Charge Pairing of Headgroups in Phosphatidylcholine Membranes: A Molecular Dynamics Simulation Study

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ABSTRACT   Molecular dynamics simulation of the hydrated dimyristoylphosphatidylcholine (DMPC) bilayer membrane in the liquid-crystalline phase was carried out for 5 ns to study the interaction among DMPC headgroups in the membrane/water interface region. The phosphatidylcholine headgroup contains a positively charged choline group and negatively charged phosphate and carbonyl groups, although it is a neutral molecule as a whole. Our previous study (Pasenkiewicz-Gierula, M., Y. Takaoka, H. Miyagawa, K. Kitamura, and A. Kusumi. 1997. *J. Phys. Chem.* 101:3677–3691) showed the formation of water cross-bridges between negatively charged groups in which a water molecule is simultaneously hydrogen bonded to two DMPC molecules. Water bridges link 76% of DMPC molecules in the membrane. In the present study we show that relatively stable charge associations (charge pairs) are formed between the positively and negatively charged groups of two DMPC molecules. Charge pairs link 93% of DMPC molecules in the membrane. Water bridges and charge pairs together form an extended network of interactions among DMPC headgroups linking 98% of all membrane phospholipids. The average lifetimes of DMPC–DMPC associations via charge pairs, water bridges and both, are at least 730, 1400, and over 1500 ps, respectively. However, these associations are dynamic states and they break and re-form several times during their lifetime.

INTRODUCTION

Phosphatidylcholine (PC) is a most prevalent phospholipid among those that make up eukaryotic cell membranes. It is also a most extensively studied phospholipid in terms of the behavior in model membranes, the dynamics and interaction of alkyl chains in particular. However, those of the PC headgroups in the polar regions of the membrane have been studied much less despite their importance in determining the membrane structure and interactions with molecules in the aqueous phase.

A PC molecule contains groups that are positively and negatively charged, whereas its net electrostatic charge is zero. Representative negatively charged groups of PC include the non-ester phosphate oxygen atoms (Ops) and the carbonyl oxygen atoms in the ester linkages between glycerol and acyl chains (Ocs). The positively charged group is the choline moiety.

Two major classes of short-distance interactions occur between the PC headgroups. One is the formation of water cross-bridges between negatively charged groups in which a water molecule is simultaneously hydrogen (H) bonded to two PC molecules. In our recent molecular dynamics (MD) simulation study of a PC bilayer membrane (Pasenkiewicz-Gierula et al., 1997), we found that ~70% of PC molecules are cross-linked by H bonded water into clusters of two to seven molecules. Of 4.5 water molecules H bonded to each

dimyristoylphosphatidylcholine (DMPC) molecule, approximately one, on average, simultaneously forms H bonds with two oxygen atoms, mainly of the phosphate and carbonyl groups, of different DMPC molecules and forms an intermolecular bridge. This result is consistent with water-mediated interactions between PC oxygen atoms deduced from experimental results by Nagle (1976), Büldt and Wohlgemuth (1981), Slater et al. (1993), and Ho et al. (1995). Such interactions were postulated by Prats et al. (1987), Sakurai and Kawamura (1987), and Teissie et al. (1990) to explain 20-fold faster lateral transfer of protons along the membrane surface than in water.

The second class of short-distance interactions that are likely to take place in the headgroup region in PC membranes involves charge associations (charge pairs) formed when the oppositely charged groups are located within 4.0 Å from one another, in analogy to salt bridges (links) in proteins (Creighton, 1983; Lounnas and Wade, 1997).

In the present study, we mainly addressed this issue. In particular, we pursued further cluster formation of PC molecules caused by charge associations in the DMPC bilayer using MD simulations.

First, we investigated the possibility of formation of charge pairs between a choline methyl group (N-CH$_3$) of one DMPC and non-ester phosphate or carbonyl oxygen atoms (Ops and Ocs, respectively) of another one. In this sense, the present research was an additional development of our previous investigation of the formation of PC clusters based on water cross-bridges. We found that, on average, 42% Ops and 28% Ocs make such charge pairs that link more than 90% of all PC molecules in the membrane.

Experimental studies of Yeagle et al. (1975, 1976, 1977) suggested the formation of intermolecular charge pairs be-

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tween the phosphate and the choline groups of neighboring phospholipid molecules in the liquid-crystalline bilayers. Although phosphate–choline charge pairs are also present in the DMPC single crystal, they are exclusively intramolecular (Hauser et al., 1981). MD simulation of negatively charged dipalmitoylphosphatidylserine (DPPS) bilayer membrane in the liquid-crystalline state showed charge interaction between the ammonium group of one DPPS molecule and oxygen atoms of adjacent DPPS molecules (Lopez Cascales et al., 1996).

In addition to identifying the short-distance PC–PC interactions in the membrane, we examined the lifetimes of the water bridges as well as of the choline–phosphate and the choline–carbonyl charge pairs. The average lifetime of water bridging of two DMPC oxygen atoms is about 50 ps. The average lifetime of a DMPC–DMPC pair linked by water molecules is $\sim$730 ps, indicating a fast exchange of H bonds and water bridges, as postulated by Prats et al. (1987) and Teissie et al. (1990). The average lifetimes of Op–N-CH$_3$ and Oc–N-CH$_3$ charge pairs are 140 and 174 ps, respectively. For a DMPC–DMPC pair linked by charge pairs, the average lifetime is 1416 ps. This indicates the presence of multiple charge pairs between DMPC molecules in most cases.

DMPC–DMPC associations linked by either or both of short-distance interactions, show higher stability. The lower limit of their average lifetime is 1500 ps, 24% of the association live longer than 3100 ps, the time window of the present analysis.

In this study, we show that 98% of DMPC molecules in the liquid-crystalline bilayer are linked via water bridges and/or choline–phosphate and/or choline–carbonyl charge pairs to form long-lived clusters. This result suggests that headgroup-headgroup interactions greatly contribute to the stability of the membrane structure.

METHODS

Simulation system

The membrane consisting of 72 (6 $\times$ 6 $\times$ 2) DMPC molecules was hydrated with 1622 water molecules and simulated for 5100 ps using AMBER 4.0 (Pearlman et al., 1991). There are $\sim$23 water molecules/ DMPC ($\sim$38% by weight), which is thought to be sufficient to fully hydrate the membrane (Gawrisch et al., 1978; Arnold et al., 1983; Nagle, 1993; Salsbury et al., 1972; Rand and Parsegian, 1989). The starting configuration was the minimized structure of Vanderkooi (1991) in the second arrangement. The system was equilibrated for 1100 ps. Details concerning the membrane construction and equilibration have been described in Pasenkiewicz-Gierula et al. (1997). Fig. 1 shows the structure and numbering of atoms in the DMPC molecule.

Simulation parameters

For DMPC, optimized potentials for liquid simulations (OPLS) parameters (Jorgensen and Tirado-Rives, 1988) were used. In the original OPLS set, the stretching, bending, and torsion parameters for the ester group were missing. These parameters were transferred from the MM3 set (Alinger et al., 1989) with the help of two calibration curves for the stretching and
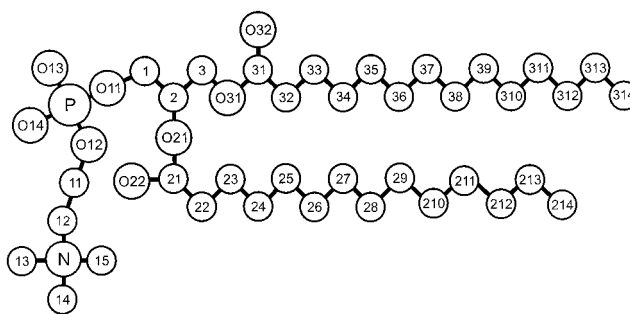

FIGURE 1 Molecular structure of DMPC with numbering of atoms (chemical symbol for carbon atoms, C, is omitted).

