surface area of the membrane and then dividing it by 28 DMPC molecules present in each leaflet. The mean surface area of the Chol molecule of 39 Å^2 was determined by Hyslop et al. (1990) in a Chol monolayer. This value is greater by 2.8 Å^2 than the value obtained by Vanderkooi (1994) in the crystal. As can be seen in Fig. 2, *b* and *c*, the surface area/DMPC and the number of *gauche* conformations/myristoyl chain asymptotically reached average values of 58.4 ± 0.7 Å^2 and 2.7 ± 0.1 after 2300 ps and 2000 ps, respectively. The distributions and errors of these and other parameters used in this as well as our previous papers (Pasenkiewicz-Gierula et al., 1997, 1999) are given in standard deviations. Other parameters, like the temperature, potential energy, and number of H bonds between DMPC and water, converged in shorter time periods. Therefore, we concluded that the DMPC-Chol bilayer membrane had reached thermal equilibrium after 2.3 ns of MD simulation. Fig. 3 is a snapshot of the DMPC-Chol bilayer membrane at 4.3 ns. Results described below are obtained from a 2.0-ns trajectory generated between 2.3 and 4.3 ns of MD simulation; the average values are ensemble and time averages.

The mean surface area/DMPC in the DMPC-Chol membrane of 58.4 ± 0.7 Å^2 is smaller than that in the pure DMPC membrane of 60.2 ± 1.0 Å^2 obtained in our previous MD simulation (Pasenkiewicz-Gierula et al., 1999). In a palmitoyloleoylphosphatidylcholine-Chol monolayer, the surface area/palmitoyloleoylphosphatidylcholine was measured to decrease by ~7 Å^2 in the presence of Chol (Hyslop

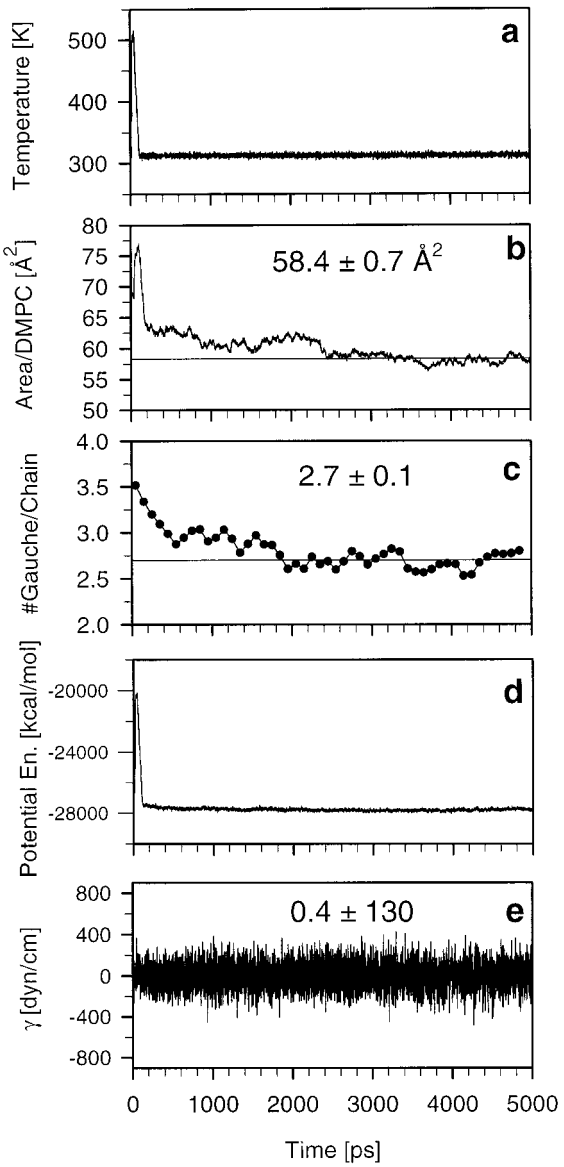

FIGURE 2 Diagrams showing the time developments of the (*a*) temperature, (*b*) surface area per DMPC (the equilibrium average surface area is 58.4 ± 0.7 Å^2), (*c*) number of *gauche* bonds per chain (the equilibrium average number is 2.7 ± 0.1), (*d*) potential energy, and (*e*) surface tension (the average equilibrium tension is −0.4 ± 130 dyn/cm). The errors are standard deviations (SD). Thin lines in *b* and *c* indicate average values of the parameters in the time range between 2300 and 5000 ps.

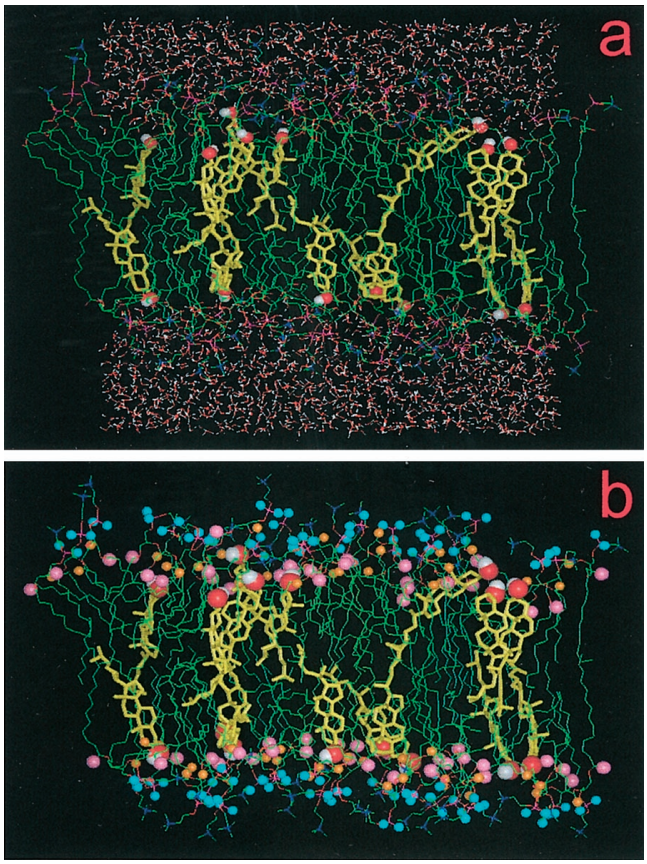

FIGURE 3 Snapshots of the DMPC-Chol bilayer membrane at 4300 ps of MD simulation. (*a*) The full system. (*b*) Water is removed to show locations of DMPC and Chol polar groups. The Chol molecules are shown as yellow sticks. The OH group of Chol is shown in standard colors as the CPK model; the nonester phosphate oxygen atoms (Op) are light blue; the carbonyl oxygen atoms O22 and O32 are light brown and pink, respectively. The spheres representing the oxygen atoms are of varying sizes—Ops are the smallest, O32s are the largest. The remaining atoms are coded in standard colors as lines.

et al., 1990). However, in that experiment the monolayer contained 50 mol % of Chol and mono-unsaturated chains, whereas the DMPC-Chol bilayer used in this study contains 22 mol% Chol and fully saturated chains. The average number of *gauche* rotamers/myristoyl chain obtained in this study is 2.7 ± 0.1, and in the pure DMPC membrane it was 2.8 ± 0.1 (Pasenkiewicz-Gierula et al., 1999); thus they do not differ significantly. However, in accordance with predictions of Ipsen et al. (1987), a decrease in the probability of *gauche* conformations in alkyl chains next to Chol was observed. Analyses of DMPC alkyl chain conformations and dynamics in the DMPC-Chol membrane will be published elsewhere.

The surface tension in the simulation box was monitored from the onset of simulation until 4300 ps (Fig. 2 *e*). After equilibration, the average surface tension (Zhang et al., 1995) is −0.4 ± 130 dyn/cm. Its nearly zero value follows from keeping the system pressure constant. Large fluctuations in this parameter (±130 dyn/cm) are caused by large fluctuations in the system pressure.

The profiles of the order parameter along the DMPC hydrocarbon chains in the DMPC and DMPC-CHOL membranes are shown in Fig. 4. The presence of Chol increased the molecular order parameter of DMPC alkyl chains at all depths in the membrane, which is in agreement with experimental data (Sankaram and Thompson, 1990; Urbina et al., 1995, 1998).

