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Review

Thermodynamics of lipid interactions in complex bilayers

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ABSTRACT

The mutual interactions between lipids in bilayers are reviewed, including mixtures of phospholipids, and mixtures of phospholipids and cholesterol (Chol). Binary mixtures and ternary mixtures are considered, with special emphasis on membranes containing Chol, an ordered phospholipid, and a disordered phospholipid. Typically the ordered phospholipid is a sphingomyelin (SM) or a long-chain saturated phosphatidylcholine (PC), both of which have high phase transitions temperatures; the disordered phospholipid is 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) or dioleoylphosphatidylcholine (DOPC). The unlike nearest-neighbor interaction free energies (ω_{AB}) between lipids (including Chol), obtained by an variety of unrelated methods, are typically in the range of 0–400 cal/mol in absolute value. Most are positive, meaning that the interaction is unfavorable, but some are negative, meaning it is favorable. It is of special interest that favorable interactions occur mainly between ordered phospholipids and Chol. The interpretation of domain formation in complex mixtures of Chol and phospholipids in terms of phase separation or condensed complexes is discussed in the light of the values of lipid mutual interactions.

Contents

Abbreviations: Chol, cholesterol; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DMPA, dimyristoylphosphatidic acid; DPPA, dipalmitoylphosphatydic acid; DLigPC, dilignoceroylphosphatidylcholine; PSM, *N*-palmitoylsphingomyelin; SSM, *N*-steroylsphingomyelin; LigSM, *N*-lignoceroylsphingomyelin; LigGalCer, lignoceroylgalactosylceramide; BSM, brain sphingomyelin; di14:1PC, dimyristoleoylphosphatidylcholine; di16:1PC, dipalmitoleoylphosphatidylcholine; di24:1PC, dinervonoylphosphatidylcholine; di18:2PC, dilinoleoylphosphatidylcholine; di18:3PC, dilinolenoylphosphatidylcholine; di20:4PC, diarachidonoylphosphatidylcholine; di22:6PC, didocosahexaenoylphosphatidylcholine; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; PPC, pressure perturbation calorimetry; T_m, main transition temperature; E/M, excimer-to-monomer ratio; ω_{AB}, nearest-neighbor interaction free energy; FRET, fluorescence resonance energy transfer; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; GUV, giant unilamellar vesicle; NNR, nearest neighbor recognition; MD, molecular dynamics

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1. Introduction

The field of inhomogeneous membranes began with the publication of phase diagrams for binary mixtures of phospholipids [1] or a phospholipid and cholesterol [2]. Coexistence regions of gel and fluid phases [1–4], or two fluids [3] were identified in those phase diagrams, demonstrating that lipids do not mix ideally in general. Rather, different membrane components or different states of a phospholipid interact differently with each other, displaying mutual preferences that determine the distribution of their nearest neighbors in the membrane. If the interactions between two types of lipids are repulsive, meaning that these two lipids interact better with like than with unlike neighbors, the lipids will demix into domains. If the interactions are strongly repulsive, a complete separation of components will occur. How strong are these lipid–lipid interactions? What values must they have for a mixture to deviate detectably from an ideal mixture? When will domains begin to form and how large do repulsive interactions need to be for complete demixing to occur? Do some components prefer to interact with unlike lipids? What are the physical origins of these interactions?

Those are the questions addressed in this review. First, the meaning of lipid interaction as used here will be precisely defined. Then, the interactions between different phospholipids or different states of a phospholipid in the plane of the membrane will be considered. Next, interactions across the bilayer are discussed. This is a topic often overlooked, but recently there has been a significant interest in the matter, mainly because of the question of whether liquid-ordered (ℓ_o) domains, rich in cholesterol (Chol), in one leaflet of the bilayer are superimposed onto ℓ_o domains in the other leaflet.

This article will then focus on its main topic, the interactions in complex bilayers containing cholesterol. Binary mixtures of Chol and phosphatidylcholine (PC) were proposed to form a monotectic system, with a region of coexistence of ℓ_o and liquid-disordered (ℓ_d) phases in the temperature–composition phase diagram [5]. A statistical–mechanical model was developed, based on microscopic interactions between Chol and the various states of PC (solid, ℓ_d, or ℓ_o). The model successfully explained the excess heat capacity as a function of temperature for PC/Chol binary mixtures obtained by differential scanning calorimetry (DSC) [6]. Namely, the high-temperature, broad transition typically observed in these mixtures was a natural outcome of the model [7]. Subsequently, the ℓ_d–ℓ_o phase coexistence model was supported by several lines of evidence [8–15].

More recently, another model was proposed (or revisited), which postulates the formation of a 'condensed' complex between Chol and PC. Unusual phase diagrams were obtained for PC/Chol monolayers, with pairs of upper miscibility critical points [16], and an abrupt increase in the chemical potential was observed when the Chol concentration just exceeded a certain stoichiometric ratio [17]. Those observations prompted McConnell and co-workers to propose that Chol forms complexes with phospholipids in defined, stoichiometric proportions [18–20]. Several of the previous observations were re-interpreted using the condensed complex model [18]. The broad high-temperature shoulder in the heat capacity functions [14] was interpreted as thermal dissociation of the complexes [19].

Other models have been proposed. Long-range, regular arrangements of lipids in 'superlattices' were invoked to explain a series of 'dips' or 'breaks' recorded in the fluorescence intensity of probes incorporated in bilayers of PC/Chol mixtures [21,22]. However, formation of those superlattices requires a large decrease in entropy, which would have to be compensated by very large and favorable nearest-neighbor interactions. An alternative to explain these observations is the 'umbrella model' [23], which is based on the idea of the need to shield the hydrophobic Chol molecule from water. Pairwise and multibody interactions were used to simulate those effects, but the values of nearest-neighbor interactions required are still very large [23]. Another problem in the proposal of regular arrangements is that the translational diffusion of the minor component would have to be very slow, but the diffusion coefficients of Chol and PC are comparable at all compositions in binary mixtures [24–26].

Which model best explains the data? The magnitudes of the interactions between phospholipids and cholesterol recently published are examined in detail. In the end, the available information from experimental and simulation studies is brought together in a self-consistent view of ternary mixtures containing cholesterol, a saturated and an unsaturated phospholipid, where the complex behavior observed is a consequence of simple lipid–lipid binary interactions.

Domains can form in membranes as a result of dynamic processes, as a stationary state generated by fluxes leading to concentration or depletion of lipid components in particular regions of the membrane. This has been modeled using aggregation kinetics for a phospholipid/cholesterol mixture, providing a possible mechanism of raft formation and recycling in cells [27]. A different approach combined the recycling idea, that is, the dynamic incorporation and release of cholesterol, with the use of realistic thermodynamic interaction parameters [28]. In this model, domain formation is observed in mixtures of cholesterol with a saturated and an unsaturated phospholipid, provided the lipid interactions are chosen so that the system is close to a phase boundary. However, for these domains to persist long enough they must be stabilized by thermodynamic interactions. Thus, the dynamic aspects of domain formation and the domain sizes at a steady state are influenced by the fluxes of components. But domains will not form to any appreciable extent and time unless the lipid interaction energies have the appropriate magnitude [29]. The definition and magnitudes of those interactions are now discussed.

2. How lipid–lipid interactions are defined and measured

The differences between lipid–lipid interactions determine their mutual preferences and, in thermodynamic equilibrium, the spatial distribution in the plane of the membrane. First, we must define what is exactly meant by lipid–lipid interactions. To do that, consider a simplified model of one of the leaflets of the lipid bilayer. Let us suppose that this leaflet (or monolayer) is formed by lipids placed on the sites of a triangular lattice. Each lipid molecule is surrounded by 6 nearest neighbors. This involves an approximation in that, in the gel state, the acyl chains, not the lipids themselves, occupy the sites of a regular triangular lattice. In reality, a phospholipid or cholesterol molecule in a fluid bilayer probably has 4 to 6 neighbors, on average. However, that approximation is acceptable because essentially similar results are obtained whether lattice points represent lipids or acyl chains [30].

Consider now a binary mixture of two different lipid species A and B, both in the fluid state, occupying the lattice sites. These could be two phospholipids, such as PC and sphingomyelin (SM), or a phospholipid and Chol. There are three types of lipid–lipid interactions, AA, AB, and BB. However, only one thermodynamic parameter is necessary to describe the mutual interactions of these two lipid species, the difference between an AB interaction and the average of AA and BB interactions [31]:

$$\omega_{AB} = g_{AB} - \frac{1}{2}(g_{AA} + g_{BB}), \quad (1)$$

where g_{AA} and g_{BB} are the Gibbs free energies of interaction between two A or two B molecules, and g_{AB} is the Gibbs energy of interaction between A and B. The parameter ω_{AB} is the unlike nearest-neighbor interaction. It represents one-half of the change in interaction Gibbs energy for the reaction shown in Fig. 1, where two pairs of like neighbors are exchanged to produce two pairs of unlike neighbors. (We seek the value per AB interaction, so we divide by 2.) It is this interaction parameter that determines whether lipids A and B mix well or separate into domains.

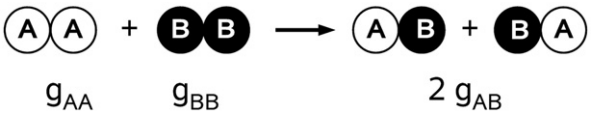

Fig. 1. Exchange reaction of two pairs of like lipids to produce two pairs of unlike lipids. Each circle represents a lipid, not an acyl chain. This reaction defines the Gibbs free energy of interaction between unlike lipids, ω_{AB}.