bending force constants. The bond angles and force constants for the choline group were set in analogy to OPLS parameters for lysine. The van der Waals parameters for the phosphorus atom were calculated based on the AMBER parameters and then re-adjusted to satisfy the proportion (O/N$\approx$S/P) between the van der Waals parameters for the oxygen (O), nitrogen (N), sulfur (S), and phosphorus (P) atoms. For water, TIP3P parameters (Jorgensen et al., 1983) were used. The united atom approximation was applied to the DMPC molecule to reduce computation time. The atomic charges of the DMPC molecule used in this simulation were taken from Charifson et al. (1990) except for the first 800 ps of the simulation. During the initial 800 ps, the charges used were those calculated in Pasenkiewicz-Gierula et al. (1997) in which the choline and carbonyl charges were similar to those from Charifson et al. (1990), whereas the charges for the phosphate group atoms were 10–20% lower. The dipole moment of the DMPC headgroup calculated for the charge distribution from Charifson et al. (1990) was 20.4 $\pm$ 2.8 Debye and was lower than that calculated for the charge distribution from Pasenkiewicz-Gierula et al. (1997) of 26.6 $\pm$ 3.3 Debye (both averaged over 500 ps between 1100 and 1600 ps). The value for the dipole moment of the DMPC headgroup given in the literature is $\sim$20 Debye (Shepherd and Büldt, 1978; Scott and Lee, 1980; Büldt and Wohlgemuth, 1981; Frischleder and Peinel, 1982; Bowen and Lewis, 1983; Taylor et al., 1990). For this reason, MD simulation from 800 ps onward was carried out using atomic charges of Charifson et al. (1990).

Simulation conditions

Three-dimensional periodic boundary conditions using the usual minimum image convention were used. The SHAKE algorithm (Ryckaert et al., 1977) was used to preserve the bond lengths of the water molecule, 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 calculation time of nonbonded interactions, each DMPC was divided into six residues. Each residue was chosen in such a way that the total electrostatic charge on the residue was close to zero and the integrity of its chemical groups was preserved (Pasenkiewicz-Gierula et al., 1997). The list of nonbonded pairs was updated every 50 steps.

Simulation was carried out at a constant pressure (1 atm) and a constant temperature (310 K = 37°C), which is above the main phase transition temperature for the DMPC bilayer ($\sim$23°C). Temperatures of the solute and solvent were controlled independently. Both the temperatures 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 system and comparison with experimental data

Fig. 2 shows the time profiles of the simulation box dimensions (Fig. 2 *a*), surface area/DMPC (Fig. 2 *b*), number of *gauche* conformations/myristoyl chain (Fig. 2 *c*), and surface tension (Fig. 2 *d*), from the onset of simulation until 5100 ps. Initial changes in the system dimensions (Fig. 2 *a*) are caused by rescaling, temporary changes of the temperature, and addition of another 600 water molecules (cf. Pasenkiewicz-Gierula et al., 1997). At the beginning of the equilibration process, the temperature of the system was raised to 550 K for 20 ps, which was effective in breaking the initial crystalline structure. The temperature was then slowly lowered to 310 K and kept constant. The potential energy of the system became stable after 500 ps of the

simulation time. Approach toward a thermally equilibrated DMPC bilayer in the liquid-crystalline phase has been described in detail (Pasenkiewicz-Gierula et al., 1997). In that paper we concluded that the membrane had reached thermal equilibrium after 1100 ps of MD simulation.

Between 1100 and 5100 ps of MD simulation, the average surface area per DMPC of 60.2 ± 1.0 Å^2, the average number of *gauche* rotamers/myristoyl chain of 2.8 ± 0.1, and the nearly zero average tilt angle of hydrocarbon chains with respect to the bilayer normal were obtained, which is consistent with experimental observations (Nagle et al., 1996; Nagle, 1993; Casal and McElhaney, 1990; Moser et al., 1989; Meier et al., 1982), theoretical calculations (Carlson and Sethna, 1987), and other simulations (Tu et al., 1995). The electron density profile of the bilayer and the profile of the order parameter are in agreement with experimental data (Levine and Wilkins, 1971; Hubbell and McConnell, 1971).

The surface tension in the simulation box was monitored from ~800 ps of the simulation time. After equilibration, the average surface tension (Zhang et al., 1995) is -0.6 ± 123 dyn/cm (Fig. 2 *d*). Its nearly zero value is in good agreement with theoretical predictions of Jähnig (1996) and MD simulation of Tu et al. (1996). Large fluctuations of this parameter are caused by large fluctuations in the system pressure.

Fig. 3 *a* shows the distribution of the orientation of the P-N vector with respect to the membrane normal (the *z* axis). The distribution of the inclination angle is broad and almost uniform in the range of angles between 0 and 100°.
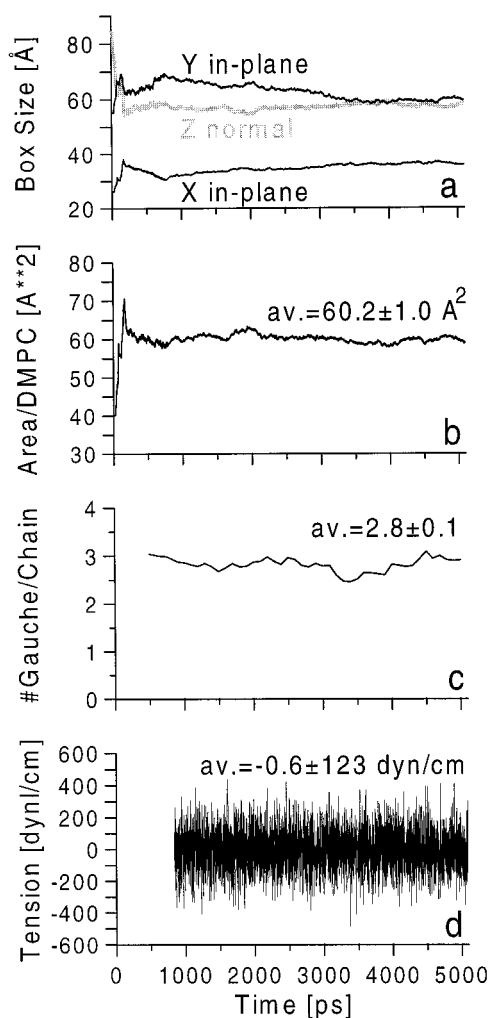
FIGURE 2   Diagrams showing the time developments of the (*a*) simulation box dimensions (along the in-plane *x*- and *y*-axes, and the bilayer normal, *z*-axis), (*b*) surface area per DMPC, the average surface area is 60.2 ± 1.0 Å, (*c*) number of *gauche* rotamers per chain, the average number is 2.8 ± 0.1, and (*d*) surface tension, the average tension is -0.6 ± 123 dyn/cm.
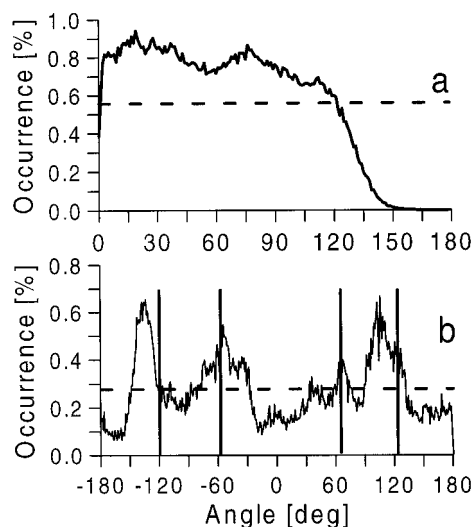
FIGURE 3   (*a*) Normalized distribution of the orientation of the phosphorus-nitrogen (P-N) vector with the bilayer normal (*solid line*). The distribution of the orientation was divided by the *sin* function to compare with the uniform distribution of angles in the range of 1–180° (*dash line*). (*b*) Normalized distribution of the orientation of the P-N vector with the in-plane *x*-axis: in the single crystal, vertical lines (for DMPC A and B in the upper and lower leaflet), and in the liquid-crystalline bilayer at 5100 ps of the simulation time-continuous line; the dash line indicates the uniform distribution of the orientation.