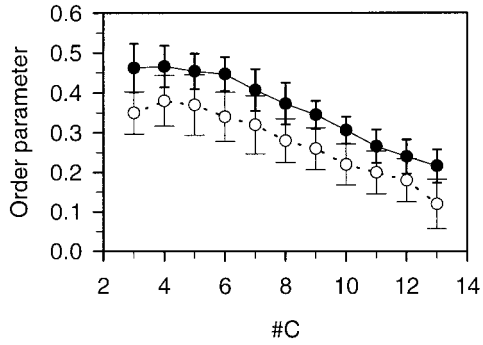

FIGURE 4 The molecular order parameter (S_{mol}) profiles calculated for the DMPC-Chol membrane (●) and for the pure DMPC membrane (○) at 310 K (averaged over β- and γ-chains, and over 2000 ps). Error bars are standard deviations.

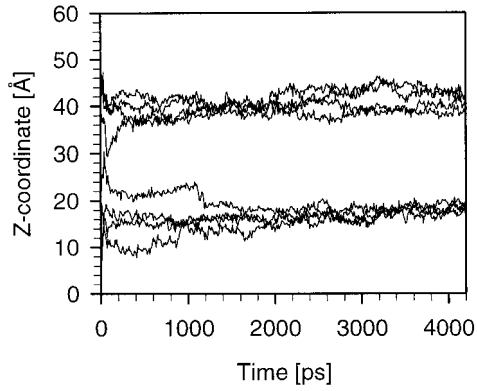

FIGURE 5 Time profiles of the vertical positions (z axis) of hydroxyl groups of eight arbitrarily chosen Chol molecules (four in each leaflet) in the DMPC-Chol membrane.

Our earlier electron paramagnetic resonance experiments on DMPC-Chol multibilayer membranes utilizing Chol analog spin-labels, cholestane and androstane, indicated that in the membrane at 35°C the probes undergo substantial wobbling in a cone (Kusumi and Pasenkiewicz-Gierula, 1988; Pasenkiewicz-Gierula et al., 1990). In the DMPC-Chol membrane containing 30 mol% Chol at 35°C, the average tilt inside the cone of the androstane long axis relative to the normal was 22.3° (Pasenkiewicz-Gierula et al., 1990). The Chol tilt in this simulation was defined as an angle between the C3-C15 vector (cf. Fig. 1 *b*) and the bilayer normal. In the simulated DMPC-Chol membrane containing ~22 mol% Chol at 37°C, the average tilt, calculated based on the cone angle formalism, is 27 ± 2°. Experimental data indicate that the angle of tilt was decreased as the Chol concentration in the membrane was increased (Murari et al., 1986; Pasenkiewicz-Gierula et al., 1990). However, the magnitude of the decrease depends on the experimental method used. The average Chol tilt angle obtained in this work is in general agreement with those estimated experimentally.

The change of the location of OH-Chols along the membrane normal (z axis) with time is shown in Fig. 5. The equilibrium atom density profiles across the membrane of OH-Chols and the carbonyl O22 and O32 atoms are shown in Fig. 6 *a*. These two figures, as well as Fig. 3, indicate that after membrane equilibration the vertical location of OH-Chols is close to that of O22 and O32. This result is in good agreement with data obtained by x-ray diffraction (McIntosh, 1978), ^{13}C NMR (Yeagle and Martin, 1976; de Kruijff, 1978), and electric measurements (Karolis et al., 1998).

The results summarized above suggest that the simulated membrane obtained here reproduces various properties of PC-Chol bilayers in the liquid-crystalline phase that have been observed experimentally. Therefore, it is concluded that this membrane provides a good model for a DMPC-Chol membrane.

In the following analyses of a 2.0-ns trajectory of the MD simulation, we focus on the atomic-level interactions of OH-Chols with PC headgroups and water molecules. One of the major issues here is to determine how these interactions contribute to the organization of the membrane/water interface and the extent to which they interfere with the formation of DMPC-DMPC water bridges and charge pairs.

In this study, we use the same geometrical definitions of H bonding, water bridging, charge pairing, and their lifetimes as in our previous papers (Pasenkiewicz-Gierula et al., 1997, 1999).

1. An H bond between OH-Chol and Op, Oc, or Ow (Ow is the oxygen atom of a water molecule) is judged to be formed when the O···O distance (r_{HB}) is less than or equal to 3.25 Å and the angle, θ, between the O···O vector and the O-H bond (O···O-H angle) is less than or equal to 35° (Table 1). The distance of 3.25 Å is the position of the first minimum in the radial distribution function (RDF) of the

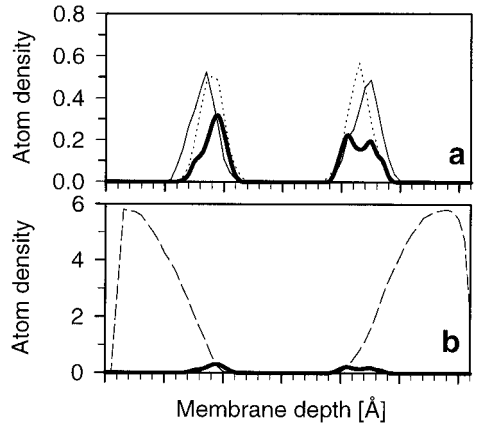

FIGURE 6 Equilibrium atom number density profiles along the bilayer normal of the Chol oxygen atoms. Och (*thick line*) is shown together with (*a*) O22 (*thin line*) and O32 (*dotted line*) and (*b*) the water oxygen atoms, Ow (*dashed line*). The center of the horizontal axis (zero) is the middle of the hydrocarbon core of the bilayer. The notation is given in Fig. 3.

TABLE 1 Characteristics of direct H bonds between DMPC oxygen atoms (nonester phosphate (Op), carbonyl (Oc: O22 and O32)) and cholesterol (CHOL); between cholesterol and water (Wat); and between cholesterol molecules

Pair	No./cholesterol (%)	No./membrane (%)	r (Å)	θ (deg)	Lifetime (ps)
A. DMPC···CHOL H bonds					
DMPC···CHOL	0.16 ± 0.06	2.5 ± 1.0 16% CHOL 4% DMPC	—	—	68 ± 140
Op···CHOL	0.05 ± 0.04 (30%)	0.76 ± 0.6 (30%) O14,O13 (15%)	2.64 ± 0.1	10.3 ± 5.8	—
Oc···CHOL	0.11 ± 0.05 (70%)	1.7 ± 0.8 (70%)	2.78 ± 0.1	13.5 ± 6.0	—
O22···CHOL	0.03 ± 0.03	0.4 ± 0.5 (19%)	2.79 ± 0.1	12.3 ± 5.4	—
O32···CHOL	0.08 ± 0.04	1.3 ± 0.6 (51%)	2.78 ± 0.1	14.0 ± 6.0	—
B. Wat···CHOL H bonds					
Wat···CHOL	1.1 ± 0.10	17.4 ± 2.7	—	—	41 ± 68
Wat···Och	0.33 ± 0.10 (30%)	5.2 ± 1.6 (30%)	2.91 ± 0.1	17.0 ± 8.3	24 ± 37
Wat···Hch	0.76 ± 0.07 (70%)	12.2 ± 1.1 (70%)	2.76 ± 0.1	12.0 ± 6.7	45 ± 90
No. single Wat···CHOL H bonds:		0.6			
No. bridging Wat···CHOL H bonds:		0.5			
C. CHOL···CHOL H bonds					
CHOL···CHOL	—	0.15 2% CHOL	—	—	—

The average numbers, geometry (distance, r, and angle, θ), and the lifetimes of H bonds are given. Och and Hch are, respectively, the oxygen and hydrogen atoms of the Chol hydroxyl group. The percentage of Chol and DMPC molecules involved in direct H bonding and the number of Chol–Chol H bonds are also given.

Chol oxygen atoms (Och) relative to Op (Oc, Ow) (cf. Fig. 7, *a–c*).

2. A water bridge between two molecules (two DMPCs, DMPC and Chol, or two Chols) is judged to be made when a water molecule is simultaneously H bonded to both molecules.