For example, in Fig. 2, the Gibbs free energy change (ΔG°) for the exchange of positions of the two central neighbors is $\Delta G^\circ = 6g_{AB} - 3g_{AA} - 3g_{BB} = 6\omega_{AB}$. The mixing entropy favors the right-side of the reaction of Fig. 2 and further mixing thereafter. But if $\omega_{AB} > 0$ the left side is favored. Whether and how much A and B demix depends on the sign and value of ω_{AB}. The more positive ω_{AB}, the more complete the separation into A and B domains. If $\omega_{AB} = 0$, random mixing occurs. If $\omega_{AB} < 0$, A and B will mix even more uniformly than in a random mixture; if ω_{AB} is very negative, a checkerboard AB pattern will form. The values of ω_{AB} between different lipid species typically vary between −300 to +300 cal/mol [30,32–44], and only in rare cases approach the value of +400 cal/mol required for complete separation of the components [45].

The values of ω_{AB} give no indication about the physical origin of these interactions, which can be very different for different lipid pairs. They include, for example, the conformational entropy of the acyl chains, London dispersion forces, and hydrogen bonds. In fact, ω_{AB} includes not only the interactions in the plane of the bilayer, but also those with water, such as hydrophobic interactions. In some cases ω_{AB} could originate solely from hydration differences. None of this information is provided by ω_{AB}, but it is not important for thermodynamics. The essential point is that ω_{AB} includes all effects that contribute to the pairwise, nearest-neighbor lipid–lipid interaction. Long-range interactions, involving more distant than nearest neighbors could be included. However, with the decay of van der Waals interactions with distance, nearest neighbors account for about 90% of the interaction [35]. An exception may occur when dealing with ionic lipid membranes in low salt conditions. This point will be discussed later. Finally, the interactions represented by ω_{AB} occur between equilibrium, average conformations of the lipid molecules, which fluctuate about their Gibbs energy minima.

Various approaches have been used to determine ω_{AB} from experimental data. One of the most sensitive methods uses DSC to measure the excess heat capacity as a function of temperature, $C_p(T)$, of pure or mixed bilayers, in combination with a calculation of $C_p(T)$ from the fluctuations in enthalpy (H). The fluctuation–dissipation theorem [31] provides the required connection,

$$C_p = \frac{\langle H^2\rangle - \langle H\rangle^2}{RT^2}. \tag{2}$$

The enthalpy fluctuations, $\langle H^2\rangle - \langle H\rangle^2$, are obtained from Monte Carlo simulations [30,32,35] using a simple lattice model of the bilayer, such as that shown in Fig. 2. For example, for a pure lipid undergoing a phase transition, this yields [30,35]

$$C_p = \frac{(\Delta H)^2\langle(\Delta n_A)^2\rangle + \omega_{AB}^2\langle(\Delta n_{AB})^2\rangle}{RT^2} \tag{3}$$

where A and B represent fluid and gel lipids, $(\Delta n_A)^2$ and $(\Delta n_{AB})^2$ are the variances in the numbers of A lipids (fluid) and AB (gel/fluid) contacts, ΔH is the transition enthalpy change, brackets denote ensemble averages, and R is the gas constant. The value of ω_{AB} determines $C_p(T)$ and can be adjusted in the calculation until a match with experiment is obtained.

Alternatively, an experimental measure of domain formation can be obtained, for example, from fluorescence resonance energy transfer (FRET) [46] or the excimer-to-monomer ratio (E/M) of a pyrene-labeled lipid [33,34], and compared with Monte Carlo simulations of bilayers. Varying ω_{AB} in the simulations until a match is obtained between the simulations and the experiment provides an estimate of ω_{AB} [33,34,46].

A completely different approach employs the nearest neighbor recognition (NNR) method, using lipids that can be covalently bonded by disulfide bridges [42,43]. The system is first allowed to equilibrate while nearest neighbors exchange disulfide bridges. The reaction is then stopped, generating a quenched mixture. The disulfide-bridged lipids are separated and the numbers of different pairs of lipids are measured [36–41,47]. The results of these experiments are expressed in terms of an equilibrium constant K for heterodimer formation, defined by [42],

$$K = \frac{[AB]_{eq}^2}{[AA]_{eq}[BB]_{eq}}. \tag{4}$$

Ideal mixing results in $K = 4$; if the homodimers are favored, $K < 4$; and if the heterodimers are favored, $K > 4$ [42]. From these data, the interactions parameters ω_{AB} between different pairs of lipids are obtained in a straightforward way [47],

$$\omega_{AB} = -\frac{1}{2}RT\ \ln(K/4) \tag{5}$$

Division of K by 4 is required because the heterodimer is statistically favored over each homodimer by a factor of 2 [42]; this is why $K = 4$ for a random mixture. This entropic factor of 4 has nothing to do with intermolecular interactions and does not enter ω_{AB} [47]. The factor of 1/2 arises because K is defined for formation of 2 dimers.

The values of ω_{AB} can also be estimated from the Gibbs energies of release and uptake of lipid components, measured by isothermal titration calorimetry (ITC) [48]. The calculation uses regular solution theory, or the Bragg–Williams approximation in statistical mechanics [31]. The basic assumption of this approximation is that the entropy of mixing is ideal. The excess Gibbs free energy of mixing (ΔG^E) arises entirely from a non-ideal mixing enthalpy (ΔH^E). This is obviously an intrinsic contradiction of the theory, but it is often a reasonable approximation, especially if the interactions are small. In regular solution theory, the excess mixing enthalpy of two components A and B is given by

$$\Delta H^E = \rho X_A X_B \tag{6}$$

where X_A and X_B are the mole fractions and ρ is an interaction parameter. In a lattice, this parameter is related to ω_{AB} by

$$\rho = z\omega_{AB}, \tag{7}$$

where z is the coordination number of the lattice (number of nearest neighbors). The values of ω_{AB} obtained from ITC data by this procedure fall within the expected ranges in spite of the approximation intrinsic to regular solution theory. When ω_{AB} is small, this is to be expected because the mixing entropy does not differ too much from the ideal value. For stronger interactions (favorable or unfavorable) ω_{AB} is overestimated in absolute value to compensate for the use of a mixing

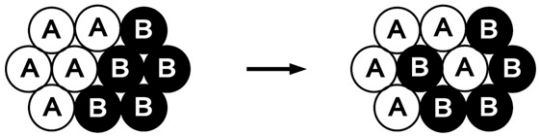

Fig. 2. Exchange between two lipids in one leaflet of a lipid bilayer, represented by a triangular lattice. The free energy difference between the initial and the final states is always a multiple of ω_{AB}.

entropy term that is too large (ideal). Any other mixing has fewer possible configurations that random mixing; hence the real mixing entropy is smaller.

Finally, non-ideality interaction parameters have also been extracted from analyses of lipid phase diagrams [49,50]. The earliest estimates were based on the calculation of phase diagrams to reproduce those obtained experimentally [49]. This calculation makes use of a non-ideality parameter, which is related to ω_{AB}, though not in a straightforward manner [51]. More recently, phase diagrams were used in combination with heat capacity curves [52–56] and regular solution theory to obtain interaction parameters. The mixing entropy is assumed ideal and the non-ideality in those mixtures, entirely enthalpic, $\Delta G^E = \Delta H^E$. This excess mixing enthalpy depends on the mole fractions of the components in each of the phases, gel and fluid, through Eq. (6) applied to each phase separately. Using the values of the transition enthalpy (ΔH) and T_m of both components, fits to experimental binary phase diagrams were obtained [50]. This approach was used by Blume's group in one of the most extensive examinations of phospholipid binary mixtures that include anionic components [52–56]. A more complicated version of regular solution theory was employed, in which the excess Gibbs energy is expanded in powers of the lipid mole fractions and only the first two terms are retained [52]. This yields, for each phase,

$$\Delta G^E = \Delta H^E = X(1-X)[\rho_1 + \rho_2(2X-1)] \quad (8)$$

where X is the mole fraction of one of the components in that phase; ρ_1 (defined within each phase) corresponds to ρ in Eq. (6); and ρ_2 is a parameter that accounts for asymmetry in the phase diagrams. The approach of Blume and collaborators was to use the first term in Eq. (8), $\rho X(1-X)$, in conjunction with ΔH and T_m for each lipid to calculate the excess heat capacity function (C_p) for an infinitely cooperative transition. The C_p thus obtained was then convoluted with a 'broadening function' and the resulting expression was used to fit the experimental C_p as a function of temperature for lipid binary mixtures [52]. In a second step, the onset and completion temperatures obtained from those fits were used in combination with the full Eq. (8) to simulate the phase diagrams of the mixtures as a function of composition and temperature. In this process the values of ρ_1 were refined and the asymmetry parameters, ρ_2, were fitted [52].

An assumption of this approach is that phase separation occurs in the transition region. This is why the parameters ρ are defined to account for interactions between two lipid components *within* each phase. Interactions at interfaces between phases are ignored. This is a problem if the components mix extensively because the interface between gel and fluid becomes very large. Unlike ω_{AB}, which is defined for each state (gel or fluid) of each lipid component, ρ is defined within each phase. Therefore it does not take into account interactions between lipids in two different states. Furthermore, the fits are not very sensitive to the values of ρ, which have, therefore a considerable uncertainty [52]. This is not very problematic to simulate phase diagrams, but it is in relating ρ_1 to lipid–lipid interactions. Assuming a lattice model for the membrane, Eq. (7) relates ω_{AB} to ρ_1.