Orientations and conformations of the DMPC headgroups in the liquid-crystalline bilayer significantly differ from those in the initial structure. Fig. 3 *b* shows the distribution of angles between the P-N vector and the in-plane *x* axis in the initial structure and in the liquid-crystalline bilayer at 5100 ps (calculated for last 300 ps, i.e., between 4800 and 5100 ps). In the former case, the vector has only four orientations (−57°, 64°, 124°, and −119° for DMPC A and B in the upper and lower leaflet, respectively). In the latter case, the angles span the whole range from −180° to 180°.

These results suggest that the simulated membrane obtained here is well equilibrated, stable over a long period of time, and reproduces various properties of PC bilayers in the liquid-crystalline phase that have been observed experimentally. Therefore, it is concluded that this membrane provides a good model for DMPC membranes, and that by analyzing it at the level of individual atoms, we can obtain experimentally inaccessible information about structure and dynamics of PC membranes.

In the present report, in analyzing trajectories of 4.0 ns MD simulation after equilibration, we concentrate on formation of charge pairs between positively charged choline methyl groups and negatively charged oxygen atoms of the phosphate or carbonyl groups in the membrane, which along with bridging by hydrogen bonded water molecules leads to formation of extended DMPC clusters. We also estimate the lifetimes for water bridging and charge pairing.

DMPC–DMPC association via charge pairs and water bridges

Interactions between Op and N-CH$_3$

The radial distribution function (RDF) of the phosphorus atoms relative to a nitrogen atom, g_{P-N}, (intramolecular distances are not included) has a distinct maximum at 4.8 Å (Fig. 4 *a*). The presence of the maximum indicates that in the membrane the choline and the phosphate groups stay in a close, preferred distance from one another. For comparison, the RDFs of Ps relative to P and Ns relative to N are also shown in Fig. 4, *c* and *d*, respectively. These functions have broader peaks at longer distances (when an intermolecular Op–Op water bridge is made, the average P-P distance is 6.65 ± 1.41 Å).

The RDF of Ops relative to a methyl group in the choline residue (Fig. 5 *a*) shows three maxima at 3.4, 5.4, and 7.4 Å, which reflects the presence of three methyl groups in a residue, and the first minimum at ∼4.6 Å. Because the sum of the van der Waals radii of Op and CH$_3$ is 3.4 Å, the pairs of Op (with a negative charge of −0.9 in units of an electronic charge (e)) and CH$_3$ (a united atom C3 with a positive charge of 0.14 (e) that contribute to the peak in RDF at 3.4 Å must be strongly interacting electrostatically with each other and form transient Op–N-CH$_3$ charge pairs.

Electrostatic interactions between closely localized, oppositely charged side chains in proteins are called salt bridges or salt links (Creighton, 1983). These interactions
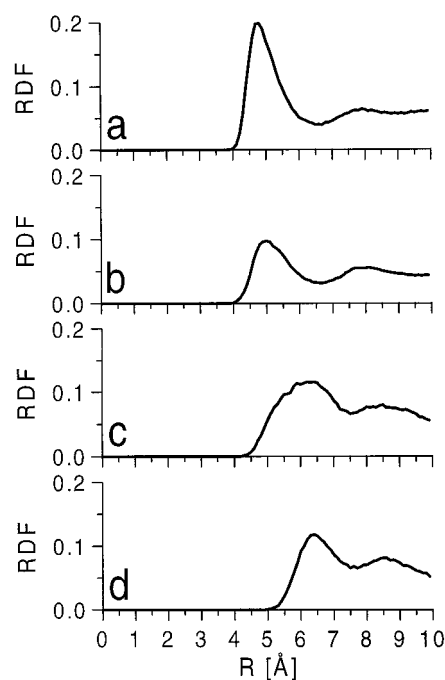
FIGURE 4   The radial distribution functions (RDF) of (*a*) the phosphorus atoms relative to a nitrogen atom, (*b*) the carbonyl carbon C21 atoms relative to a nitrogen atom, (*c*) the phosphorus atoms relative to a phosphorus atom (when an intermolecular Op–Op water bridge is made, the average P-P distance is 6.65 ± 1.41 Å), (*d*) the nitrogen atoms relative to a nitrogen atom. In all cases the RDFs were calculated only for the intermolecular distances.

contribute to protein stability to different extent (Honig and Hubbell, 1984; Lounnas and Wade, 1997). By analogy to the definition of salt links, introduced by Lounnas and Wade (1997) to assess their contribution to cytochrome P450cam
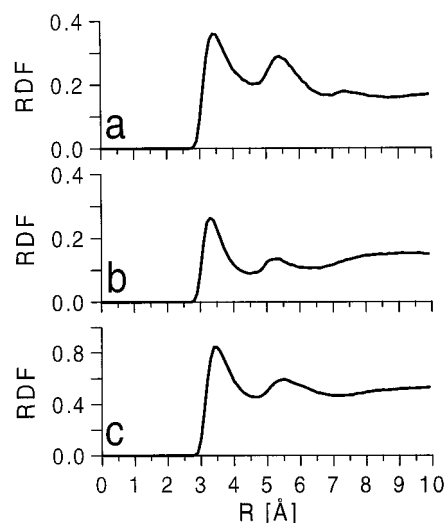
FIGURE 5   The radial distribution functions (RDF) of (*a*) the nonester phosphate oxygen atoms (Op) relative to a methyl group of the choline moiety (N-CH$_3$), (*b*) the carbonyl oxygen atoms (Oc) relative to an N-CH$_3$, (*c*) the water oxygen atoms (Ow) relative to an N-CH$_3$. In all cases the RDFs were calculated only for the intermolecular distances.

stability, we call a pair of Op and N-CH$_3$, which are located within 4.0 Å from each other (which is less than the position of the first minimum in the RDF of 4.6 Å) a charge pair. A sphere of radius 4.0 Å centered on N-CH$_3$ is called here an interaction sphere.

As can be seen in Fig. 5, *a* and *c*, the RDFs of Ops and the water oxygen atoms, Ows, relative to a choline methyl group are very similar, particularly in the positions of the major peaks (~3.5 Å) and the first minimum (~4.6 Å). Thus, the interaction spheres around N-CH$_3$ for Ops and Ows coincide, which is consistent with our finding that Op, Ow, and N-CH$_3$ moieties mutually interact and compete (results will be published elsewhere).

On average, 61 Ops are located inside the interaction spheres of 216 N-CH$_3$, which constitutes 42% of all 144 Ops in the membrane (Table 1). For comparison, 503 water molecules are located inside the interaction spheres of N-CH$_3$s (31% of all water molecules in the membrane). Of them, 143 are simultaneously H bonded to Ops and 360 interact solely with the N-CH$_3$ groups.

The average distance between Op and N-CH$_3$ is 3.57 ± 0.25 Å in intermolecular charge pairs and 3.74 ± 0.20 Å in intramolecular charge pairs (Table 2).

On average, 93 Op–N-CH$_3$ charge pairs were found; 97% of them are intermolecular and only 3% are intramolecular (Table 1). The average number of intermolecular Op–N-CH$_3$ charge pairs in the membrane is 90, which is more than 61 Ops located inside the interaction spheres of N-CH$_3$. This means that one Op can simultaneously make pairs with two or more N-CH$_3$. An example of an Op that simultaneously forms charge pairs with three N-CH$_3$ is given in Fig. 6 *a*. An example of intramolecular Op–N-CH$_3$ charge pair is given in Fig. 6 *c*.

The snapshots of Op–N-CH$_3$ charge pairs that lasted for at least 60% of the 10 ps period at the beginning of the of analysis (2000 ps) and at the time of 3500 ps, are shown in Fig. 7. The lifetime of the pairs is discussed below.