3. A charge pair between oppositely charged groups on two separate molecules (either two DMPCs or DMPC and Chol) is judged to be formed when the two groups are located within 4.0 Å of each other (cf. Fig. 7 *d*).

4. DMPC-Chol association via H bonding and charge pairing is dynamic. To calculate the lifetime of the association, each DMPC-Chol pair was monitored every 1.0 ps for the time from its first appearance (after equilibration of the system) until the final time of 4300 ps. In this analysis, if the association was temporarily broken but reformed within 60

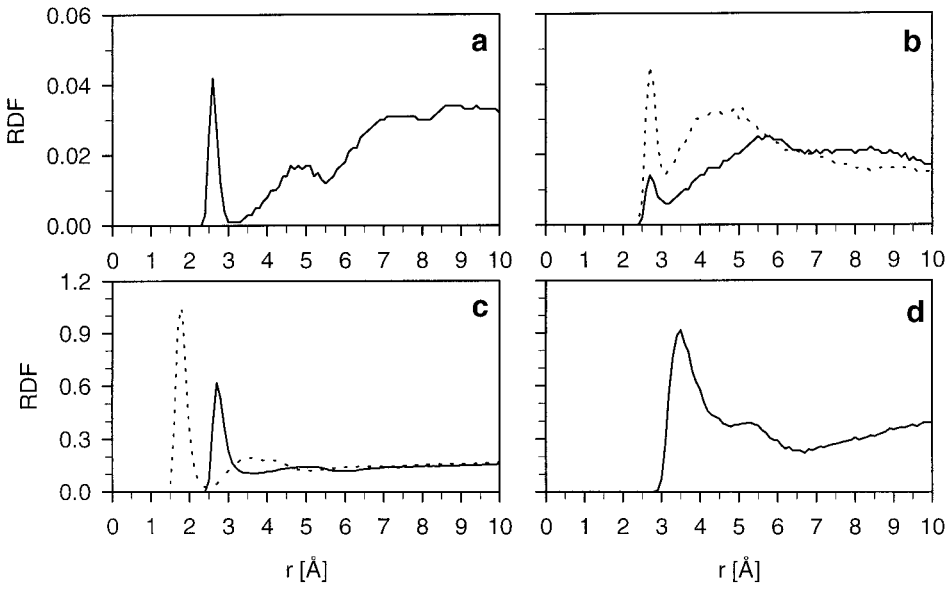

FIGURE 7 The radial distribution functions (RDF) of Ochs relative to (*a*) Ops, (*b*) O22 (——), and O32 (– – –). The RDFs of Ows relative to (*c*) Och (——) and the Chol hydroxyl hydrogen atom, Hch (– – –). (*d*) The RDFs of Ochs relative to a choline methyl group, N-CH_3. The notation is given in Figs. 3 and 6.

ps between the same molecules, the break was ignored, whereas a break longer than 60 ps was treated as the final decay.

H bonds formed by Chol

Direct H bonding between OH-Chol and DMPC

The equilibrium RDFs of the Chol oxygen atoms relative to Op and to O22 and O32, are shown in Fig. 7, *a* and *b*, respectively. Their shapes indicate a formation of H bonds between Chol and DMPC, which is not extensive. The probability of finding a Chol molecule directly H bonded to DMPC oxygen atoms (Op, Oc···Chol) is only 16% on average. Thus 2.5 of 16 Chol molecules are H bonded to DMPC oxygen atoms at a given instant. Of these H bonds, 70% are formed with Oc (O22 and O32) and 30% with Op (O14 and O13) (Table 1). An example of a DMPC-Chol pair that is linked by a direct H bond (DMPC···Chol) is shown in Fig. 8 *a*. The average lifetime of DMPC···Chol is 68 ps (Table 1). Snapshots of DMPC···Chol H bonds at 3300 ps and 4300 ps are shown in Fig. 9 in light green.

H bonding between OH-Chol and water

Fig. 7 *c* shows the equilibrium RDFs of the water oxygen atoms (Ow) relative to the oxygen atom (Och) (*solid line*) and the hydrogen atom (Hch) (*dotted line*) of OH-Chol. Their shapes indicate that Chol forms H bonds with water more extensively than with DMPC. On average, there are 1.1 H bonds with water/Chol; 30% of them are made via Och (water···Och H bonds) and 70% via Hch (water···Hch H bonds) (Table 1). The average lifetimes of the H bonds are 24 and 45 ps, respectively (Table 1). On average, 84% of the Chol molecules present in the membrane make H bonds with water; the multiplicity of the bonds is given in Table 2.

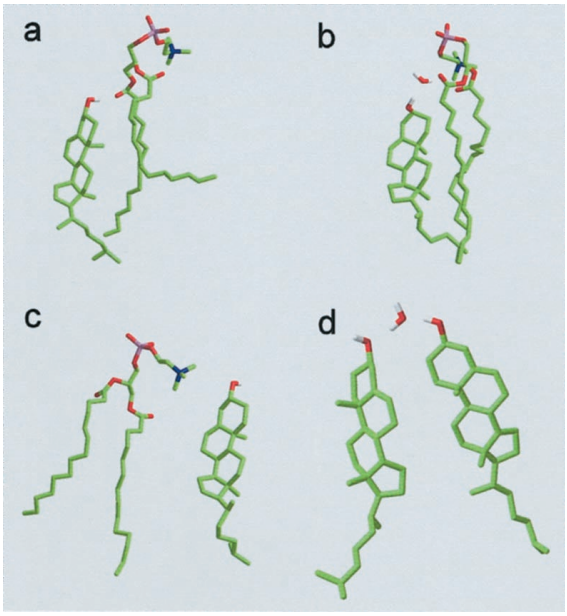

FIGURE 8 Examples of DMPC-Chol and Chol-Chol pairs in the membrane. (*a*) A direct H bond between Oc and the hydroxyl group of Chol (OH-Chol). (*b*) A water bridge between Oc and OH-Chol. (*c*) A charge pair between Och and N-CH_3. (*d*) A water bridge between two Chol molecules. The notation is given in Figs. 3 and 6. The image was produced with MolScript (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997).

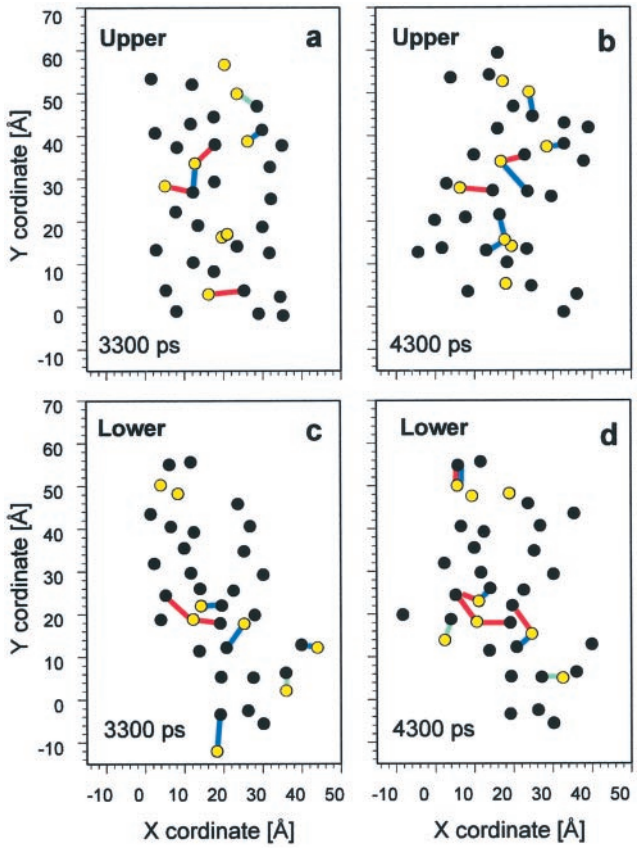

FIGURE 9 DMPC-Chol cross-links due to direct H bonding (*light green*), water bridging (*blue*), and charge pairing (*red*) for the upper layer at 3300 (*a*) and 4300 ps (*b*) and for the lower layer at 3300 (*c*) and 4300 ps (*d*). A black dot shows the location of the phosphorus atom of DMPC in the *x, y* plane. A yellow dot shows the location of the oxygen atom of Chol in the *x, y* plane.