A compilation of values of ω_{AB} is presented in Tables 1 and 2. Some important points should be noted. In mixtures that involve zwitterionic lipids or cholesterol (Table 1) the absolute value of ω_{AB} is typically between 100–300 cal/mol. These are small Gibbs energies, of at most half the thermal energy at room temperature (RT=600 cal/mol). At about room temperature, ω_{AB}=+400 cal/mol in a two component system leads to complete phase separation [45], and ω_{AB}=+330 cal/mol (or ω_{AB}=0.528 RT) between gel and fluid states is the minimum necessary for a first-order phase transition to occur in

Table 1
Unlike nearest-neighbor interaction free energies (ω_{AB}) for several lipid pairs

Lipid A	Lipid B	T (°C)	Chol (mol %)	state (A/B)	ω_{AB} (cal/mol)	Ref.
DMPC	DSPC	–	–	ℓ_d	+80	[32]
DMPC	DSPC	–	–	s	+140	[32]
DMPC	DMPC	–	–	ℓ_d/s	+320	[32]
DPPC	DPPC	–	–	ℓ_d/s	+280	[30,35]
DSPC	DSPC	–	–	ℓ_d/s	+350	[32]
DMPC	DSPC	–	–	s/ℓ_d	+370	[32]
DMPC	DSPC	–	–	ℓ_d/s	+410	[32]
POPC	POPS	25	–	ℓ_d	+240	[33,34]
DOPC	POPS	25	–	ℓ_d	+260	[34]
di16:1PC	POPS	25	–	ℓ_d	+280	[34]
di14:1PC	POPS	25	–	ℓ_d	+340	[34]
SM	POPC	25	–	ℓ_o/ℓ_d	+300	[46]
SM	POPC	37	–	ℓ_d	0	[48]
SM	Chol	25	–	ℓ_o	−350	[46]
SM	Chol	37	–	ℓ_o	−600	[48]
POPC	Chol	25	–	ℓ_d	+200	[46]
POPC	Chol	37	–	ℓ_d	+200	[48]
DMPC[a]	DSPC	60	–	ℓ_d	0	[36,38,41,42]
DMPC	POPC	40	–	ℓ_d	+30	[37]
DSPC	POPC	60	–	ℓ_d	+70	[37]
DMPC	DOPC	40	–	ℓ_d	0	[37]
DMPC	DOPC	60	–	ℓ_d	0	[37]
DPPC	DOPC	55	–	ℓ_d	+70	[37]
DSPC	DOPC	60	–	ℓ_d	+110	[37]
DMPC	Chol	60	16	ℓ_d	−50	[39]
DPPC	Chol	60	10	ℓ_d	20	[47]
DSPC	Chol	60	16	ℓ_d	−20	[39]
DMPC	Chol	30	15	ℓ_d	0	[40]
DMPC	Chol	60	40	ℓ_o	−80	[39,41]
DPPC	Chol	60	40	ℓ_o	−100	[47]
DSPC	Chol	60	40	ℓ_o	−180	[39,41]
DMPC	Chol	30	40	ℓ_o	−110	[40]
DPPC	Chol	45	40	ℓ_o	−190	[47]
DSPC	Chol	30	40	ℓ_o	−240	[40]
DSPC	Chol	30	15	s	+370	[40]
DSPC	DLPC	60	40	ℓ_o	+130	[41]

The calculation of ω_{AB} from ITC data [48] uses Eq. (7) with z=6.
[a] The interactions listed from this point on in the Table are determined with the NNR method using PC analogues, sometimes in mixtures with actual PC.

Table 2
Unlike nearest-neighbor interaction free energies obtained from fits of phase diagrams, as a function of pH

Lipid A	Lipid B	pH	ω_{AB} gel phase	(cal/mol) fluid phase	Ref.
DMPC	DPPC	7	+20	0	[55]
DMPE	DPPE	7	+20	−10	[55]
DMPG	DPPG	2	+170	+120	[55]
		7	0	0	[55]
DMPA	DPPA	4	−80	−100	[55]
		7	+70	+40	[55]
DMPC	DMPG	2	−280	−250	[54]
		7	−20	−40	[54]
DPPC	DPPG	2	+40	+130	[54]
		7	0	0	[54]
DPPC	DMPG	2	+30	+120	[54]
		7	+70	0	[52,54]
DMPC	DPPG	2	−230	−300	[54]
		7	−100	−190	[54]
DMPC	DMPA	4	−80	+40	[53]
		7	−100	−90	[53]
DPPC	DPPA	4	−30	+130	[53]
		7	−240	−230	[53]
DPPC	DMPA	4	+80	+220	[53]
		7	−50	0	[52,53]
DMPC	DPPA	4	−70	+50	[53]
		7	−190	−130	[53]
DMPE	DMPG	2	0	+40	[56]
		7	−130	−60	[56]
DPPE	DPPG	2	+110	+110	[56]
		7	−150	−110	[56]
DMPE	DPPG	2	+80	+30	[56]
		7	−100	−70	[56]
DPPE	DMPG	2	−50	−80	[56]

The values of ω_{AB} were calculated from ρ_1 (Eqs. 7 and 8, with z=6).

dipalmitoylphosphatidylcholine (DPPC) [57]. Most unlike lipid–lipid interactions are repulsive ($\omega_{AB}>0$), that is, lipids prefer to interact with like neighbors. Some, however, are attractive ($\omega_{AB}<0$), the interaction between Chol and SM or saturated PC being an especially important example. In mixtures of an anionic and a zwitterionic lipid (Table 2), ω_{AB} is often negative, particularly when the acidic lipid is deprotonated (pH 7), which is a consequence of the electrostatic repulsion between negatively charged, like lipids.

3. Interactions between phospholipids in the plane of the membrane

In one of the first papers attempting to estimate the magnitudes of lipid–lipid interactions in binary mixtures of phospholipids [49], a non-ideality parameter, defined as the ratio of AB pairs in a real system to an ideally mixed system, was adjusted to obtain a match with the experimental phase diagrams of dimyristoylphosphatidylcholine (DMPC)/distearoylphosphatidylcholine (DSPC), DMPC/DPPC, and DPPC/DSPC. This non-ideality parameter has been empirically related to ω_{AB}, but a theoretical relation between the two does not exist [51]. The values obtained for the non-ideality parameter correspond to $\omega_{AB}\approx 300$ to 600 cal/mol in the gel phase and ≈100 to 200 cal/mol in the fluid phase [51]. Using those values, computer simulations were used to model lipid bilayers on a lattice, which showed the formation of domains [51].

The excess heat capacity function, $C_p(T)$, of the gel/fluid phase transition of DPPC SUVs was obtained by DSC. Matching $C_p(T)$ calculated from Monte Carlo simulations (Eqs. (2 and 3)) to the experimental heat capacity yielded $\omega_{AB}=282$ cal/mol [30,35,57]. The effect of treating lattice points in Monte Carlo simulations as whole phospholipids, uncoupled chains, or physically and thermodynamically (infinitely) coupled chains was also examined [30]. The exact values of ω_{AB} vary, but the conclusions are qualitatively unaltered. If the lattice sites are whole lipids, $\omega_{AB}=282$ cal/mol-lipid; if the sites are uncoupled acyl chains, $\omega_{AB}=300$ cal/mol-chain; and if the sites are thermodynamically coupled chains, $\omega_{AB}=175$ cal/mol-chain [30]. That is, if the chains are infinitely coupled a smaller unfavorable interaction between gel and fluid states is sufficient to achieve the same degree of segregation of gel and fluid domains. This concept will become important later in the discussion of the effect of pairing SM and Chol in their interaction with unsaturated lipids, such as 1-palmitoyl-2-oleoylphosphatidylcholine (POPC).

Mixtures of DMPC/DSPC are almost ideal in the fluid state, but show a broad gel/fluid coexistence region, and largely demix in the gel state [4,58]. Detailed Monte Carlo simulations of the excess heat capacity function of these mixtures led to determination of ω_{AB} for all possible pairs of gel and fluid DMPC and DSPC [32]. Those values are also listed in Table 1, but note that they are calculated per mol of (uncoupled) chains. (Half of the ΔH per mol of lipid for the gel/fluid transition was used in those calculations [32].) The values are ≈+350 cal/mol-chain for gel/fluid interaction of pure DMPC or DSPC; +80 for DMPC/DSPC interaction in the fluid phase; +130 for DMPC/DSPC interaction in the gel phase; and ≈+400 cal/mol-chain for the fluid state of one lipid interacting with the gel state of the other. As with DPPC, the values of these unlike interactions would be 5–10% smaller if the simulations were performed with lattice sites representing entire lipids instead of uncoupled chains.

A similar approach was used in the simulation of the phase diagrams of a series of homologous binary mixtures of phosphatidylcholines with variable chain length mismatch [59]. Good agreement with the experimental phase diagrams of DMPC/DSPC, DMPC/DPPC, and dilauroylphosphatidylcholine (DLPC)/DSPC was obtained using an interaction parameter related to the extent of hydrophobic mismatch of the acyl chains. This is essentially equivalent to making ω_{AB} proportional to the acyl chain length difference for each pair of lipids. The concept of hydrophobic mismatch was also used in interpreting the experimental E/M ratio of an acyl chain-labeled pyrene-PC, which clustered maximally when the host lipid was dimyristoleoylphosphatidylcholine (di14:1PC), and decreased as the acyl chain of the monounsaturated PC increased, from di14:1PC to dinervonoylphosphatidylcholine (di24:1PC) [60]. However, as discussed below, there is more to lipid–lipid interactions than hydrophobic mismatch.