Interactions between Oc and N-CH$_3$

The RDF of the carbonyl carbon atoms relative to a nitrogen atom (intramolecular distances are not included), g_{C-N}, is shown in Fig. 4 *b*. It has a distinct maximum at 5.0 Å. As

TABLE 1   Numbers of Op–N-CH$_3$ and Oc–N-CH$_3$ inter- and intramolecular charge pairs (ch-p) in the bilayer membrane built of 72 DMPC molecules

Oxygen atom	Op	Oc	Op + Oc
Number (%) of atoms in N-CH$_3$			
Interaction sphere	61 (42%)	41 (28%)	102 (35%)
Number of charge pairs			
Total	93	77	170
Intermolecular (%)	90 (97%)	60 (78%)	150 (88%)
Intramolecular (%)	3 (3%)	17 (22%)	20 (12%)
Percent of intermolecular ch-p	60%	40%	
Percent of intramolecular ch-p	15%	85%	

TABLE 2   Inter- and intramolecular distances between charged groups involved in charge pairing (ch-p) in the DMPC bilayer membrane

	Distance from N-CH$_3$ (Å)	
Oxygen atom	Intermolecular ch-p	Intramolecular ch-p
Op	3.57 ± 0.25	3.74 ± 0.20
Oc	3.52 ± 0.26	3.54 ± 0.25

one can see in Fig. 5 *b* and read from Table 1, the percentage of Ocs that make charge pairs with N-CH$_3$ in the membrane is smaller than that of Ops (28 vs. 42%). Of all intermolecular charge pairs N-CH$_3$ forms, 60% are with Ops and 40% are with Ocs. However, Ocs make intramolecular charge pairs more frequently (in 22% of cases) than Ops (in 3% of cases), but intramolecular charge pairs constitute merely 12% of all charge pairs formed in the membrane. The average distance between Oc and N-CH$_3$ comprising of an intermolecular charge pair is 3.52 ± 0.26 Å and is almost the same as that between Op and N-CH$_3$ (Table 2). An example of an Oc that simultaneously forms charge pairs with two N-CH$_3$s is given in Fig. 6 *b*. An example of intramolecular Oc–N-CH$_3$ charge pair is given in Fig. 6 *d*. The snapshots of Oc–N-CH$_3$ that lasted for at least 60% of the 10 ps period at the time of 2000 ps and of 3500 ps, are shown in Fig. 8.

DMPC–DMPC interactions via charge pairs

The average number of all intermolecular charge pairs (Op–N-CH$_3$ and Oc–N-CH$_3$) in the membrane built of 72 DMPC molecules is 150, whereas the number of DMPC molecules linked via charge pairs is 67, and they constitute 93% of all DMPC molecules in the membrane (Table 3). The average number of all intramolecular charge pairs in the membrane is 20 (Table 1).

DMPC–DMPC interactions via water bridges

Intermolecular water bridges between DMPC molecules in each layer of the membrane at two time points separated by 1500 ps (2000 and 3500 ps) are shown in Fig. 9. The water bridges that, during 10 ps (2000–2010 ps and 3490–3500 ps), lasted for at least 60% of the time are drawn with a solid line and the ones that lasted for 40–60% of the time are drawn with a dash line. The ones that lasted for less than 40% of the time are not shown in the figure. This procedure followed from the fact that bridges can temporarily break. A double (triple) line indicates a double (triple) bridge. The location of the headgroup is represented by that of the phosphorus (P) atom. The display of intermolecular bridges under periodic boundary conditions requires different arrangements of the P atoms at the two time points. In the figure, the DMPC oxygen atoms involved in water bridging are not discriminated, i.e., bridges linking phosphate non-
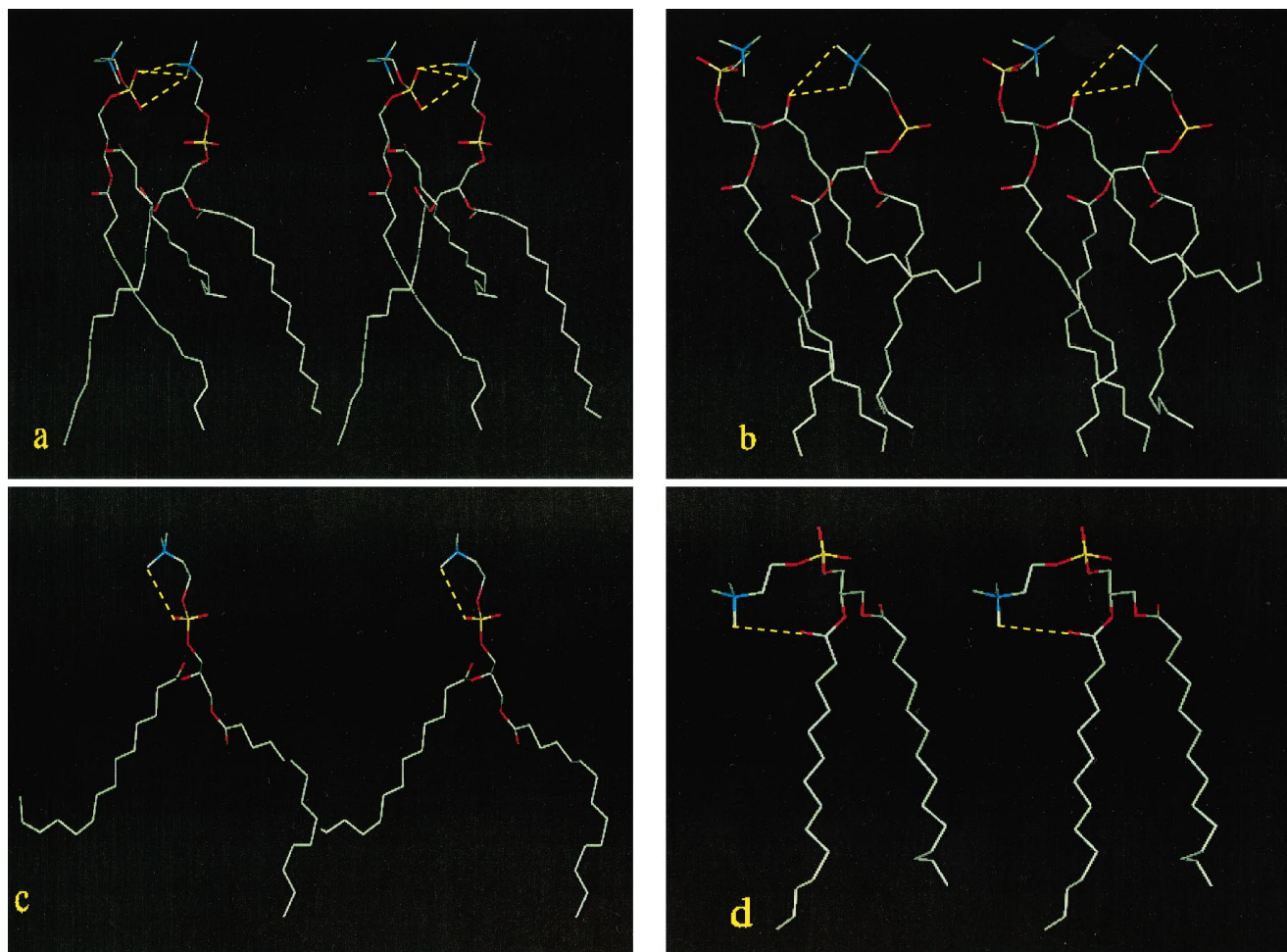
FIGURE 6   Stereo views of representative charge pairs. (*a*) An intermolecular Op–N-CH$_3$ triple pair. (*b*) An intermolecular Oc–N-CH$_3$ double pair. (*c*) An intramolecular Op–N-CH$_3$ single pair. (*d*) An intramolecular Oc–N-CH$_3$ single pair.

ester and carbonyl oxygen atoms (Op–Oc bridges) are marked in the same way as Op–Op bridges.