Water bridging between OH-Chol and DMPC

In the pure DMPC bilayer membrane, a pair of DMPC molecules can be linked by a water bridge (Pasenkiewicz-Gierula et al., 1997). Because Chol forms on average H bonds with 1.1 water molecules, water bridging between DMPC and Chol was examined. The snapshots of DMPC-Chol pairs linked by water bridges at 3300 ps and 4300 ps are shown in Fig. 9 in blue.

TABLE 2 H bonds formed between cholesterol and water

A. Simple H bonds between CHOL and water						
No. H bond/CHOL		0	1	2	3	
% CHOL		16	59	24	1	
B. DMPC–CHOL water bridges						
DMPC						
oxygen atom	Op:	O14	O13	Oc:	O22	O32
% H bonds	42	21	21	58	14	44
No. H bonds	2.8	1.4	1.4	3.9	1.0	2.9
CHOL						
CHOL atom	Och		Hch			
% H bonds	16		84			
No. H bonds	1.3		6.8			

A. Simple H bonds: Percentage of Chol molecules forming zero, one, two, and three H bonds with water. B. Water bridges: Percentage and numbers of H bonds formed with nonester phosphate (Op) and carbonyl (Oc) oxygen atoms of DMPC, and with oxygen (Och) and hydrogen (Hch) atoms of the Chol hydroxyl group.

TABLE 3 DMPC–Chol and DMPC–DMPC water bridges and water bridged pairs

Pair	No./Membrane (%) (No./Molecule)	Lifetime (ps)
DMPC-Chol membrane		
Op,Oc–Chol	6 bridges (0.38 bridges/Chol)	40 ± 70
	85% single, 15% multiple	
DMPC–Chol	6 pairs (0.38 pairs/Chol)	500 ± 500
	5.2 Chol (33%)	
	5.7 DMPC (10%)	
Op,Oc–Op,Oc	28 bridges (0.5 bridges/DMPC)	27 ± 65
	69% single, 31% multiple	
DMPC–DMPC	24 pairs (0.43 pairs/DMPC)	530 ± 600*
	35.5 DMPC (64%)	(8% ≥ 2000 ps)
Chol–Chol	0.5 bridges (0.03 bridges/Chol)	—
	6% Chol	
DMPC–DMPC–Chol	3.45 DMPC (6%)	—
Pure DMPC membrane†		
Op,Oc–Op,Oc	41 bridges (0.57 bridges/DMPC)	50 ± 50
	61% single, 39% multiple	
DMPC–DMPC	40 pairs (0.55 pairs/DMPC)	730 ± 900*
	55 DMPC (76%)	(7.5% ≥ 3100 ps)

Numbers of water bridges, bridged molecules (%), and the lifetimes of the water bridging are given (Op is a nonester phosphate oxygen atom, Oc is a carbonyl oxygen atom, and Och and Hch are the oxygen and hydrogen atoms of the Chol hydroxyl group, respectively).
*Only lower limit estimate; see text (in parentheses, the percentage of lifetimes longer than the time window used for analysis is given).
†Pasenkiewicz-Gierula et al. (1999).

A water molecule can bridge DMPC to Chol in two ways—either via its two hydrogen atoms (16% of cases) or via one of its hydrogen atoms and the oxygen atom (84% of cases) (Table 2). In the former case the water molecule is H bonded to Chol via Och, in the latter, via Hch. In any case, one of the water hydrogen atoms is H bonded to either Op (42% of cases) or Oc (58% of cases) of the DMPC molecule (Table 2). Fig. 8 *b* shows an example of a water bridge between Oc and Hch.

The average number of Op(Oc)-Chol water bridges is ~0.4/Chol (six per membrane; five of them are single and 0.5 are double), and they link, on average, 5.2 Chol molecules to 5.7 DMPC molecules (Table 3) (two DMPC molecules can be linked to one Chol; cf. Fig. 9). Thus, 33% of all Chol molecules in the membrane are water bridged to 10% of DMPCs (Table 3). The average lifetime of a single Op(Oc)-Chol water bridge is 40 ps, and the average lifetime of a DMPC-CHOL pair bridged by a water molecule is 500 ps (Table 3).

DMPC-Chol charge pairs

In the DMPC-Chol membrane the negatively charged oxygen atom of the Chol hydroxyl group can interact with a positively charged methyl group of the choline moiety (N-CH_3) of PC, to form charge pairs analogous to DMPC-DMPC charge pairs, particularly Oc–N-CH_3 pairs (Pasenkiewicz-Gierula et al., 1999). The RDF of N-CH_3s relative to Och (Fig. 7 *d*) indicates the formation of Och–N-CH_3 charge pairs (the first peak in the RDF corresponds to the Och–N-CH_3 distance of 3.6 ± 0.2 Å). The snapshots of DMPC-Chol links via charge pairs at 3300 ps and 4300 ps are shown in Fig. 9 in red. An example of a DMPC-Chol charge pair is given in Fig. 8 *c*.

On average, there are 0.5 Och–N-CH_3 charge pairs per Chol molecule (8.5 per membrane; four of them are single, the rest are multiple) in the DMPC-Chol membrane (Table 4). Thus, compared with DMPC, Chol forms half as many charge pairs per molecule. The pairs directly link 5.5 Chol molecules to 5.1 DMPC molecules (two DMPC molecules can be linked to one Chol molecule and vice versa; cf. Fig. 9). The Chol molecules that are charge paired to DMPCs constitute 35% of all Chol molecules in the membrane. The average lifetime of a single Och–N-CH_3 charge pair is 550 ps (Table 4). For a DMPC-Chol pair linked by charge pairs, because of the finite analysis time, only the lower limit of the average lifetime can be estimated, and it is equal to 1100 ps (17% of lifetimes are longer than 2000 ps, the time window of the present analysis) (Table 4).

The hydroxyl group of Chol, when fully hydrated, is able to form three H bonds with water (two via the oxygen and one via the hydrogen atom). However, the average number of H bonds made between water and OH-Chol in the DMPC-Chol membrane of 1.1/Chol is less than that (Table 1). This is caused in part by the fact that in the membrane, the Chol hydroxyl groups are located at the border of the membrane hydrophobic region (as O32s) and are not fully hydrated (Fig. 6 *b*), and in part by competition between water molecules and N-CH_3 groups to interact with the oxygen atom of Chol (DMPC-Chol charge pairing). When Och participates in charge pairing with N-CH_3, it makes H bonds with water in only 23% of cases; otherwise, it makes H bonds with water in 40% of cases.

TABLE 4 DMPC–Chol and DMPC–DMPC charge pairs

Charge pair	No./Membrane (%) (No./Molecule)	Lifetime (ps)
DMPC-Chol membrane		
Och—N-CH_3	8.5 pairs (0.53 pairs/Chol)	550 ± 500
	46% single, 54% multiple	
DMPC–Chol	6 pairs (0.38 pairs/Chol)	1100 ± 600*
	5.5 Chol (34%)	(17% ≥ 2000 ps)
	5.1 DMPC (9%)	
Op,Oc–N-CH_3	92 pairs (1.64 pairs/DMPC)	175 ± 250
	25% single, 75% multiple	
Op–N-CH_3	51 pairs (55%)	140 ± 140
Oc–N-CH_3	41 pairs (45%)	220 ± 330
DMPC–DMPC	48 pairs (0.86 pairs/DMPC)	950 ± 750*
	51 DMPC (91%)	(18.5% ≥ 2000 ps)
DMPC–DMPC–Chol	2.6 DMPC (5%)	—
Pure DMPC membrane†		
Op,Oc–N-CH_3	150 pairs (2.08 pairs/DMPC)	—
	24% single, 76% multiple	
Op–N-CH_3	90 pairs (60%)	140 ± 225
Oc–N-CH_3	60 pairs (40%)	174 ± 325
DMPC–DMPC	77 pairs (1.07 pairs/DMPC)	1400 ± 1000*
	67 DMPC (93%)	(18% ≥ 3100 ps)

Numbers of charge pairs, charge paired molecules, and the lifetimes of pairing are given (Op is a nonester phosphate oxygen atom, Oc is a carbonyl oxygen atom, N-CH_3 is a methyl group of the choline moiety, and Och is the oxygen atom of the Chol hydroxyl group).
*Only lower limit estimate; see text (in parentheses, the percentage of lifetimes longer than the time window used for analysis is given).
†Pasenkiewicz-Gierula et al. (1999).