Mixtures of PC and phosphatidylserine (PS) in the fluid state have also received considerable attention. By comparing the excess chemical potential of PS, in the presence of Ca^{++}, estimated from experiment and from Monte Carlo simulations on a lattice, a value of $\omega_{AB}\approx 0.4$–0.6 RT was obtained [44], which corresponds to ≈+300 cal/mol. This is close to a recent estimate from molecular dynamics (MD) simulations, which in the presence of Ca^{++} (to eliminate the PS–PS electrostatic repulsion) yielded $\omega_{AB}\approx 0.3$–0.6 RT [61]. In a third study a value of $\omega_{AB}=240$ cal/mol was obtained [33], which is close to the two other estimates [44,61]. Here the E/M ratio of a pyrene-phosphatidylglycerol (pyrene-PG) lipid incorporated in POPC/POPS mixtures was measured upon addition of a PS-binding protein, the C2A motif of synaptotagmin I. In the presence of salt (but absence of Ca^{++}), the protein induced an increase in E/M, suggesting formation of PS domains. Monte Carlo simulations mimicking this experiment were performed using ω_{AB} for the PS/PC interaction as the only adjustable parameter. With a value of $\omega_{AB}=+240$ cal/mol the simulations produced results that closely matched the observed increase in E/M and showed that the protein was able to induce PS domains provided a weak repulsion existed between PC and PS [33]. Furthermore, E/M of pyrene-PG was measured in mixtures of PC with POPS, varying the chemical structure of the PC, for a series of monounsaturated PC, from di14:1PC to di24:1PC, and for a series of polyunsaturated PC, dilinoleoylphosphatidylcholine (di18:2PC), dilinolenoylphosphatidylcholine (di18:3PC), diarachidonoylphosphatidylcholine (di20:4PC), and didocosahexaenoylphosphatidylcholine (di22:6PC) [34]. In all cases, E/M decreased as the acyl chain of the PC increased [34]. Monte Carlo simulations reproduced the experimental trend by increasing ω_{AB} from 240 cal/mol in POPC/POPS (matching chains) to 340 cal/mol in di14:1PC/POPS (largest chain mismatch) [34]. This suggested a simple relation between ω_{AB} and the acyl chain hydrophobic mismatch, as in different PC mixtures [59]. However, according to the hydrophobic mismatch concept [62,63], in mixtures of monounsaturated PC with POPS, ω_{AB} should be large for the shorter lipids (di14:1PC), reach a minimum at POPC (identical acyl chains), and increase again as the PC acyl chains became longer. Instead, the E/M decreased monotonically with the chain length of the host PC. Furthermore, membranes of all the polyunsaturated PC examined have about the same hydrophobic thickness, but the E/M varies significantly [34]. Thus, ω_{AB}, which is roughly proportional to E/M [33], increases as the T_m of the lipid decreases. It appears to be largely determined by the conformational entropy and the van der Waals interactions of the unsaturated PC [34].

Lipid interactions in lipid mixtures containing anionic lipids have been examined as a function of pH. They include binary mixtures of PC, phosphatidylethanolamine (PE), PG, and phosphatidic acid (PA) in combinations where the acyl chains or the headgroups vary [52–56]. Table 2 lists values of ω_{AB} calculated from ρ_1 using Eqs. (7 and 8), for a series of phospholipid binary mixtures. The reader is referred to those studies for the values of ρ_2. The effect of mixing lipids with the same headgroup but chains that differ by two methylene groups was examined in DMPC/DPPC, dimyristoylphosphatidylethanolamine (DMPE)/dipalmitoylphosphatidylethanolamine (DPPE), dimyristoylphosphatidylglycerol (DMPG)/dipalmitoylphosphatidylglycerol (DPPG), and dimyristoylphosphatidic acid (DMPA)/dipalmitoylphosphatidic acid (DPPA) [55]. The different possible combinations of headgroups and chain length were also examined for PC/PG [52,54], PC/PA [52,53], and PE/PG mixtures [56]. The caveats discussed in

section 2 regarding the calculation of ω_{AB} from these data should be kept in mind. In particular, as indicated by the original authors, they have a considerable uncertainty [52]. In addition, some of these experiments were performed under low salt conditions, which mean that longer range contributions to the apparent ω_{AB} are an additional factor. Nevertheless, some interesting conclusions can be reached. First, the interaction free energies that those models yield are of the order of 100 cal/mol. Second, at pH 7, the interaction between an anionic and a zwitterionic phospholipid tends to be favorable ($\omega_{AB}<0$), a consequence the repulsion between two anionic, like lipids. Third, when the pH is lowered, the anionic lipid becomes more protonated and the effect of repulsion decreases; it can then be overcome by the effect of chain length mismatch, which is unfavorable, and lipid headgroup. As a rule, ω_{AB} is more negative at pH 7 than in acidic conditions.

4. Interactions across the bilayer

Lipid interactions across the bilayer, from one leaflet (or monolayer) to the other, have received far less attention over the years. Once in a while, the subject has been approached by different researchers and with different methods. Recently, a surge of interest has occurred in connection with the question of the coherence of the ℓ_d and ℓ_o phases across the membrane in cholesterol-containing systems.

The phase transition of small unilamellar vesicles (SUV) can be followed by the linewidth of the ^{1}H-NMR signal of the choline methyl groups as a function of temperature [64,65]. In the presence of millimolar quantities of lanthanides, such as 6 mM Pr3+, the phase transition temperature of DPPC SUVs increases from 37 °C to about 40 °C. If the lanthanide is added only to the extravesicular solution, the two monolayers undergo separate phase transitions. Two separate signals are then obtained, with different linewidths [64,65], indicating that the transitions of the two monolayers are thermodynamically uncoupled.[1] But under certain conditions the addition of the metal ion to the outside causes a narrowing of the signal arising from the inner monolayer, which is indicative of some level of communication between the two leaflets of the membrane [64]. Similar results were obtained for DMPC SUVs, indicating that the two leaflets of the bilayer are thermodynamically uncoupled or weakly coupled [66].

In the extreme case of a very asymmetric lipid, in SUVs of *N*-lignocerylsphingomyelin (LigSM), the transitions of the outer and inner layers are coupled, though not perfectly [65]; but in vesicles of stearoyl SM (SSM), no coupling was observed. This suggested that the very long lignoceryl chain is involved in interdigitation across the bilayer. Yet, interdigitation seems to be a relatively unimportant curiosity. In mixtures of DPPC with 20 mol% *N*-lignocerylgalactosylceramide (LigGalCer), at a temperature where gel, rich in ceramide, coexists with fluid, rich in DPPC, diffusion of a fluid lipid probe spanning one monolayer was much faster than diffusion of a probe spanning both monolayers of the membrane [67], clearly beyond the small difference in the diffusion coefficients of the two probes measured in homogeneous bilayers [68]. This indicated that the LigGalCer domains are not superimposed across the bilayer, even though they could interdigitate. Conversely, with the same experimental approach, in mixtures of DMPC/DSPC the gel domains were found to be exactly superimposed across the bilayer, thus showing coupling in the absence of interdigitation [67]. The probable reason is the size of the gel domains, which are large in DMPC/DPPC but small in LigGalCer/DPPC. If the cross-sectional area of the domains is small, the interfacial energy in the bilayer midplane is also small, and the driving force for coupling is not sufficient to overcome the configurational entropy of domain distribution, which favors lack of coherence. In mixtures of DLPC/DPPC, which have the same chain length difference as DMPC/DSPC, it was shown by fluorescence microscopy that the gel and fluid phases are coherent across the bilayer, gel matching gel and fluid matching fluid [69], in agreement with the diffusion data in DMPC/DSPC [67].

On the other hand, in NNR experiments, in mixtures of DMPC and DSPC analogues in the fluid state (60 °C), there appears to be a preference for DMPC analogues in one monolayer to be apposed to DSPC analogues in the other monolayer [38]. Thus, those experiments indicate that the lipids are paired across the bilayer in a complementary way, as if to keep a constant bilayer thickness. Furthermore, the preferential interaction free energy across the bilayer between unlike phospholipids was estimated to be ≈−100 cal/mol of lipid [70]. The tendency for complementary matching was also seen in MD simulations using coarse grained models of lipids [71]. In mixtures of model lipids with the equivalent of 12 carbons and 24 carbons in their acyl chains, mimicking DLPC and dilignoceroylphosphatidylcholine (DLigPC), a very clear (anti) correlation was observed across the bilayer, in which DLPC in one layer most often apposed DLigPC in the other layer. The same trend, albeit to a lesser extent, was observed for mixtures of models of DLPC/DSPC.

In mixtures of phospholipids and cholesterol, an important question is whether the ℓ_o phase is coherent across the bilayer, as was proposed based on measurements of bilayer thickness using the ^{2}H-NMR order parameter [9,11]. The concept is that Chol is positioned like a phospholipid in the ℓ_o phase, in either monolayer of the membrane, but may partially enter the bilayer midplane in the more disorganized ℓ_d phase [9]. This idea provides a simple explanation for the phase separation as a way to minimize hydrophobic thickness mismatch at the ℓ_d/ℓ_o interface. Further evidence for this positioning of Chol was provided by the enhancement of relaxation rates of the carbon nuclei of Chol in ^{13}C-NMR when a spin-label phospholipid probe was incorporated in DPPC/Chol membranes. In the ℓ_o phase the sterol rings showed the largest effect from the spin label, but its alkyl tail showed little effect; in the ℓ_d phase, the alkyl tail showed a significant effect, indicating proximity to the fifth position of the phospholipid probe, where the spin label was attached [11].

Using the NNR method, if about 30 mol% Chol is added to mixtures of phospholipid analogues of DMPC and DSPC, the transbilayer complementarity of those two PC analogues is more pronounced, probably because of ordering of the DSPC analogue by Chol, which enhances the effect on thickness mismatch [72]. Note that these results are obtained in fluid membranes.

More recently, fluorescence microscopy was used to visualize domains in asymmetric bilayers of mixtures of diphytanoylphosphatidylcholine/DPPC/Chol [73]. These planar membranes were assembled from two monolayers with different compositions. If one monolayer has as a composition that produces domains it can induce domain formation in an apposing monolayer with a composition that would not form domains on its own. The results indicate that the ℓ_o and ℓ_d phases are exactly superimposed across the membrane and that the coupling between monolayers is strong enough to drive phase separation in a monolayer with a composition that does not support domain formation if a bilayer were formed with that composition. Conversely, if a weaker domain-forming composition is chosen and assembled into a bilayer with the monolayer that does not form domains, then domains are not detected in either monolayer. Thus, the coupling is strong enough to abolish phase separation in a monolayer with a composition that would normally support it. However, it is important to note that this is not simply a result of

[1] Thermodynamic coupling means that the two monolayers communicate, but it says nothing about superposition (coherence) of ordered or disordered domains across the bilayer.

lipid rearrangements within each monolayer, but significant lipid exchanges occur between the two monolayers driven by the necessity to obtain, in the end, phase coherence across the bilayer [73]. In essence this is in agreement with the proposal of phase coherence across the bilayer based on NMR [9,11]. Some other possibilities have been recently discussed [74]. Yet, the strength of the coupling and its physical origin remain to be established.