The average number of intermolecular water bridges in the DMPC membrane built of 72 molecules is 51. As water bridges link DMPC molecules into clusters and some linkages go via 2 or even 3 bridges (Fig. 9) (the average number of double and triple bridges in the membrane is 7.3 and 0.8, respectively), the average number of DMPC molecules linked by water is 55, which constitutes 76% of all DMPC molecules in the membrane (Table 3). The size of the water-bridged clusters ranges from 2 to 24 DMPC molecules, however, 2-molecule clusters are rare. The results qualitatively agree with the ones obtained in our previous paper (Pasenkiewicz-Gierula et al., 1997) in which 70% of DMPC molecules were linked into clusters of 2 to 7 DMPC molecules. However, previous results were obtained for much shorter MD simulation; apparently, re-arrangement of DMPC headgroups to participate in the cluster formation is a lengthy process, the rotational correlation time of the P-N vector is 3.7 ns (Pasenkiewicz-Gierula and Róg, 1997). The time dependence of water bridging is discussed below.

DMPC–DMPC interactions via charge pairs and/or water bridges

The average number of DMPC–DMPC pairs formed via charge pairs and/or water bridges in the 72 DMPC molecule membrane is 97, whereas the number of DMPC molecules linked via these short-distance interactions is 71 and they constitute 98% of all DMPC molecules in the membrane (Table 3). The snapshots of DMPC–DMPC links via water bridges and/or charge pairs that lasted for at least 60% of the 10 ps period at the time of 2000 ps and of 3500 ps are shown in Fig. 10.

Lifetime of DMPC–DMPC associations

The above analysis indicates that in a fully hydrated phospholipid bilayer membrane, at a given time, the headgroups of DMPC molecules extensively interact with one another via water bridges and/or charge pairs. Here we describe the lifetimes of these interactions within the time window of 3100 ps, between 2000 and 5100 ps of the simulation.
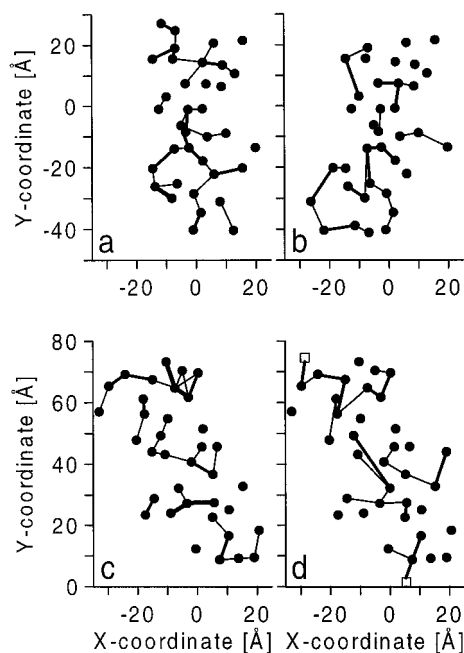
FIGURE 7　Intermolecular Op–N-CH$_3$ charge pairs for the upper layer at 2000 ps (*a*) and 3500 ps (*b*) and for the lower layer at 2000 (*c*) and 3500 ps (*d*). Pairs that, during 10 ps (2000–2010 ps or 3490–3500 ps), lasted for at least 60% of the time are displayed. The multiplicity of the pair is represented by the thickness of the line. ● shows the location of the phosphorus atom in the *x, y*-plane. A pair of open squares represents images of "real" PC molecules to show a link between headgroups of adjacent simulation boxes via periodic boundary conditions.

Lifetime of charge pairs between headgroups

As described previously, in the liquid-crystalline state, charge pairs are formed between a nonester phosphate oxygen, Op, and a methyl group of the choline residue, N-CH$_3$, and between a carbonyl oxygen, Oc, and N-CH$_3$. The average number of intermolecular Op–N-CH$_3$ pairs in the present membrane is 90 and of Oc–N-CH$_3$ pairs is 60 (Table 1) (the number of Ops, Ocs, and N-CH$_3$ in the membrane is 144, 144, and 216, respectively — we do not discriminate between O13 and O14 and between O$_2$2 and O32). The average number of DMPC–DMPC charge pairs, linked via either Op–N-CH$_3$ pairs or Oc–N-CH$_3$ pairs, is 67 (Table 3).

Op–N-CH$_3$ charge pairs. To calculate the lifetime, a history of each charge pair, formed during a period between 2000 and 4000 ps, was monitored for the time from its first appearance till the final time of 5100 ps, every 1.0 ps. In each case it was established which Op and N-CH$_3$ of two DMPC were charge paired. In this analysis, if a pair was temporarily broken but re-formed within 60 ps between the same pair of Op and N-CH$_3$, the break was ignored, whereas a break longer than 60 ps was treated as the final decay of the pair. The limiting value of 60 ps was chosen because 60 ps was the time of the longest firm pairing (binding without breakage) between breaks. Besides, occurrence of breaks longer than 60 ps was below 6%. The same cutoff time of 60 ps for breaks is used in the analyses of charge pairing and water bridging described below.
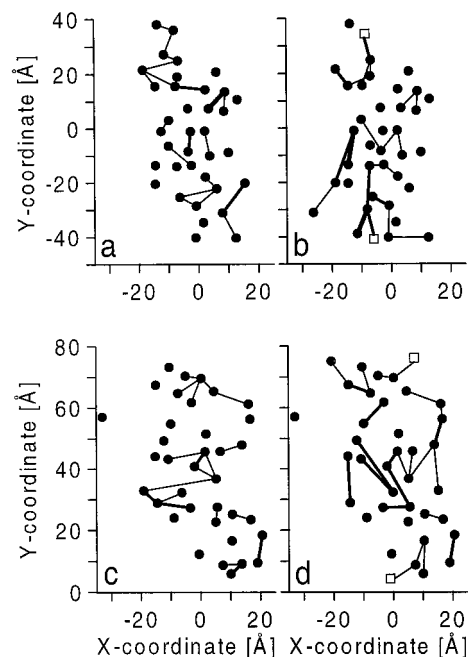

FIGURE 8　Intermolecular Oc–N-CH$_3$ charge pairs. For other explanations see Fig. 7.

Fig. 11 *a* shows the distribution of the lifetimes of Op–N-CH$_3$ pairing. On average, an Op–N-CH$_3$ charge pair lives for 140 ± 225 ps, during which it goes from on to off to on pairing state 18 times. The frequency of breaks is ~1.3 × 10^{11} per second. The term, firm pairing, was used for bonding without breakage (bonding between breaks, but only observed every 1 ps). The average firm pairing time of Op–N-CH$_3$ charge pair is 3.4 ps, and the average temporary break time is 3.8 ps (Table 4).

Oc–N-CH$_3$ charge pairs. The lifetime of Oc–N-CH$_3$ charge pairs was established in an analogical way as for Op–N-CH$_3$ pairs.

Fig. 11 *b* shows the distribution of the lifetime of Oc–N-CH$_3$ pairing. The average lifetime, time of firm pairing between breaks, temporary break time and number, and frequency of breaks are given in Table 4.

The overall behavior of Oc–N-CH$_3$ pairing is similar to that of Op–N-CH$_3$ pairing. However, the average lifetime (174 vs. 140 ps) and the firm pairing time (4.5 vs. 3.4 ps) are longer, the temporary break time (3.1 vs. 3.8 ps) is shorter; the frequencies of breaks are the same.