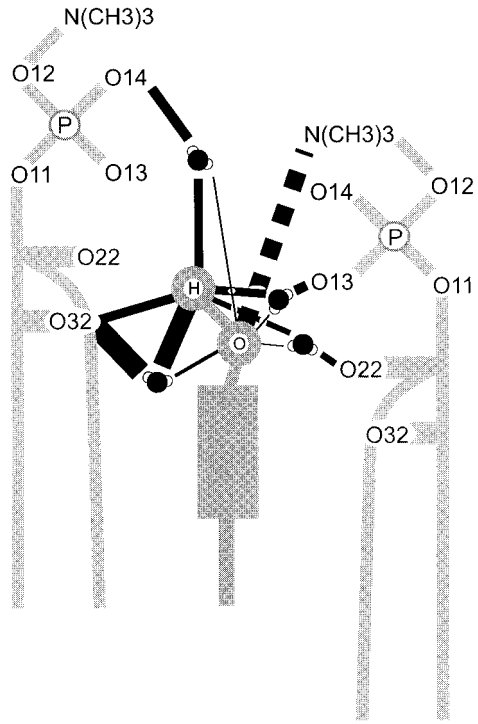

FIGURE 10 Partners with which the hydroxyl group of Chol is linked to a DMPC molecule. A solid line indicates a direct H bond, a dashed line indicates a charge pair, and a water molecule indicates a water bridge. The thickness of the line represents the probability of the link. Light gray indicates DMPC molecules; dark gray indicates a Chol molecule.

DMPC-Chol pairs formed via H bonds and/or charge pairs and/or water bridges

The results obtained so far indicate that in the DMPC-Chol membrane, 16% of Chol molecules are directly H bonded (Table 1), 33% are water bridged (Table 3), and 34% are charge paired to DMPC molecules (Table 4). Partners with which the hydroxyl group of Chol is linked to a DMPC are summarized in Fig. 10. The percentage of Chol molecules that are linked to DMPC molecules in the membrane is not a simple sum of the above numbers because 26% of Chol molecules that are charge paired are simultaneously water bridged (1.5 of 5.5), and ~7% of them are directly H bonded to DMPC (0.4 of 5.5). Chol molecules that are water bridged practically do not simultaneously make direct H bonds with DMPC. Consequently, 70% of Chol molecules are linked to 21% of DMPC molecules in the membrane at any instant (Table 6 and Figs. 9 and 11). Moreover, transient DMPC-Chol associations involve all Chol molecules in the membrane. During the analysis time of 2.0 ns, every Chol molecule has been associated with a DMPC for at least 20% of this time and, on average, for 70% of this time. The lower limit of the average lifetime of DMPC-Chol pairs is 920 ps (Table 7). Snapshots of the pattern of DMPC-Chol links via any of the short-distance interactions at 3300 ps and 4300 ps are shown in Fig. 11 in red.

Chol-Chol interaction

In the initial structure of the DMPC-Chol membrane, the Chol molecules were placed almost uniformly in the *x*, *y* plane (parallel to the membrane surface) and were well separated from one another by DMPC molecules. In the equilibrated membrane, Chol molecules linked via a direct H bond or via a bridging water molecule were found but were rare. In the former case, there were on average 0.01/Chol (0.15/membrane), and, in the latter case, 0.03/DMPC (0.5/membrane) of such interactions at any instant (Tables 1 and 3). Fig. 8 *d* shows an example of two Chol molecules bridged by a water molecule.

DMPC-DMPC interactions in the presence of Chol

Our previous studies of the water/membrane interface in the liquid crystalline DMPC bilayer membrane (Pasenkiewicz-Gierula et al., 1997, 1999) indicate that, on average, each DMPC makes 5.4 H bonds with water, but only 4.5 water molecules are H bonded to each DMPC. Approximately 20% of these water molecules are simultaneously H bonded to oxygen atoms of two DMPC molecules making water bridges. In effect, 76% of all DMPC molecules in the membrane are linked by water into clusters of various sizes.

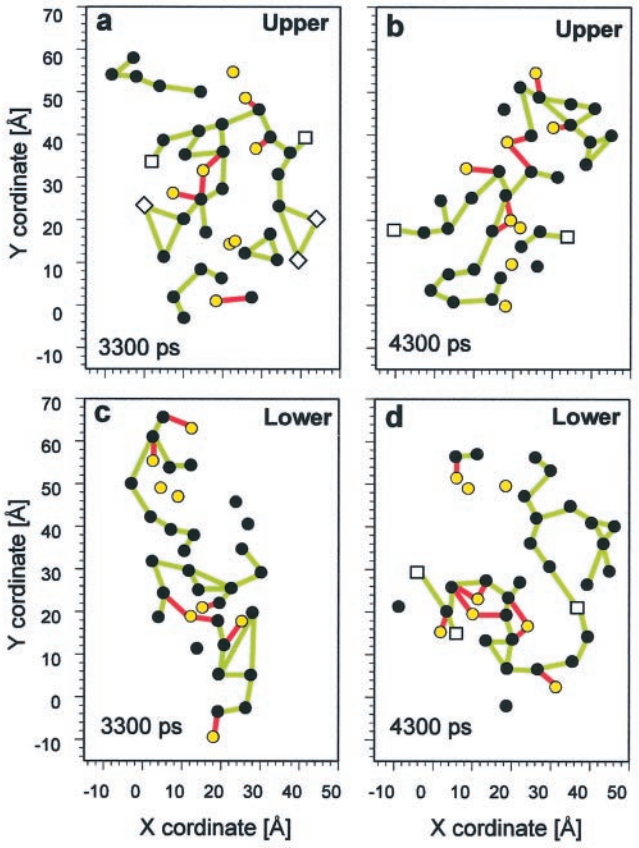

FIGURE 11 DMPC-DMPC cross-links (*light green*) and DMPC-Chol cross-links (*red*) for the upper layer at 3300 (*a*) and 4300 ps (*b*) and for the lower layer at 3300 (*c*) and 4300 ps (*d*). A black dot shows the location of the phosphorus atom of DMPC in the *x, y* plane; a yellow dot shows the location of the oxygen atom of Chol in the *x, y* plane. Pairs of open symbols represent images of "real" PC molecules to show links between DMPCs of adjacent simulation boxes via periodic boundary conditions.

The zwitterionic groups of PCs interact directly via Coulombic interaction to form charge pairs. This interaction involves 93% of DMPC molecules. Both water bridging and charge pairing link, on average, 98% of DMPC molecules, for an average duration of over 1500 ps.

In the present study, we examined the extent to which the interactions between water and DMPC and between DMPC molecules are preserved in the DMPC-Chol membrane. The average number of water molecules H bonded to DMPC was found to increase slightly (7%) with the inclusion of Chol in the DMPC bilayer membrane (Table 5). The increase occurred for all oxygen atoms in DMPC. In particular, a higher access of water (15%) was observed in the region of the carbonyl groups (Table 5). This is consistent with our previous experimental finding (Kusumi et al., 1986), as well as that of Ho et al. (1995). The average numbers of intermolecular Op, Oc-Op, Oc water bridges that link DMPC molecules and DMPC molecules bridged by water, as well as the occurrence of multiple bridges, decreased by ~15% in the presence of 22 mol% Chol

TABLE 5 Numbers of water molecules H bonded to DMPC oxygen atoms (nonester phosphate (Op), carbonyl (Oc), and ester phosphate (Ope)) in DMPC-Chol and pure DMPC membranes (average over 2000 ps)

	Number of H bonded water molecules	
DMPC oxygen atoms	DMPC-Chol Membrane	Pure DMPC Membrane
Op	3.9 ± 0.12	3.7 ± 0.10
Oc	0.92 ± 0.08	0.80 ± 0.07
Ope	0.47 ± 0.07	0.43 ± 0.07
Total	4.8 ± 0.16	4.5 ± 0.12
Total number of H bonds/DMPC	5.7 ± 0.20	5.4 ± 0.14

(Table 3). The lower limit of the average lifetime of DMPC-DMPC links via water bridges in the presence of Chol is 530 ps (Table 3). About 6% of DMPC molecules are simultaneously water bridged to DMPCs and to Chol (Table 3). The average number of intermolecular Op, Oc–N-CH_3 charge pairs linking DMPC molecules decreased by 20% in the presence of 22 mol% Chol (Table 4). However, the fractions of DMPC molecules that are charge paired, as well as the occurrence of multiple charge pairs, are similar in the two membranes (Table 4). The lower limit of the average lifetime of DMPC-DMPC links via charge pairs in the presence of Chol is 950 ps (Table 4). About 5% of DMPC molecules are simultaneously charge paired to DMPCs and to Chol (Table 4).