A few recent theoretical papers have addressed the interleaflet coupling, using somewhat different approaches to express the free energy, but conceptually similar order parameters, related to Chol concentration [75–77]. With sufficiently strong interleaflet coupling the ordering effect of Chol in one monolayer is transmitted across the bilayer, inducing phase separation, albeit weaker, in the previously uniform apposing monolayer [75]. The number of phases that exist depend on the strength of the coupling parameter [76]. A mean-field approach with regular solution theory leads to similar conclusions [77]. A range of outcomes in terms of phases and their coherence across the bilayer is possible, which depends on the degree of coupling between the monolayers. The results from these calculations produce bilayer configurations [76,77] clearly reminiscent of the pictures obtained by fluorescence microscopy [73].

In summary, in binary mixtures of saturated PC with gel/fluid coexistence, domains appear to be exactly superimposed across the bilayer [67,69]. Those domains are large, which probably provides a sufficient driving force for coherence even if the coupling per lipid is weak. Very strong coupling is ruled out because the phase transitions of the leaflets in SUVs of pure phospholipids can be made to occur separately, though not independently [64–66]. But in DPPC/LigGalCer 80:20, where the domains are small, they are not coherent across the bilayer [67]. In mixtures of pairs of phospholipids in the fluid phase, the tendency appears to be for complementary matching across the bilayer [38,70,71]. In any case, the coupling per lipid is certainly weak, about −100 cal/mol [70]. In PC/Chol mixtures coupling is sufficient to drive domain coherence across the bilayer [9,11,73]. Whether Chol plays a role in this coupling by rapid exchange between the two leaflets (flip-flop), by simply ordering the lipids, or by inducing thickness mismatch is an open question. In any case, its role in ordering the PC in the ℓ_o phase appears to be important and is consistent with the superposition of large, ordered, gel domains between the two leaflets [67,69].

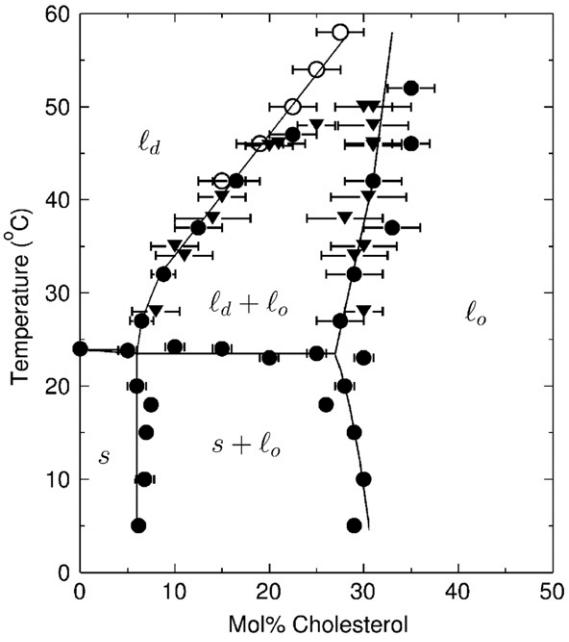

Fig. 3. DMPC/cholesterol phase diagram [12]. Reprinted (with modifications) with permission from Biochemistry 31, 6739–47. Copyright (1992) American Chemical Society.

5. Mixtures of cholesterol and phospholipids

Binary mixtures of phospholipids and cholesterol were some of the first for which phase diagrams became available [2]. Both DMPC/Chol and DPPC/Chol systems included a region of coexistence between a fluid and a solid phase. The fluid phase was later named liquid-disordered (ℓ_d) and what was originally called solid became known as liquid-ordered (ℓ_o) [5]. In two influential papers [5,7], a theoretical model was proposed that produced monotectic phase diagrams of PC/Chol mixtures with the same general features as the experimental ones, including a ℓ_d/ℓ_o phase coexistence region. Subsequently, more evidence for similar phase diagrams in phospholipid/Chol mixtures became available [8,10–15]. An example of this type of phase diagram is shown in Fig. 3 for DMPC/Chol [12]. The basis for phase separation in these mixtures is the preference of Chol to interact with the ordered phospholipid acyl chains. That is, Chol interacts more favorably with the ℓ_o state of the phospholipid (typically PC or SM) than with the ℓ_d state. In PC/Chol mixtures, increasing the cholesterol content leads to an increase in bilayer thickness [9]. Hence, it was proposed that phase separation occurs to minimize the regions of hydrophobic mismatch present at the ℓ_d/ℓ_o interface.

However, the description of binary mixtures of a high-melting phospholipid, such as a saturated PC or SM, with Chol in terms of ℓ_d/ℓ_o phase separation has been questioned [78,79]. It is clear that domains exist in LUVs of PC/Chol in a significant region of temperature and composition, but those domains have not been observed by fluorescence microscopy in GUVs [79–81]. This indicates that either the domains are smaller in GUVs than in LUVs (which is possible, but a large difference seems unlikely), or else those inhomogeneities are smaller than about 1 μm in length. Should they be called phases? In my opinion, it is not yet clear whether the heterogeneities in PC/Chol mixtures should be classified as phases or not. The size of these domains is certainly smaller than the wavelength of visible light, but this is not a valid criterion for defining a phase. A thermodynamic criterion is necessary. It is interesting to quote what one of the classics on the phase rule says on the matter: 'Although a phase is homogeneous, it is not necessarily continuous. It may be broken up into numerous crystals or drops' [82]. The question of what can be called phase separation was addressed previously [29]. The criterion is that the relative contribution of the interface to the domain energy must be small [83]. Otherwise, the energy density of the phase would depend on domain size. In any case, domains clearly exist in some temperature–composition regions of these systems [12], which reveal the non-ideal interactions between PC and Chol.

In mixtures of POPC/Chol there is certainly no extensive ℓ_d/ℓ_o phase separation. Whether significant domain heterogeneity even exists is not clear. Earlier reports favored the existence of domains [84–86], but the most recent evidence indicates that if domains exist they are very small [46,87–90]. Namely, no micrometer-size domains are observed by fluorescence microscopy [79,87]; a peptide sensitive to ℓ_d/ℓ_o coexistence fails to detect any in POPC/Chol mixtures [89]; and the first moment of the ^{2}H-NMR spectrum and the area expansion modulus increase linearly with Chol content in POPC bilayers, with no indication of saturation even at 30 mol% Chol, which is consistent with very small domains or compositional fluctuations, but no phase separation [88]. Pressure perturbation calorimetry (PPC) data have been interpreted in terms of two competitive models, one assuming phase separation and the other, a homogeneous mixture [90]. The real data lie in-between these two extremes, suggesting a gradual change of the membrane as Chol concentration increases.

In contrast with binary mixtures of Chol and phospholipids, it is clear that phase separations occur in some ternary mixtures of Chol

with two phospholipids, one with a low T_m, typically an unsaturated PC, such as POPC or DOPC, and another with a high T_m, typically SM or a long-chain saturated PC, such as DPPC or DSPC. Large scale (≥1 μm) ℓ_d/ℓ_o phase separations have been directly observed in these systems by fluorescence microscopy [79–81,87,91], although recent work suggests that in some cases very large domains may result from light-induced coalescence of domains that are initially smaller than the visible range [92]. In any case, domains in these mixtures have also been detected by fluorescence spectroscopy [85], in particular by FRET [46,81,86,93], or by a peptide that is sensitive to ℓ_d/ℓ_o coexistence [89]. Theoretical work has predicted the existence of liquid–liquid phase separation in these ternary systems [94,95]. Abstracting from disagreements on the exact location of the phase boundaries, on which systems exhibit ℓ_d/ℓ_o phase separations, and on what scale these separations occur, a reasonable consensus has actually emerged on the broad location of a region of ℓ_d/ℓ_o coexistence (Fig. 4) in the phase diagrams of ternary mixtures of Chol/(SM or saturated PC)/(DOPC or POPC). Our present concern is to establish the magnitudes of the Gibbs energies (ω_{AB}) corresponding to the molecular interactions that cause domain formation or phase separation.

6. Interactions in the ℓ_d phase: low cholesterol content

The temperature dependence of ω_{AB} between DPPC and Chol was examined by the NNR method [47]. In the ℓ_d phase, in DPPC/Chol 90:10, $\omega_{AB}\approx 0$, independent of temperature, which indicates that the enthalpy of the DPPC/Chol interaction is also $\Delta H_{AB}\approx 0$ [47]. Judging from NNR experiments with DMPC, DPPC, and DSPC analogues [39,40,47], the interaction between Chol and saturated PC in the ℓ_d state is always close to ideal ($\omega_{AB}\approx 0$). But Chol interacts less favorably with unsaturated PC [96], and very unfavorably with polyunsaturated PC [97], as a consequence of an extremely high flexibility of the unsaturated acyl chains [98], which makes for a large entropic penalty if they are placed next to Chol. The interaction of Chol with POPC must therefore be unfavorable in the ℓ_d phase, but small in magnitude, with ω_{AB} probably between 100 and 200 cal/mol [46].

The interactions between Chol, SM, and POPC have been estimated on the basis of the heats of transfer from ITC experiments [48]. Regular solution theory was used to calculate the Gibbs energies of interaction, assuming that the lipids mix ideally in the corresponding binary mixtures. This is equivalent to the Bragg–Williams approximation in statistical mechanics [31]. It leads to an overestimation of the absolute values of ω_{AB}, to compensate for the assumed random mixing of the components. Nevertheless, especially if ω_{AB} is small in absolute value, the approximation is reasonable because mixing is not too different from random. Larger values of ω_{AB} have a bigger error, but the signs and the approximate magnitude are still correct. A better approach, however, would be to calculate the ω_{AB} directly using Monte Carlo simulations, which yield the exact mixing entropy. The interaction parameter obtained from those ITC data for POPC/Chol in the ℓ_d phase at 37 °C [48] corresponds to $\omega_{AB}=+200\pm100$ cal/mol.