TABLE 3　Numbers of DMPC pairs and DMPC molecules linked by charge pairs, water bridges, and both types of short-distance interactions in the membrane built of 72 DMPC molecules

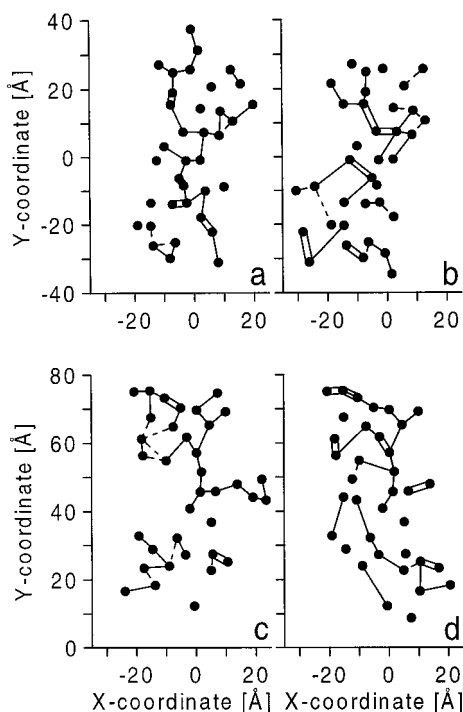| Links	Number (%) of linked DMPC molecules	Number of DMPC pairs
By charge-pairs	67 (93%)	67
By water bridges	55 (76%)	40
By both	71 (98%)	97

FIGURE 9   Cross-bridges of H-bonded water molecules between DMPC molecules for the upper layer at 2000 ps (*a*) and 3500 ps (*b*) and for the lower layer at 2000 ps (*c*) and 3500 ps (*d*). Bridges that, during 10 ps (2000–2010 ps or 3490–3500 ps), lasted for at least 60% of the time are drawn with a solid line and the ones that lasted for 40–60% of the time are drawn with a dash line. Double (triple) lines indicate double (triple) bridges. ● shows the location of the phosphorus atom in the *x, y*-plane.

A slightly more persisting Oc–N-CH$_3$ than Op–N-CH$_3$ association might results from a smaller competition from water for N-CH$_3$ to interact with Oc than with Op (results will be published elsewhere).

The above analyses indicate that charge pairing should be looked at as a rather dynamic than a static state. Op–N-CH$_3$ and Oc–N-CH$_3$ stay as pairs for a considerably long time but during this time they go from on to off to on pairing states several times. This may result from 1) very limited large distance and/or angle mobility and 2) quite substantial wobbling in a confined space of the two groups forming a pair, particularly of N-CH$_3$.

DMPC-DMPC charge pairs.   At the time of 2000 ps, all DMPC–DMPC pairs that were formed through electrostatic interactions between charged groups of DMPC (N-CH$_3$, Oc, Op) were selected (the pool of DMPC–DMPC charge pairs, 67 pairs (Table 3)). It was also established which DMPC molecules make each pair, however it was not checked which of the charged groups were involved in making the pair. Over the time of 3100 ps, every 1.0 ps, a fraction of DMPC–DMPC pairs remaining in the pool was determined. If a pair was temporarily broken but re-formed within 60 ps, it was included in the pool again.

Fig. 11 *c* shows the distribution of lifetimes of DMPC–DMPC charge pairs. Eighteen percent of the lifetimes is longer than 3100 ps, the time window of the present anal-

ysis; it allows to estimate only the lower limit of the lifetime of 1416 $\pm$ 1058 ps as in the averaging this 18% of cases were assumed to have lifetime of 3100 ps. The times of firm pairing and temporary breaks as well as the number and frequency of breaks are given in Table 4.

For DMPC–DMPC charge pairs the average lifetime (1416 ps) as well as the firm pairing time (12 ps) are significantly longer and the average temporary break time (2.3 ps) is significantly shorter than those for Op–N-CH$_3$ (140, 3.4, and 3.8 ps, respectively) or Oc–N-CH$_3$ (174, 4.5, and 3.1 ps, respectively) charge pairs. This most likely results from two facts: 1) two DMPC molecules can be linked by more than one charge pair (only 36 out of 150 are single Op–N-CH$_3$ or Oc–N-CH$_3$ pairs) and 2) one broken pair is readily replaced by another one. In effect, many of the headgroups remain associated through Coulombic interaction for the period of time much longer than one nanosecond.

### Lifetime of DMPC–DMPC associations via water bridges

In this paragraph, the lifetimes of water bridges formed between phosphate nonester and carbonyl oxygen atoms and the lifetime of DMPC–DMPC bridged pairs are given. The average number of H bonded water molecules in the membrane is 323 (4.5/DMPC) and, on average, 64 of them (~20%) make bridges (Pasenkiewicz-Gierula et al., 1997). The average number of DMPC–DMPC pairs bridged by water is 40 (Table 3).

Water bridges.   The lifetime of water bridges was determined in a similar way as the lifetime of charge pairs. During a period between 2000 and 4000 ps, a history of each water bridge was monitored for the time from its first appearance till the final time of 5100 ps, every 1.0 ps. In each case it was established which oxygen atoms of two DMPC were bridged by a given water molecule. The term, firm bridging, was used for bonding between breaks (but only observed every 1 ps). Consistent with the analysis of charge pairs, a break longer than 60 ps was treated as the final decay of the bridge.

Fig. 12 *a* shows the distribution of bridging lifetimes. The average lifetime is 49 $\pm$ 49 ps.

Fig. 12, *b* and *c* show distributions of time of firm water bridging between breaks of a pair of oxygens (*b*) and of temporary break in the bridging (*c*). The average firm bridging time is 6.5 ps and the average temporary break time is 2.8 ps. The average number of breaks during the lifetime is 5, which gives ~1.0×10^{11} breaks per second; this value is very similar to the value for charge pair break frequency of 1.3×10^{11}/s (Table 4). Most likely, the breaks result from a relative motion of the H bonded water molecule and its pair DMPC oxygens, which temporarily places their relative orientation and/or distance outside the limiting values set as H-bonding criteria (Op⋯Ow distance less or equal to 3.25 Å and Op⋯Ow-H angle less or equal to 35° (Pasenkiewicz-Gierula et al., 1997)). A bridge is finally broken when the

FIGURE 10 DMPC–DMPC links via water bridges and/or charge pairs for the upper layer at 2000 ps (*a*) and 3500 ps (*b*) and for the lower layer at 2000 ps (*c*) and 3500 ps (*d*). Links that, during 10 ps (2000–2010 ps or 3490–3500 ps), lasted for at least 60% of the time are displayed. The charge pairs (either Op–N-CH$_3$ or Oc–N-CH$_3$) are drawn with a black line; water bridges are drawn with a red line. ● shows the location of the phosphorus atom in the *x, y*-plane. Pairs of open symbols represent images of "real" PC molecules to show links between headgroups of adjacent simulation boxes via periodic boundary conditions.
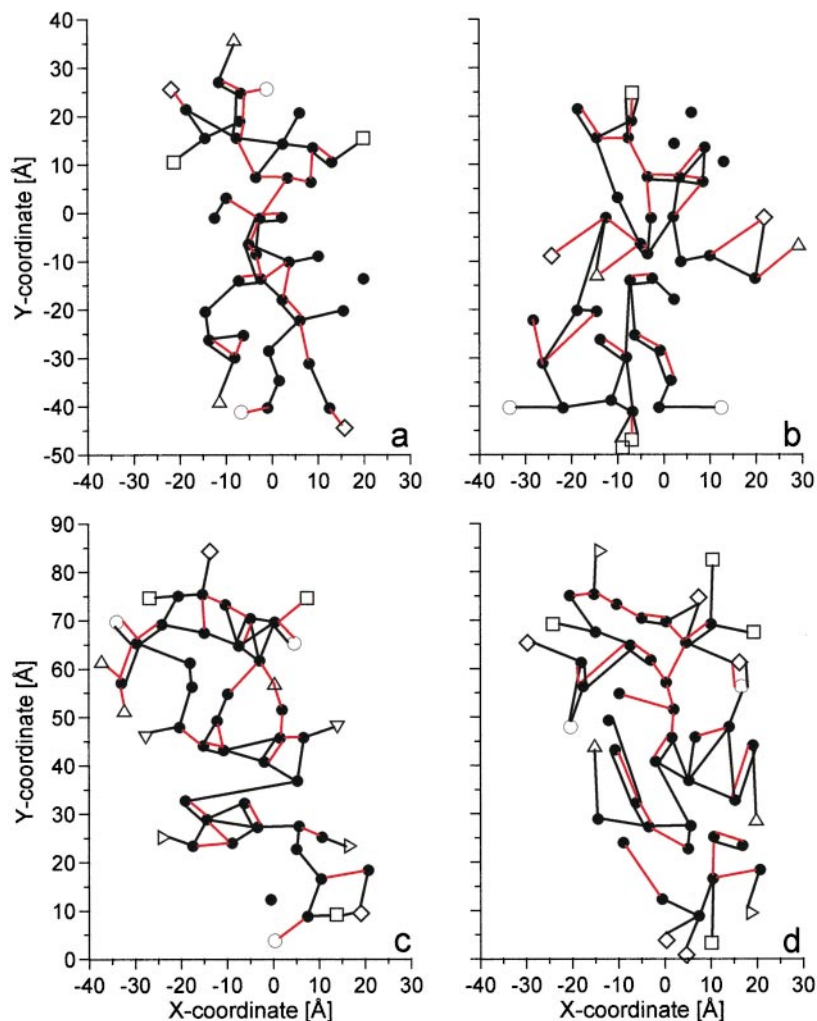
water molecule has been away from at least one of its original pair-oxygens for a time longer than 60 ps, i.e., makes an H bond with only one or none of the oxygens it originally bridged. Water bridging seems a less dynamic state than charge pairing, probably because a water molecule, because of its much less limited mobility, easily diffuses away from its H bonding partner. Thus, both the number of breaks and the average lifetime of H bonds are much smaller than those of charge pairs.