Taking account of both charge pairs and water bridges, each DMPC molecule makes, on average, 2.2 and 2.7 links with other DMPC molecules in the DMPC-Chol and pure DMPC membranes, respectively (Table 6). The lower limit of the average lifetime of a DMPC-DMPC pair in the DMPC-Chol membrane is 1100 ps (Table 7).

In the DMPC-Chol membrane, ~20% of DMPC molecules are simultaneously linked to DMPC and Chol (Table

TABLE 6 Average numbers of DMPC and Chol molecules as well as DMPC–DMPC and DMPC–Chol associations linked by all types of short-distance interactions in the DMPC-Chol membrane (56 DMPC and 16 Chol molecules) and in the pure DMPC membrane (72 DMPC molecules)

Links	No. (%) of molecules	No. of pairs/ membrane (/lipid)
DMPC-Chol membrane		
DMPC–Chol	11.8 (21%) DMPC	14 (0.3/DMPC)
	11.2 (70%) Chol	14 (0.9/Chol)
DMPC–DMPC	54 (96%)	62 (2.2/DMPC)
DMPC–DMPC–Chol	11.2 (20%) DMPC	13
	10.7 (67%) Chol	13
DMPC–DMPC or DMPC–Chol	54.6 (97.5%) DMPC	69 (2.5/DMPC) (1.9/(DMPC + Chol))
Pure DMPC membrane*		
DMPC–DMPC	71 (98%)	97 (2.7/DMPC)

*Pasenkiewicz-Gierula et al. (1999).

TABLE 7 Times characterizing DMPC–DMPC and DMPC–Chol associations linked by all types of short-distance interactions in the DMPC-Chol membrane (56 DMPC and 16 Chol molecules) and in the pure DMPC membrane (72 DMPC molecules)

Association	Average lifetime (ps)	Firm bonding (ps)	Break time (ps)	No. (frequency) of breaks (1/s)
DMPC-Chol membrane				
DMPC–Chol	920 ± 650* (12.7% ≥ 2000 ps)	7.1	2.4	96 (1.0×10^{11})
DMPC–DMPC	1100 ± 800* (26.5% ≥ 2000 ps)	11.7	3.1	72 (0.6×10^{11})
Pure DMPC membrane†				
DMPC–DMPC	1500 ± 1160* (24% ≥ 3100 ps)	14.1	2.6	89 (0.6×10^{11})

*Only lower limit estimate; see text (in parentheses, the percentage of lifetimes longer than the time window used for analysis is given).
†Pasenkiewicz-Gierula et al. (1999).

6), and 1% are linked solely to Chol (isolated DMPC-Chol pairs; on average, there is less than one such pair in the membrane). Thus 97.5% of DMPC molecules are linked to either DMPC or Chol. When both DMPC-DMPC and DMPC-Chol links are included, then the average number of links formed by each DMPC molecule is 2.5/DMPC. Because Chol forms far fewer links, the overall average of intermolecular links/lipid decreases to 1.9 in the DMPC membrane containing 22 mol% Chol.

Fig. 11 is a snapshot of interlipid links via short-distance interactions for the upper and lower leaflets of the bilayer at 3300 ps and 4300 ps. The pattern of DMPC-Chol links via direct H bonds, water bridges, and/or charge pairs is shown in red (Chol is represented by *yellow dots* and DMPC by *black dots*). The pattern of DMPC-DMPC links via water bridges and/or charge pairs is shown in green. On average, a Chol molecule is linked with 0.75 DMPC molecules, 0.15 by direct H bonds, 0.33 by water bridges, and 0.34 by charge pairs (some links are via both water bridges and charge pairs), and with 0.04 Chol molecules, 0.01 via direct H bonds and 0.03 CHOL via water bridges.

Comparison of the network of DMPC-DMPC links in the DMPC-Chol membrane (*green lines* in Fig. 11) with that in the pure DMPC membrane (figure 10 of Pasenkiewicz-Gierula et al., 1999) shows that in both membranes almost all DMPC molecules are linked with one another; however, the links in the presence of Chol are less branched.

DISCUSSION

A computer model of the hydrated DMPC membrane containing 22 mol% Chol was found to be stable on the nanosecond time scale and to reproduce several experimental results well. A 2.0-ns MD trajectory of the well-equilibrated membrane was analyzed to determine the effects of Chol on the membrane/water interfacial region. This region is not easily accessible to experimental studies. The present study addressed three problems: 1) atomic-level interactions between polar groups of DMPC and Chol and between Chol and water, 2) modifications of DMPC-water and DMPC-DMPC interactions in the presence of Chol, and 3) network of interlipid links involving both DMPC and Chol molecules (DMPC-DMPC-Chol links).

Direct H bonds between PC and Chol molecules in the PC-Chol membrane have been a controversial issue over the years. It has been proposed that Chol makes H bonds with the carbonyl oxygen atoms of PC, but no conclusive experimental data have been published (for a review, see McMullen and McElhaney, 1996). In the present work, direct H bonding was found to be infrequent: of 0.75 DMPC molecules linked to a Chol molecule, only 0.16 were linked via a H bond. Direct H bonding requires a certain relative orientation and distance between OH-Chol and Op or Oc. The rigid structure of Chol, the large size of DMPC, and their dynamics probably preclude a close approach of the Chol hydroxyl group to the DMPC headgroup in the membrane over a time period long enough to form a stable H bond.

The hydrogen atom of the Chol hydroxyl group participates in Chol-water interactions more often than the oxygen atom—over two times more often in the case of simple H bonding and over five times more often in the case of water bridging. The lifetime of water···Och is two to three times shorter than that of water···Hch. The difference in the occurrence and stability of the two types of H bonds with water might result, in addition to other factors (Murari et al., 1986), from a competition between a water molecule and a choline group to interact with Och. When Och makes a charge pair with N-CH_3, the number of H bonds between Och and water is two times smaller than otherwise. A similar competition between a water molecule and N-CH_3 was observed in the pure DMPC membranes (results to be published). Formation of charge pairs between DMPC and Chol observed in this study is consistent with the inward orientation of the PC choline group observed by Tu et al. (1998).

In the simulated DMPC-Chol membrane, the number of H bonded water molecules to the PC headgroups is 7% higher than that in the pure DMPC membrane (Table 5). This likely results from larger DMPC-DMPC spacing (Yeagle et al., 1977; Ho et al., 1995; Hyslop et al., 1990). Furthermore, each Chol molecule is H bonded to 1.1 water molecules, of which 0.5 participate in DMPC-Chol bridging. As OH-Chols are located in the region of DMPC carbonyl groups, increased H bonding is observed, particularly in this region of the DMPC-Chol membrane. These findings are in accord with experimental results (Kusumi et al., 1986; Ho et al., 1995).

As can be seen in Fig. 11, Chol is often an "end" molecule in the lipid-lipid network in the DMPC-Chol mem-

brane. In this membrane, 70% of Chol molecules are linked via short-distance interactions to DMPC at any instant (Table 6). On average, each of these 70% of molecules makes 1.3 links with DMPC, while an average Chol molecule makes 0.9 such links. In effect, the all-membrane average number of interlipid links is 1.9/lipid (either DMPC or Chol) (0.9/Chol) (Table 6). However, this does not destroy the extended network of lipid-lipid links in the interfacial region observed in the pure DMPC membrane (Pasenkiewicz-Gierula et al., 1999), where the average number of interlipid links is 2.7/lipid (Table 6). Both formation of an extended network of interlipid links in the DMPC-Chol membrane and its less branched nature than in the pure DMPC membrane agree with experimental data (Slater et al., 1993).