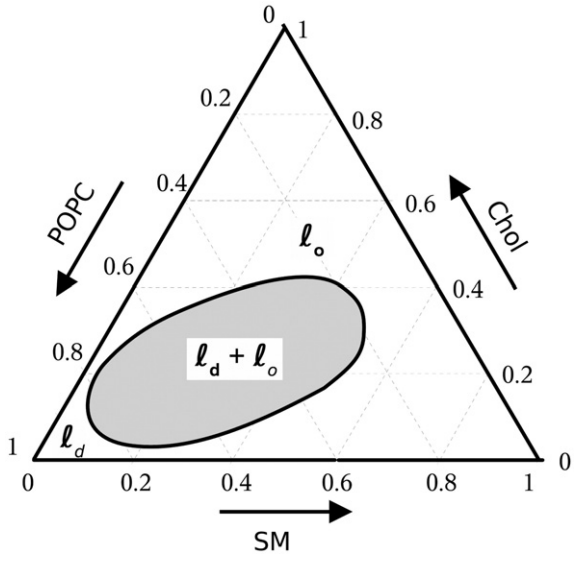

Fig. 4. Schematic phase diagram for ternary mixtures of an ordered phospholipid (SM), a disordered, unsaturated phospholipid (POPC), and Chol. The closed-loop ℓ_d/ℓ_o coexistence region is observed in several systems. Note that at a sufficiently low temperature a triangular region of ℓ_d/ℓ_o/solid coexistence will occur close to the lower (horizontal axis), in addition to two-phase solid/fluid areas.

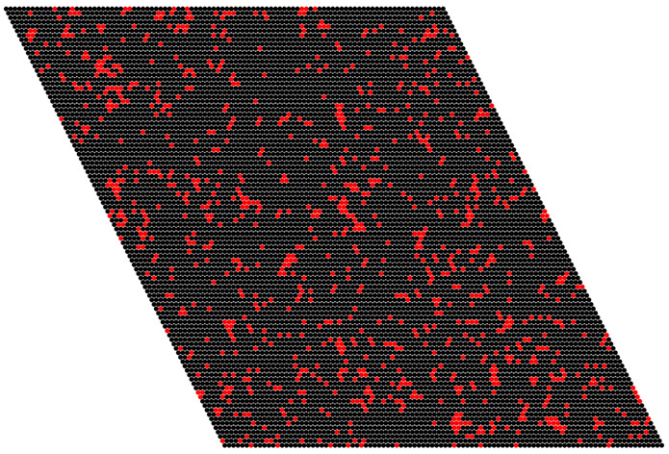

Fig. 5. Snapshot of a Monte Carlo simulation of POPC/Chol 90:10, with $\omega_{AB}=+200$ cal/mol with the program used by Frazier et al. [46].

Based on PPC experiments, at low Chol content, the condensing effect of Chol is weaker than predicted by random mixing, indicating some heterogeneity, which is, however, not as extensive as expected if phase separation occurred [90]. This is consistent with a small but unfavorable interaction between Chol and disordered POPC. Using a combination of FRET and Monte Carlo simulations [46], the POPC/Chol interaction parameter was estimated to be $\omega_{AB}=+200$ cal/mol in the ℓ_d phase, consistent with the value from ITC [48]. This interaction leads to a mixing that is almost indistinguishable from ideal, as illustrated in Fig. 5.

Recent MD simulations examined the transfer of Chol from a POPC bilayer to a stearoyl sphingomyelin (SSM) bilayer in the limit of very low Chol content [99]. The Gibbs energy for transfer of Chol is $\Delta G=-1.6\pm0.8$ kcal/mol at 46 °C and $\Delta G=-1.1\pm0.6$ kcal/mol at 56 °C. Thus, Chol prefers SSM to POPC bilayers. The entropy of transfer corresponds to $T\Delta S=-13$ kcal/mol (at 56 °C) [99], which is unfavorable, and the transfer is exothermic with $\Delta H=-14$ kcal/mol. A value of $\Delta H=-10$ kcal/mol is estimated from ITC [103] by extrapolating to zero Chol content the enthalpies of Chol transfer from POPC to SM bilayers containing 20 and 30 mol% Chol. Thus, the results of ITC experiments and MD simulations are consistent with each other. The major source of the negative entropy change of transfer from POPC to SM bilayers appears to be the rotational distribution of Chol. In SSM bilayers, the distribution of the tilt angle of Chol relative to the membrane normal is narrow indicating a well-defined, close to vertical, sterol orientation [99]. But in POPC, in the limit of low Chol concentration, the distribution is much broader and the average tilt angle is much larger [99]. When Chol is transferred from POPC to SM membranes, its rotational entropy decreases significantly [99].

Recalling that the MD simulations were performed in the low limit of Chol content, the Gibbs energies of transfer provide a way to

estimate ω_{AB} between Chol and SM in the ℓ_d state.[2] Thus, with $\Delta G^{PC\rightarrow SM}_{Chol} = -1.15$ kcal/mol at 56 °C, and −1.55 kcal/mol at 46 °C from the MD simulations [99], the free energy of transfer per interaction, assuming 6 nearest neighbors, is $\Delta G^{PC\rightarrow SM}_{Chol}/6 = -190$ kcal/mol at 56 °C, −260 at 46 °C, and, by extrapolation, −320 cal/mol at 37 °C. Using Eq. (14), this estimate yields ω_{AB} values for the SM/Chol interaction in the ℓ_d phase between ~ −100 cal/mol at 56 °C and ~−200 at 37 °C, which is still slightly favorable, more than for DPPC/Chol, where $\omega_{AB}\approx 0$ [47], but much less favorable than for the SM/Chol interaction in the ℓ_o phase (≈−350 cal/mol, see below).

7. Interactions in the ℓ_o phase: high cholesterol content

The interaction between Chol and an ordered phospholipid, such as SM or a saturated PC, is especially important in the ℓ_o phase, where the Chol content is high. The temperature dependence of ω_{AB} for DPPC/Chol was determined by the NNR method. Unlike in the ℓ_d phase where it is independent of temperature, ω_{AB} varies significantly with temperature in the ℓ_o phase [47]: at 65 °C the interaction is marginally favorable, with $\omega_{AB}=-100$ cal/mol, but at 45 °C it almost doubles (−190 cal/mol), and extrapolation to 20 °C yields $\omega_{AB}\approx -320$ cal/mol. Using the van't Hoff equation, this temperature dependence corresponds to an interaction enthalpy $\Delta H_{AB}=-2$ kcal/mol for DPPC/Chol [47]. Similarly, for DSPC/Chol, $\omega_{AB}\approx -350$ cal/mol based on extrapolation [46] from high-temperature data [29,40]. Those favorable interaction free energies are at the high-end limit of what is usually observed with lipids. It is highly significant that they occur between saturated PC and Chol in the ℓ_o phase. The interaction of Chol with SM is at least as favorable as with DPPC or DSPC, and probably more so [96,100–102]. On this basis, a conservative estimate for the interaction of Chol with ordered SM is $\omega_{AB}=-350$ cal/mol [46]. Based on ITC data, and assuming 6 neighbors/molecule, $\omega_{AB}=-600\pm300$ cal/mol for SM/Chol in the ℓ_o phase [48]. This is fairly good agreement considering the uncertainty involved and that the values are obtained by completely unrelated approaches.

The thermodynamics of uptake and release of Chol from POPC/Chol and SM/Chol membranes were measured using ITC [103]. The changes in the thermodynamic functions of transfer of Chol from POPC/Chol 70:30 to SM/Chol 70:30, calculated from those ITC data, are essentially constant between 37 °C and 50 °C. Both the enthalpy and Gibbs energy of transfer are favorable: $\Delta H^{PC\rightarrow SM}_{Chol} = -3$ kcal/mol and $\Delta G^{PC\rightarrow SM}_{Chol} = -1$ kcal/mol [103]. Thus, the entropy change is unfavorable, with $T\Delta S^{PC\rightarrow SM}_{Chol} = -2$ kcal/mol. The unfavorable entropy changes arise because of the rotational freedom of Chol, which is larger in the POPC than in the SM membrane [99]. It is interesting that, based on MD simulations, transfer of Chol from POPC to SM in the limit of low Chol has a much larger temperature dependence, with $\Delta H^{PC\rightarrow SM}_{Chol} = -14$ kcal/mol [99] instead of −3 kcal/mol with 30 mol% Chol, obtained by ITC [103].

Although the POPC/Chol interaction is weakly repulsive in the ℓ_d phase, with $\omega_{AB}=+200$ cal/mol [46,48], if Chol is forced into POPC bilayers at 30 mol% or more, the interaction may become more favorable, as the POPC chains become more ordered. In fact, ω_{AB} for POPC/Chol in the ℓ_o phase can be estimated from the above data, as follows. The free energy of transfer of Chol from POPC/Chol 70:30 to SM/Chol 70:30 is $\Delta G^{PC\rightarrow SM}_{Chol}\approx -1$ kcal/mol [103]. Assuming a uniform lipid distribution in the membrane (which is strictly incorrect, but probably not a bad approximation in this case) and 6 nearest neighbors per molecule, this corresponds to ≈−240 cal/mol. Hence, the difference between the interactions of Chol with SM and POPC, $\omega^{SM/Chol}_{AB}-\omega^{POPC/Chol}_{AB}\approx -350$ cal/mol.[3] Substituting $\omega^{SM/Chol}_{AB}\approx -350$ cal/mol. [46] this yields $\omega^{POPC/Chol}_{AB}\approx 0$ in POPC/Chol 70:30, which is more favorable than in the ℓ_d phase. It is worth noting that with POPC/Chol interactions of +200 cal/mol in the ℓ_d phase and ~0 in the ℓ_o phase, phase separation cannot occur in POPC/Chol binary mixtures, in agreement with recent experiments [87–90].