DMPC–DMPC water-bridged pairs. At the time of 2000 ps all DMPC–DMPC pairs that were bridged by water molecules were selected (the pool of DMPC–DMPC bridged pairs, 40 pairs (Table 3)). It was also established which DMPC molecules make each pair, but exchanges of water molecules bridging the pair were allowed. Over the time of 3100 ps, every 1.0 ps a fraction of DMPC–DMPC bridged pairs remaining in the pool was determined.

Fig. 12 *d* shows the distribution of the lifetimes of DMPC–DMPC bridged pairs. As for water bridges, the lifetime was defined as the time after which a DMPC–DMPC pair is broken for a time longer than 60 ps. Approximately 7.5% of the DMPC–DMPC bridged pairs lives

longer than 3100 ps, the time window of the present analysis. This allows only to estimate the lower limit for the average lifetime of a DMPC–DMPC bridged pair, which is 730 ± 887 ps (Table 4).

The average time of firm bridging, temporary break time, and the number and frequency of breaks are given in Table 4.

A much longer average lifetime of DMPC–DMPC pairs bridged by water (730 ps) than water bridges (49 ps) indicates that although bridging bonds may decay relatively fast, in their place other ones are formed either through water or bonds exchange. In effect, several headgroups of DMPC remain water bridged for times longer than 1 ns. The frequencies of breaks of DMPC–DMPC pairs bridged by water and by charge pairs are practically the same and equal to 0.7×10^{11}/s.

Lifetime of DMPC–DMPC links via charge pairs and/or water bridges

All DMPC–DMPC links via charge pairs and water bridges were found at the time of 2000 ps (the pool of 97 DMPC–
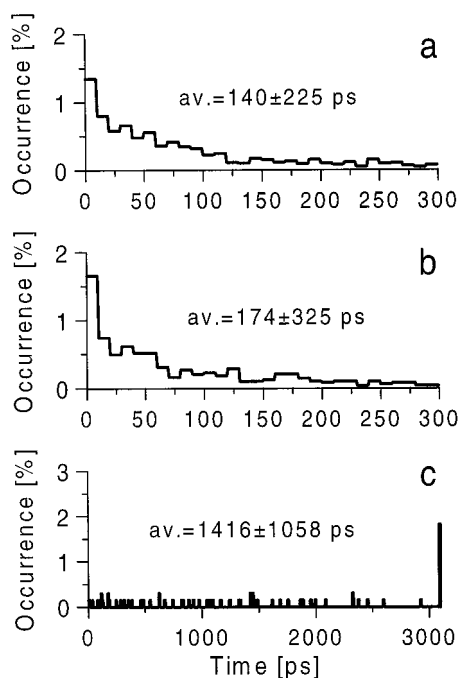
FIGURE 11   Intermolecular charge pairs. Normalized distributions of (*a*) the Op–N-CH$_3$ charge pairing lifetime, the average lifetime is 140 ± 225 ps, (*b*) the Oc–N-CH$_3$ charge pairing lifetime, the average lifetime is 174 ± 325 ps, (*c*) the DMPC–DMPC pairing lifetime, the lower limit of the average lifetime is 1416 ± 1058 ps. In *a* and *b*, the *x* axis is expanded by a factor of 10.

DMPC links (Table 3)), and the lifetime analysis of the links was carried out as described above.

Fig. 13 shows the distribution of the lifetimes of DMPC–DMPC links. Approximately 24% of them stayed longer than 3100 ps, the time window of the present analysis. This only allows estimation of the lower limit of their average lifetime, which is 1500 ± 1165 ps (Table 4). This value is only slightly greater than that for charge pairing or water bridging probably because of the insufficient length of the trajectory.

The average time of firm bonding, temporary break time, and the number and frequency of breaks are given in Table 4, and their distributions are shown in Fig. 13.

DMPC–DMPC links via both types of short-distance interactions (charge pairing and water bridging) have longer average lifetime (1500 ps) and firm bonding time (14 ps), and lower frequency of breaks (0.6 × 10^{11}/s) than DMPC–DMPC links via a single type of short-distance interactions (either charge pairing or water bridging) (Table 4). However, in these analyses, we were able to establish only the lower limits of the lifetimes, so exact comparison is not possible. Nevertheless, 24% of the DMPC–DMPC links via both types of short-distance interactions survives for the time longer than 3100 ps, whereas in the case of charge pairing and water bridging 18% and 7.5% survive longer than 3100 ps, respectively (Figs. 11 *c*, 12 *d*, and 13 *a*).

DISCUSSION

Headgroup-headgroup interactions: structural properties

Our previous work (Pasenkiewicz-Gierula et al., 1997) indicated that 76% of DMPC molecules in the bilayer are linked by single or multiple water bridges. In the present research we showed that DMPC headgroups also interact directly via Coulombic attraction between positively charged choline methyl groups of one DMPC molecule and negatively charged phosphate or carbonyl oxygen atoms of another molecule. Out of 170 charge pairs present in the bilayer at any instant, 150 (88%) are intermolecular and 20 (12%) are intramolecular. The intermolecular charge pairs link 93% of the DMPC molecules into clusters of varying sizes.

Water bridges and charge pairs form an extended network of interactions among PC headgroups. These interactions link 98% of all PC molecules in the membrane; on average, merely one PC molecule is not linked to the remaining ones.

In the present study, we used the atomic charges established by Charifson et al. (1990) and a united atom approximation. These choices would affect the charge pairing observed in this work, which is discussed below.

Both in H bonding (water bridging) and charge pairing, the dominant interaction is the Coulomb attraction, which depends on the charge distribution on the PC molecule, and surrounding water. We compared formation of H bonds between PC and water for two charge distributions; one calculated by us (Pasenkiewicz-Gierula et al., 1997) and the other by Charifson et al. (1990) (see Methods). The total numbers of H-bonded water molecules per DMPC were near 4.5 for both charge distributions, thus both distributions well reproduced experimental estimates (Arnold et al., 1983; Gawrisch et al., 1978; Nagle, 1993). But we are unable to quantitatively estimate the influence of different point charges on charge pairing. However, because the membrane obtained in this simulation well reproduced almost all experimental parameters examined in this series of study, including H bonds with water, we believe that the choice of charges made here is reasonable. Application of the united atom approximation to the DMPC molecule introduces slightly different charge distribution on the choline group and neglects a slightly polar character of the N-CH$_3$ groups. This might modify to some extent the Coulombic interactions of the choline group. However, both in the all-atom (Alper et al., 1993) and the united-atom (Pasenkiewicz-Gierula et al., 1997) models, water orientation around the choline group is similar and typical of the clathrate structure. Thus, we believe that the united atom approach did not significantly modify the Coulomb interactions of the choline group.