In the present research, we chose ~22 mol% for the Chol concentration. At ~20 mol% Chol, even at temperatures below the phase transition temperature of the DMPC membrane, DMPC and Chol are mixed well, as seen in the phase diagram of the binary mixtures of DMPC and Chol (Recktenwald and McConnell, 1981). Therefore, it is an excellent Chol concentration for the studies of the basic features of DMPC-Chol interaction. When the Chol content in the membrane is increased, it is expected that the DMPC-DMPC distance will increase, which in turn decreases the number of DMPC-DMPC links via water bridges and charge pairs, and that DMPC-Chol and Chol-Chol links will become more frequent.

CONCLUSIONS

1. A computer model of the fully hydrated liquid-crystalline DMPC-Chol bilayer membrane containing 22 mol% Chol that is stable on the nanosecond time scale and reproduces several properties of an experimental model was constructed. The bilayer reached thermal equilibrium after 2.3 ns of MD simulation.

2. At the membrane/water interface, the hydroxyl group of Chol interacts with oxygen atoms of PC via direct H bonds (16% of Chol molecules) and water bridges (33% of Chol molecules) and with the choline moiety via charge pairs (34% of Chol molecules). At any instant, 70% of Chol molecules are linked to 21% of DMPC molecules for an average time of ~900 ps. On average, there are 0.9 DMPC-Chol links per Chol.

3. On average, each Chol molecule forms H bonds with 1.1 water molecules; 0.5 of them are involved in DMPC-Chol bridging. In 70% of cases, the H bonds are made via the hydrogen atom of the Chol hydroxyl group (water···Hch H bonds).

4. Both direct H bonds and water bridges between DMPC and Chol are formed predominantly via the γ-chain carbonyl oxygen atom of DMPC; however, water bridges are over twice as frequent as H bonds.

5. The average number of water molecules H bonded to the DMPC headgroup is 7% higher in the DMPC-Chol bilayer than that in the pure DMPC bilayer.

6. In the interfacial region of the DMPC-Chol bilayer, an average DMPC molecule forms 2.2 and 0.3 links with DMPC and Chol molecules, respectively (2.5 links/DMPC).

7. An average lipid (DMPC or Chol) molecule makes 1.9 links with other lipids in the membrane (1.9 links/lipid). At any instant, 97.5% of DMPC and 70% of Chol molecules are involved in these links.

This work was supported in part by a grant from the Polish Science Foundation (BIMOL 103/93), grant 6P04A05715 from the Polish Committee for Scientific Research, and by grants-in-aid from the Japanese Ministry of Education.

REFERENCES

Berendsen, H. J. C., J. P. M. Postma, W. F. van Gunsteren, A. DiNola, and J. R. Haak. 1984. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* 81:3684–3690.

Bicknell-Brown, E., and K. G. Brown. 1980. Raman studies of lipid interactions at the bilayer interface: phosphatidylcholine-cholesterol. *Biochem. Biophys. Res. Commun.* 94:638–645.

Biosym/MSI. 1995. Discover User Guide. Biosym/MSI, San Diego.

Bittman, R., S. Clejan, S. Lund-Katz, and M. C. Phillips. 1984. Influence of cholesterol on bilayers of ester- and ether-linked phospholipids: permeability and ^{13}C-nuclear magnetic resonance measurements. *Biochim. Biophys. Acta.* 772:117–126.

Bloom, M., E. Evans, and O. G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Q. Rev. Biophys.* 24:293–397.

Bloom, M., and O. G. Mouritsen. 1995. The evolution of membranes. *In* Structure and Dynamics of Membranes. R. Lipowsky and E. Sackmann, editors. Elsevier, Amsterdam. 65–95.

Bush, S. F., H. Levin, and I. W. Levin. 1980. Cholesterol-lipid interactions: an infrared and Raman spectroscopic study of the carbonyl stretching mode region of 1,2-dipalmitoyl phosphatidylcholine bilayers. *Chem. Phys. Lipids.* 27:101–111.

Charifson, P. S., R. G. Hiskey, and L. G. Pedersen. 1990. Construction and molecular modeling of phospholipid surfaces. *J. Comp. Chem.* 11: 1181–1186.

Cheetham, J., E. Wachtel, D. Bach, and R. M. Epand. 1989. Role of the stereochemistry of the hydroxyl group of cholesterol and the formation of nonbilayer structures in phosphatidylethanolamines. *Biochemistry.* 28:8928–8934.

Davis, P. J., and K. M. W. Keough. 1983. Differential scanning calorimetric studies of aqueous dispersions of mixtures of cholesterol with some mixed-acid and single acid phosphatidylcholine. *Biochemistry.* 22: 6334–6340.

Davis, P. J., and K. M. W. Keough. 1984. Scanning calorimetric studies of aqueous dispersions of bilayers made with cholesterol and pair of positional isomers of 3-sn-phosphatidylcholine. *Biochim. Biophys. Acta.* 778:305–310.

de Kruijff, B. 1978. ^{13}C NMR studies on [4-^{13}C]cholesterol incorporated in sonicated phosphatidylcholine vesicles. *Biochim. Biophys. Acta.* 506: 173–182.

de Kruyff, B., R. A. Demel, A. J. Slotboom, L. L. M. van Deenen, and A. F. Rosenthal. 1973. The effect of the polar headgroup in the lipid-cholesterol interaction: a monolayer and differential scanning calorimetry study. *Biochim. Biophys. Acta.* 307:1–19.

Demel, R. A., K. R. Bruckdorfer, and L. L. M. van Deenen. 1972. Structural requirements of sterols for the interaction with lecithin at the air-water interface. *Biochim. Biophys. Acta* 255:311–320.

Edholm, O., and A. M. Nyberg. 1992. Cholesterol in model membrane. *Biophys. J.* 63:1081–1089.

Egberts, E., S-J. Marrik, and H. J. C. Berendsen. 1994. Molecular dynamics simulation of a phospholipid membrane. *Eur. Biophys. J.* 22: 432–436.

El-Sayed, M. Y., T. A. Guion, and M. D. Fayer. 1986. Effect of cholesterol on viscoelastic properties of dipalmitoylphosphatidylcholine multibilayers as measured by a laser-induced ultrasonic probe. *Biochemistry.* 25:4825–4832.

Ho, C., S. J. Slater, and C. D. Stubbs. 1995. Hydration and order in lipid bilayer. *Biochemistry.* 34:6188–6195.

Hyslop, P. A., B. Morel, and R. D. Sauerheber. 1990. Organization and interaction of cholesterol and phosphatidylcholine in model bilayer membrane. *Biochemistry.* 29:1025–1038.

Ipsen, J. H., G. Karlström, O. G. Mouritsen, H. Wennerström, and M. J. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta.* 905:162–172.

Jorgensen, W. L., J. Chandrasekhar, J. D. Madura, R. Impey, and M. L. Klein. 1983. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 79:926–935.

Jorgensen, W. L., and J. Tirado-Rives. 1988. The OPLS potential functions for proteins: energy minimizations for crystals of cyclic peptides and crambin. *J. Am. Chem. Soc.* 110:1657–1666.

Karolis, Ch., H. G. L. Coster, T. C. Chilcott, and K. D. Barrow. 1998. Differential effects of cholesterol and oxidised-cholesterol in egg lecithin bilayers. *Biochim. Biophys. Acta.* 1368:247–255.

Keough, K. M. W., B. Giffin, and P. L. J. Matthews. 1989. Phosphatidylcholine-cholesterol interactions: bilayers of heteroacid lipids containing linoleate lose calorimetric transitions at low cholesterol concentration. *Biochim. Biophys. Acta.* 983:51–55.

Kraulis, P. 1991. MolScript: a program to produce both detailed and schemate plots of proteins. *J. Appl. Crystallogr.* 24:946–950.