MD simulations have shown that Chol interacts with saturated acyl chains preferentially over monounsaturated chains [104,105]. In SSM/Chol 66:34 mixtures the energies of interaction of Chol with SM or POPC are similar [104]; if anything, they are slightly more favorable with POPC, but with a broader distribution. The interaction between the phospholipid headgroup and the Chol ring system is stronger with POPC than with SM, leading to a larger tilt angle of Chol relative to the bilayer normal. Therefore, in SSM/Chol 66:34 bilayers [104], the distribution of the tilt angle of Chol is narrow, around a close-to-vertical sterol orientation. In POPC/Chol 66:34 the distribution is a little broader than in SSM/Chol or SSM bilayers [104]. But the energy distribution functions for SM/Chol and POPC/Chol bilayers are both unimodal. It is expected that formation of condensed complexes between SM and Chol would lead to bimodal distributions, the lower energy peak corresponding to the complex, the higher energy peak, to non-associated SM and Chol. Not only is this not observed, but the distributions look similar for POPC/Chol and SM/Chol [104]. These results do not provide evidence for the existence of well-defined condensed complexes.

The difference between the interaction energies of Chol with SSM and POPC in bilayers with 34% Chol is +1 kcal/mol [99]. In a first reading this might suggest that Chol transfer from POPC/Chol 66:34 to SSM/Chol 66:34 is endothermic with $\Delta H\approx +1$ kcal/mol, contrary to experiment, which gives $\Delta H=-2$ kcal/mol by extrapolation to SM/Chol 66:34 from data of SM/Chol 80:20 and 70:30, at 50 °C [103]. The situation, however, is more complicated because the +1 kcal/mol in the MD simulations is not the entire enthalpy of transfer [99]. The complete process involves not only breaking POPC/Chol interactions and making SM/Chol interactions, but also breaking or making Chol/Chol interactions and breaking or making POPC/POPC and SM/SM

[2] The Gibbs energy of Chol transfer from POPC to SM ($\Delta G^{PC\rightarrow SM}_{Chol}$) is the difference between the interactions broken and established in the POPC and the SM membranes. For simplicity in notation, let POPC be A, SM be B, and Chol be C. Then, using Eq. 1 with z as the number of nearest neighbors,

$$\Delta G^{PC\rightarrow SM}_{Chol} = z(g_{BC}-g_{AC}+g_{AA}-g_{BB}) \quad (9)$$

$$= z\left(\omega_{BC}-\omega_{AC}+\frac{1}{2}(g_{AA}-g_{BB})\right) \quad (10)$$

$$= z(\omega_{BC}-\omega_{AC}+\delta), \quad (11)$$

where

$$\delta = \frac{1}{2}(g_{AA}-g_{BB}). \quad (12)$$

Hence,

$$\Delta G^{PC\rightarrow SM}_{Chol}/z = \omega_{BC}-\omega_{AC}+\delta \quad (13)$$

Now, δ must be a small positive number because the interactions between SM molecules are stronger than between POPC molecules ($g_{AA}>g_{BB}$). But, as a difference between two lipid–lipid interactions in the ℓ_d phase, $g_{AA}-g_{BB}$ is expected to fall within the typical range for ω_{AB} in the fluid state, about 100–300 cal/mol (Table 1); since this is divided by 2, it seems that $\delta\sim 100$ cal/mol is a good estimate. Using Eq. (13) with $z=6$, $\omega_{AC}=200$ cal/mol for POPC/Chol [46,48], and $\delta\sim 100$ cal/mol, one obtains

$$\omega_{BC}\approx \Delta G^{PC\rightarrow SM}_{Chol}/6 + 100 \text{ cal/mol}. \quad (14)$$

[3] See previous footnote. In this case, because only 70% of the contacts are to phospholipid, and inserting $z=6$ for the number of nearest neighbors,

$$\Delta G^{PC\rightarrow SM}_{Chol} = 0.7\times 6(g_{BC}-g_{AC}+g_{AA}-g_{BB}) \quad (15)$$

$$-1 \text{ kcal/mol} = 4.2(\omega_{BC}-\omega_{AC}+\delta) \quad (16)$$

$$-240 \text{ cal/mol} = \omega_{BC}-\omega_{AC}+\delta, \quad (17)$$

with $\delta\approx 100$ cal/mol, as before.

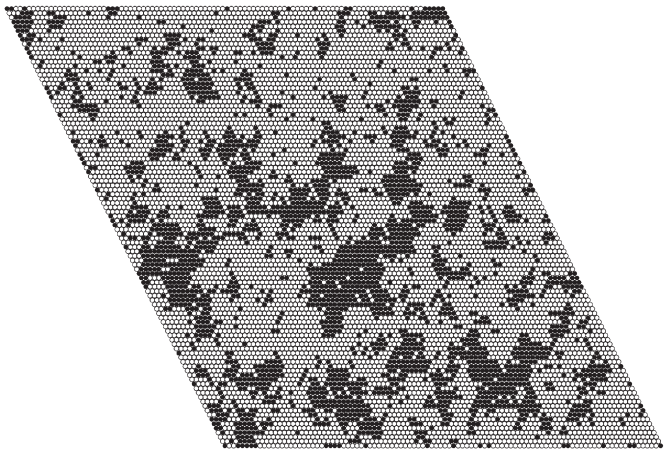

Fig. 6. Snapshot of a Monte Carlo simulation of SM/POPC 70:30, with ω_{AB}=+300 cal/mol. Reprinted with permission from Biophys. J. [46].

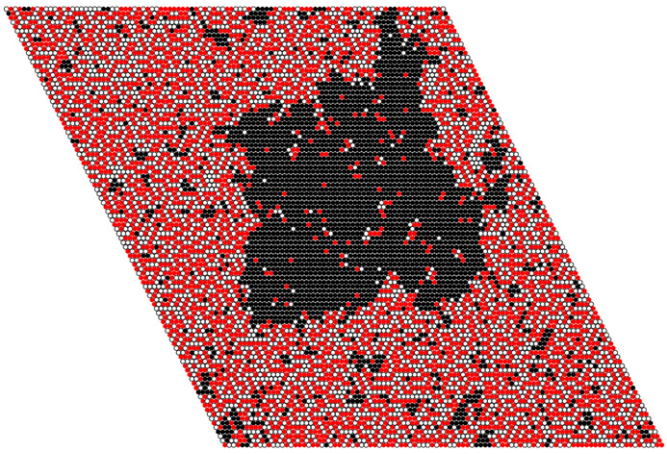

Fig. 8. Snapshot of a Monte Carlo simulation of SM/POPC/Chol 35:30:35, with ω_{AB} parameters of −350 cal/mol (SM/Chol), +300 cal/mol (SM/POPC), and +200 cal/mol (POPC/Chol). Reprinted with permission from Biophys. J. [46].

interactions. Perhaps the most important point is that these enthalpy changes, either positive or negative, are quite small.

8. Interactions between SM and POPC

In the ℓ_d state, the interaction between POPC and SM is probably small, $\omega_{AB}\approx 0$, as for most saturated PC (Table 1). In fact, from heats of transfer determined by ITC, $\omega_{AB}=0\pm100$ cal/mol for POPC/SM [48]. If SM were in the gel state and POPC in the fluid state, $\omega_{AB}>+300$ cal/mol would be expected because SM (gel) and POPC (fluid) probably interact worse than the gel and fluid states of DPPC, for which ω_{AB}=+280 cal/mol. The interaction between ordered SM (ℓ_o state), and disordered POPC, is probably between ω_{AB}=+250 and +300 cal/mol [46]. It should be noted that in the Monte Carlo simulations of Frazier et al. [46] POPC was assumed to be in the ℓ_d state but the SM was ordered. Therefore, the SM/POPC interaction was ω_{AB}=+300 cal/mol, which is very different from zero, unlike in the ITC experiments, where SM and POPC were both in the ℓ_d phase [48].

9. Ternary mixtures of DPPC/DOPC/cholesterol

The thermodynamics of lipid interactions are somewhat different in the ternary system DPPC/DOPC/Chol, which clearly shows phase separation [106]. Based on the phase diagram and calculated tie-lines, the Gibbs energy of transfer of Chol between ℓ_d and ℓ_o phases was estimated in DPPC/DOPC/Chol 35:35:30 [106]. At 20 °C, ΔG=−0.26 kcal/mol and at 25 °C, ΔG=−0.38 kcal/mol. These numbers are much smaller in absolute value than for Chol transfer between POPC and SM [103]. Furthermore, they exhibit the opposite temperature dependence, indicating an endothermic process, as if the transfer of Chol to the DPPC-rich phase were in this case favored by entropy. This can be understood because of the restrictions in the conformations of the DOPC acyl chains imposed by Chol; the oleoyl chains are intrinsically more disordered than the palmitoyl chains and placing Chol next to them results in an entropic penalty that is relieved when Chol is transferred to the DPPC-rich phase. Such constraints may not apply to the same degree to POPC. In any case, it should be remarked that, unlike in the separate mixtures of SM/Chol 70:30 and POPC/Chol 70:30, the concentrations of Chol in the two coexisting phases in DPPC/DOPC/Chol are, of course, not identical: ~30 mol% in ℓ_o and ~20 mol% in ℓ_d [106]; therefore, comparison between the two systems must be made with some caution. It has been suggested that the major driving force for phase separation or domain formation in DPPC/DOPC/Chol is probably the very unfavorable interaction between DPPC and DOPC, whereas in SM/POPC/Chol mixtures it probably arises from the differential effect of Chol on the SM and POPC [103].

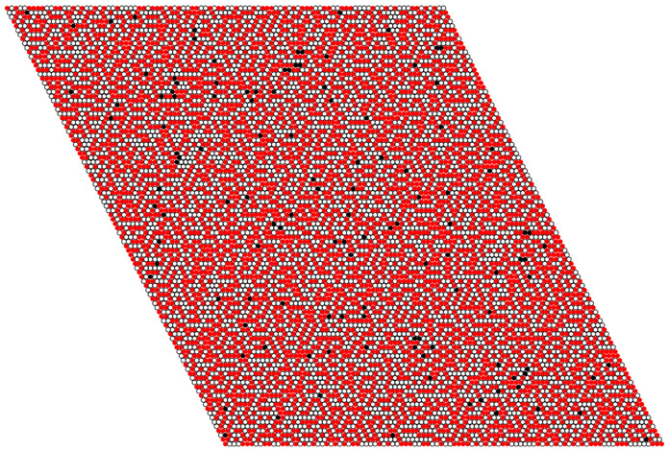

Fig. 7. Snapshot of a Monte Carlo simulation of SM/Chol 50:50, with ω_{AB}=−350 cal/mol. Reprinted with permission from Biophys. J. [46].