There have been considerable debates on the proper value for surface tension to be used in membrane simulations. The average surface tension observed in this simulation was ~0

TABLE 4   Times characterizing Op(Oc)–N-CH₃ charge pairing, Op (Oc) water bridging, and DMPC–DMPC associations (assoc.) linked by charge pairs, water bridges, and both types of short-distance interactions in DMPC bilayer membrane

Interaction	Average lifetime (ps)	Firm bonding (ps)	Temporary break time (ps)	Number (frequency) of breaks (1/s)
Op–N-CH$_3$ charge pairing	140 ± 225	3.4	3.8	18 (1.3 × 10^{11})
Oc–N-CH$_3$ charge pairing	174 ± 325	4.5	3.1	22 (1.3 × 10^{11})
DMPC–DMPC assoc. via charge pairs	1416 ± 1058*	12.0	2.3	99 (0.7 × 10^{11})
Op (Oc) water bridges	49 ± 49	6.5	2.8	5 (1.0 × 10^{11})
DMPC–DMPC assoc. via water bridges	730 ± 887*	10.6	4.0	50 (0.7 × 10^{11})
DMPC–DMPC assoc. via both interactions	1500 ± 1165*	14.1	2.6	89 (0.6 × 10^{11})

*Only lower limit estimates.

dyn/cm after equilibration, which is consistent with the fact that no surface tension was artificially applied.

Both water bridges and charge pairs are present in the DMPC single crystal (Hauser et al., 1981), in which 1) all DMPC molecules along one crystallographic axis are linked via water H bonded to the phosphate oxygen atoms to form infinite ribbons and 2) all the choline and phosphate groups

form intramolecular charge pairs. However, the carbonyl groups are involved neither in water bridging nor charge pairing in the DMPC single crystal.

^{31}P[^{1}H] nuclear Overhauser effects studies of liquid-crystalline phospholipid bilayers (Yeagle et al., 1975, 1976, 1977; Yeagle, 1978) demonstrated a strong interaction between the phosphate group and the methyl groups of the choline moiety of different lipid molecules. The result was further supported by the observations that increasing amounts of cholesterol weakened intermolecular headgroup interactions because of the spacing effect (Yeagle et al., 1975, 1977).
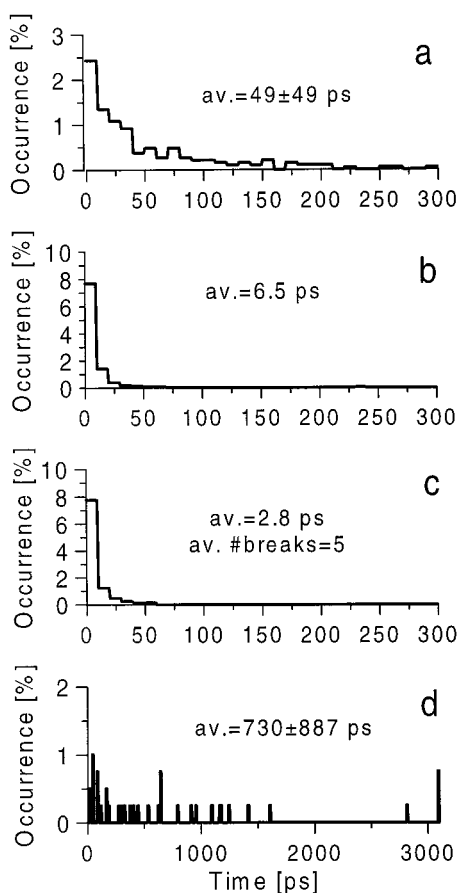

FIGURE 12   Intermolecular water bridges. Normalized distribution of (*a*) the bridging lifetime, the average lifetime is 49 ± 49 ps; (*b*) the time of firm bridging between breaks, the average firm bridging time is 6.5 ps; (*c*) the break time, the average break time is 2.8 ps and the average number of breaks is 5; (*d*) the lifetime of DMPC–DMPC bridged pairs, the lower limit of the average lifetime is 730 ± 887 ps. In *a*, *b*, and *c*, the *x* axis is expanded by a factor of 10.
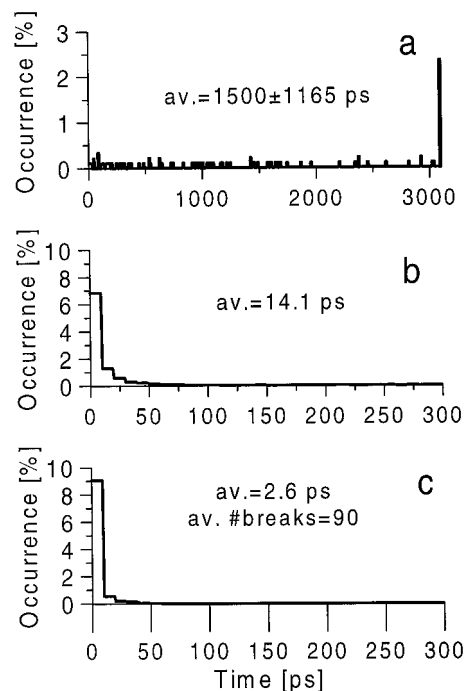
FIGURE 13   DMPC–DMPC links. (*a*) Normalized distribution of the lifetime of DMPC–DMPC links via water bridges and/or charge pairs. The lower limit of the average lifetime is 1500 ± 1165 ps. (*b*) Normalized distribution of the time of firm link between breaks; the average firm link time is 14.1 ps. (*c*) Distribution of the break time, the average break time is 2.6 ps and the average number of breaks is 90. In *b* and *c*, the *x* axis is expanded by a factor of 10.

Headgroup-headgroup interactions: lifetimes

In the liquid-crystalline DMPC bilayer membrane, the average lifetime of DMPC–DMPC pairs bridged by one or more water molecules is much longer than that of bridging H bonds (730 vs. 50 ps). This indicates that bridging water molecules and/or bridging bonds linking two DMPC molecules are effectively exchanged. This result is in accord with experimental predictions of Prats et al. (1987), Sakurai and Kawamura (1987), and Teissie et al. (1990), based on the observation of 20-fold faster lateral transfer of protons along the membrane surface than in water.

The average lifetimes of the Op–N-CH$_3$ and Oc–N-CH$_3$ charge pairs are about three times longer than that of the water bridges ($\sim$170 vs. 50 ps), and the average lifetime of DMPC–DMPC associations via charge pairs is two times longer than that of DMPC–DMPC associations via water bridges (1420 vs. 730 ps). In addition, 93% of DMPC molecules are linked by charge pairs, whereas 76% are linked by water bridges. These suggest that the contribution of the charge pairs to the membrane stability in the liquid-crystalline phase is more significant than that of water bridges. It should be stressed that both water bridges and charge pairs are breaking and re-forming dynamically. The ranges are for firm bonding time from 3.4 to 12.0 ps, for break time from 2.3 to 4.0, and for frequency of breakage from 0.7×10^{11} to 1.3×10^{11} 1/s. In the present research, if a water bridge or charge pair temporarily breaks but re-forms within 60 ps between the same pair of DMPC atoms/groups, it is counted as unbroken.

Analysis of DMPC–DMPC associations via charge pairs and/or water bridges shows that headgroups of 71 of 72 DMPC molecules (98%) are linked in a network for an average time of at least 1500 ps. Each individual pair of DMPC molecules is, on average, firmly bonded for 14 ps between 2.6 ps breaks. The longest acceptable break time is 60 ps. The break frequency is 0.6×10^{11}/s.

Results presented in this study allow us to postulate that in a PC bilayer membrane PC headgroup–headgroup interactions, both via water bridges and via Coulombic attraction, contribute to the stability of the membrane in the liquid-crystalline phase. These interactions involve 98% of DMPC molecules in the membrane and are stable in the nanosecond time scale.

In the presence of ions, the situation may be quite different. The charge pairs could be formed by way of ions, as was shown to take place between cationic surfactant and chloride counterions in a recent MD simulation study of Tobias and Klein (1996). A systematic study on the effect of varying concentration of sodium chloride on PC membranes is underway in our laboratories.

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