Kusumi, A., and M. Pasenkiewicz-Gierula. 1988. Rotational diffusion of a steroid molecule in phosphatidylcholine membranes: effects of alkyl chain length, unsaturation, and cholesterol as studied by a spin-label method. *Biochemistry.* 27:4407–4415.

Kusumi, A., W. K. Subczynski, M. Pasenkiewicz-Gierula, J. S. Hyde, and H. Merkle. 1986. Spin-label studies of phosphatidylcholine-cholesterol membranes: effects of alkyl chain length and unsaturation in the fluid phase. *Biochim. Biophys. Acta.* 854:307–317.

Kusumi, A., M. Tsuda, T. Akino, S. Ohnishi, and Y. Terayama. 1983. Protein-phospholipid-cholesterol interaction in the photolysis of invertebrate rhodopsin. *Biochemistry.* 22:1165–1170.

McIntosh, T. S. 1978. The effect of cholesterol on the structure of phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 513:43–58.

McMullen, T. P. W., and R. N. McElhaney. 1996. Physical studies of cholesterol-phospholipid interactions. *Curr. Opin. Colloid Interface Sci.* 1:83–90.

Merritt, E. A., and D. J. Bacon. 1997. Raster3D: photorealistic molecular graphics. *Methods. Enzymol.* 277:505–524.

Mouritsen, O. G., and K. Jørgensen. 1994. Dynamical order and disorder in lipid bilayers. *Chem. Phys. Lipids.* 73:2–35.

Murari, R., M. P. Murari, and W. J. Baumann. 1986. Sterol orientations in phosphatidylcholine liposomes as determined by deuterium NMR. *Biochemistry.* 25:1062–1067.

Pasenkiewicz-Gierula, M., W. K. Subczynski, and A. Kusumi. 1990. Rotational diffusion of a steroid molecule in phosphatidylcholine-cholesterol membranes: fluid phase micro-immiscibility in unsaturated phosphatidyl-choline-cholesterol membranes. *Biochemistry.* 29: 4059–4069.

Pasenkiewicz-Gierula, M., W. K. Subczynski, and A. Kusumi. 1991. Influence of phospholipid unsaturation on the cholesterol distribution in membranes. *Biochimie.* 73:1311–1316.

Pasenkiewicz-Gierula, M., Y. Takaoka, H. Miyagawa, K. Kitamura, and A. Kusumi. 1997. Hydrogen bonding of water to phosphatidylcholine in the membrane as studied by a molecular dynamics simulation: location, geometry, and lipid-lipid bridging via hydrogen-bonded water. *J. Phys. Chem.* 101:3677–3691.

Pasenkiewicz-Gierula, M., Y. Takaoka, H. Miyagawa, K. Kitamura, and A. Kusumi. 1999. Charge pairing of headgroups in phosphatidylcholine membranes. A molecular dynamics simulation study. *Biophys. J.* 76: 1228–1240.

Pearlman, D. A., D. A. Case, J. C. Caldwell, G. L. Seibel, U. C. Singh, P. K. Weiner, and P. A. Kollman. 1991. AMBER 4.0. University of California, San Francisco.

Recktenwald, D. J., and H. M. McConnell. 1981. Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol. *Biochemistry.* 20: 4505–4510.

Robinson, A. J., W. G. Richards, P. J. Thomas, and M. M. Hann. 1995. Behavior of cholesterol and its effect on head group and chain conformations in lipid bilayers: a molecular dynamics study. *Biophys. J.* 68:164–170.

Ryckaert, J. P., G. Cicotti, and H. J. C. Berendsen. 1977. Numerical integration of the Cartesian equations of motion of a system with constraints: molecular dynamics of *n*-alkanes. *J. Comp. Phys.* 22: 327–341.

Sackmann, E. 1995. Biological membranes architecture and function. *In* Structure and Dynamics of Membranes. R. Lipowsky and E. Sackmann, editors. Elsevier, Amsterdam. 1–64.

Sankaram, M. B., and T. E. Thompson. 1990. Modulation of phospholipid acyl chain order by cholesterol. A solid state ^{2}H nuclear magnetic resonance study. *Biochemistry*. 29:10676–10684.

Scott, H. L. 1991. Lipid-cholesterol interactions. Monte Carlo simulation and theory. *Biophys. J.* 59:445–455.

Scott, H. L., and S. Kalaskar. 1989. Lipid chains and cholesterol in model membranes: a Monte Carlo study. *Biochemistry.* 28:3687–3691.

Scott, H. L., and W. S. McCullough. 1993. Lipid-cholesterol interactions in the $P_{\beta'}$ phase: application of statistical mechanical model. *Biophys. J.* 64:1398–1404.

Shieh, H-S., L. G. Hoard, and C. E. Nordman. 1981. The structure of cholesterol. *Acta Crystallogr*. B37:1538–1543.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* 387:569–572.

Slater, S. J., C. Ho, F. J. Taddeo, M. B. Kelly, and C. D. Stubbs. 1993. Contribution of hydrogen bonding to lipid-lipid interactions in membranes and the role of lipid order: effects of cholesterol, increased phospholipid unsaturation, and ethanol. *Biochemistry.* 32:3714–3721.

Smaby, J. M., H. L. Brockman, and R. E. Brown. 1994. Cholesterol's interfacial interactions with sphingomyelins and phosphatidylcholines: hydrocarbon chain structure determines the magnitude of condensation. *Biochemistry.* 33:9135–9142.

Smaby, J. M., M. M. Momsen, H. L. Brockman, and R. E. Brown. 1997. Phosphatidylcholine acyl unsaturation modulates the decrease in the interfacial elasticity induced by cholesterol. *Biohys. J.* 73:1492–1505.

Subczynski, W. K., W. E. Antholine, J. S. Hyde, and A Kusumi. 1990. Microimmiscibility and three-dimensional dynamic structures of phosphatidylcholine-cholesterol membranes: translational diffusion of a copper complex in the membrane. *Biochemistry.* 29:7936–7945.

Subczynski, W. K., J. S. Hyde, and A Kusumi. 1989. Oxygen permeability of phosphatidylcholine-cholesterol membranes. *Proc. Natl. Acad. Sci. USA.* 86:4474–4478.

Subczynski, W. K., A. Wisniewska, J-J. Yin, J. S. Hyde, and A. Kusumi. 1994. Hydrophobic barriers of lipid bilayer membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol. *Biochemistry.* 33:7670–7681.

Tu, K., M. L. Klein, and D. J. Tobias. 1998. Constant-pressure molecular dynamics investigation of cholesterol effects in a dipalmitoylphosphatidylcholine bilayer. *Biophys. J.* 75:2147–2156.

Urbina, J. A., B. Moreno, W. Arnold, C. H. Taron, P. Orlean, and E. Oldfield. 1998. A carbon-13 nuclear magnetic resonance spectroscopic study of inter-proton order parameter: a new approach to study order and dynamics in phospholipid membrane system. *Biophys. J.* 75:1372–1383.

Urbina, J. A., S. Pekerar, H-b. Le, J. Patterson, B. Montez, and E. Oldfield. 1995. Molecular order and dynamics of phosphatidylcholine bilayer membrane in the presence of cholesterol, ergosterol and lanosterol: a comparative study using ^{2}H-, ^{13}C- and ^{31}P-NMR spectroscopy. *Biochim. Biophys. Acta.* 1238:163–176.

Vanderkooi, G. 1994. Computation of mixed phosphatidylcholine-cholesterol bilayer structures by energy minimization. *Biophys. J.* 66: 1457–1468.

Yeagle, P. L., W. C. Hutton, G. Huang, and R. B. Martin. 1977. Phospholipid head-group conformations; intermolecular interactions and cholesterol effects. *Biochemistry.* 16:4344–4349.

Yeagle, P. L., and R. B. Martin. 1976. Hydrogen-bonding of the ester carbonyls in phosphatidylcholine bilayers. *Biochem. Biophys. Res. Commun.* 69:775–780.

Zhang, Y., S. E. Feller, B. R. Brooks, and R. W. Pastor. 1995. Computer simulation of liquid/liquid interfaces. I. Theory and application to octane/water. *J. Chem. Phys.* 103:10252–10266.