10. Ternary mixtures of SM/POPC/cholesterol

The lipid interactions in SM/POPC/Chol mixtures were examined by a combination of experimental FRET measurements with Monte Carlo simulations on a two-dimensional lattice, using a simple Ising model [46]. FRET was measured between two fluorophores that partition into the ℓ_d phase (POPC-rich) and simulated in the same mixtures, adjusting the interaction parameters of the three lipid pairs, (SM/Chol, SM/POPC, POPC/Chol) to obtain agreement between simulated and experimental values of the fluorescence energy transfer efficiency. If ω_{AB} for POPC/Chol is set to +200 cal/mol, the distribution of lipids in a binary mixture is not very different from ideal mixing (Fig. 5) [46]. A stronger repulsive interaction, ω_{AB}=+300 cal/mol for SM/POPC, leads to formation of small domains in a binary mixture, but not to phase separation (Fig. 6). A favorable interaction, ω_{AB}=−350 cal/mol for SM/Chol, results in a distribution that is more uniform than ideal, with a tendency to form unlike, AB pairs in SM/Chol binary mixtures (Fig. 7). In none of these binary mixtures is phase separation observed [46]. However, when the three components are mixed together, phase separation is observed, as shown in Fig. 8 for SM/Chol/POPC 35:35:30 [46].

If the SM/POPC interaction is decreased to ω_{AB}=+250 cal/mol, the picture changes: large domains are still observed but not phase separation (Fig. 9) [46]. These changes in domain sizes are revealed by the domain distribution function of POPC molecules (Fig. 10). With fixed values for the POPC/Chol and SM/Chol interactions (ω_{AB}=+200 and −350 cal/mol, respectively) and ω_{AB}=300 cal/mol for SM/POPC, the distribution is clearly bimodal, indicating phase separation (Fig. 10, black symbols); if ω_{AB} is decreased to 270 cal/mol, the peak corresponding to large domains becomes broader (Fig. 10, red symbols); and if ω_{AB}=250 cal/mol for the SM/POPC interaction, the distribution becomes very broad with no maximum (Fig. 10, gray symbols). Those changes are caused by a reduction of ω_{AB} for SM/POPC by just 50 cal/mol [46]. Decreasing the absolute values of the other parameters would have comparable effects. This suggests that observation of large-scale phase separation in mixtures of SM/POPC/Chol by fluorescence microscopy is sensitive to the SM structure, the presence of impurities, and oxidation of small amounts of components, as all those factors can be expected to change the apparent interactions by amounts equivalent to 50 cal/mol in ω_{AB}. This explains why micrometer size domains are observed in some samples but not in others, even if very similar [80,92]. Since the SM/DOPC interaction is certainly more repulsive than the SM/POPC interaction, larger domains are observed in ternary mixtures of SM/Chol with DOPC than with POPC [80]. In general, because the values of ω_{AB} are small, the domains formed are only marginally stable. They can easily be changed or actively regulated by moderate perturbations, such as the local incorporation of lipids or binding of peripheral proteins [33,34]. This is very different from the irreversible formation of lipid domains, leading to static structures, which would occur if the magnitudes of lipid–lipid interactions were large.

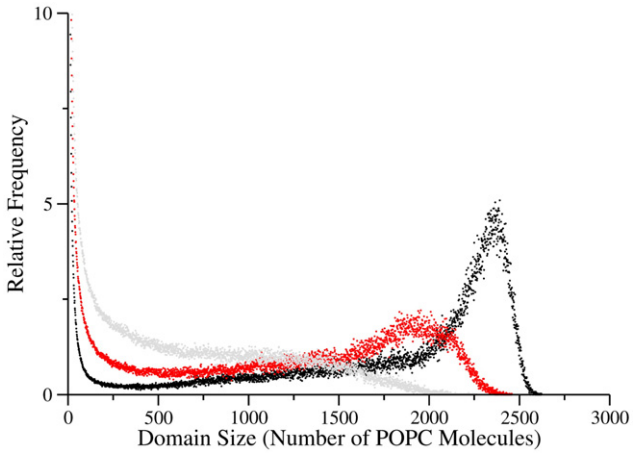

Fig. 10. Distribution function of POPC domains in SM/POPC/Chol 35:30:35, with ω_{AB} parameters of −350 cal/mol (SM/Chol), +200 cal/mol (POPC/Chol), and for SM/POPC, +300 (black), +270 (red), and +250 cal/mol (gray). Reprinted (with modifications) with permission from Biophys. J. [46].

11. Conclusion: complex behavior from simple interactions

Non-ideal mixing of phospholipids and Chol above the T_m of the phospholipid was described as phase separation between two liquids, ℓ_d and ℓ_o, where Chol interacts favorably with the ordered state but unfavorably with the disordered state [5,7]. In the ternary mixtures examined recently, the ordered lipid is usually SM or a long-chain, saturated PC, and the disordered lipid is usually POPC or DOPC. Alternatively, the interactions between ordered phospholipids and Chol have been interpreted in terms of formation of condensed complexes [18,20,94,107]. In the condensed complex model, the two fundamental chemical components in a ternary mixture of SM/POPC/Chol are the SM/Chol complex and POPC. There is a repulsive interaction between SM/Chol complexes and POPC, and the mixing entropy between these two 'components' is assumed ideal, according to regular solution theory. The Monte Carlo simulations of Frazier et al. [46] can be interpreted in terms of liquid–liquid phase separation. Large regions of ℓ_o (rich in SM/Chol) and ℓ_d (rich in POPC) phases are shown to coexist in SM/POPC/Chol mixtures (Fig. 8). On the other hand, consistent with complex formation, there is indeed a tendency for occurrence of pairs of Chol (red) and SM (white) molecules (Fig. 7).

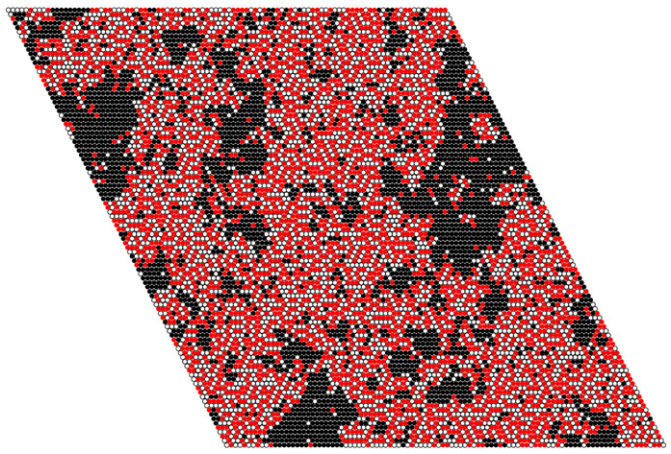

Fig. 9. Snapshot of a Monte Carlo simulation of SM/POPC/Chol 35:30:35, with ω_{AB} parameters of −350 cal/mol (SM/Chol), +250 cal/mol (SM/POPC), and +200 cal/mol (POPC/Chol). Reprinted with permission from Biophys. J. [46].

The condensed complex model has had some important successes: it explains the monolayer phase diagrams with a pair of upper miscibility critical points [16] and the sudden increase in the rate of β-cyclodextrin-induced Chol desorption at the stoichiometric concentration [17], which reflects the increase in Chol chemical potential when free PC is no longer available to form complexes [20]; it describes the experimental deuterium NMR signals in sterol/phospholipid bilayers [108]; and predicts the occurrence of a closed loop in the ternary mixtures of phospholipids and cholesterol [94,107].

However, as evident from several different theoretical and experimental approaches described here, the Gibbs energies of interaction between lipids are typically of the order of a few hundred calories per mol, most positive but some negative. The condensed complex model postulates values of free energy and enthalpy that are about one order of magnitude larger. The model uses an equilibrium constant for complex formation that corresponds to a Gibbs energy of about −3 kcal/mol (with one Chol and one phospholipid per complex) [107] to −4 kcal/mol (two phospholipids and one Chol per complex) [108], at room temperature. In contrast, an SM/Chol interaction parameter of only ω_{AB}=−350 cal/mol, in combination with repulsive interactions of +200 and +300 cal/mol between POPC/Chol and SM/POPC, is sufficient to achieve phase separation in ternary systems [46]. Similarly, the enthalpy of formation of a condensed complex has been estimated to be between ΔH_{AB}=−9 kcal/mol (one phospholipid per complex) [107] and −19 kcal/mol (two phospholipids per complex) [108]. However, experimentally, from the temperature dependence of ω_{AB} of DPPC/Chol in the ℓ_o phase, a value of ΔH_{AB}=−2 kcal/mol was obtained [47]. Furthermore, $\Delta H \approx -10$ kcal/mol of phospholipid for complex formation is larger (in absolute value) than ΔH for the $\ell_d \rightarrow$ gel phase transition of DPPC (−8.7 kcal/mol [4]) and SM (≈−7 kcal/mol for PSM and brain SM [109]), which correspond to a greater ordering of the phospholipid acyl chains than the $\ell_d \rightarrow \ell_o$ transition. Indeed, the condensed complex model does not perform very well in simulating the experimental heat capacity curves of phospholipid/Chol mixtures in a quantitative manner, although qualitatively it correctly predicts some of the main features [19,108]. In addition, a 'spurious transition' (which is not supported by

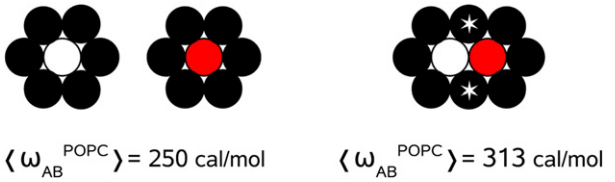

Fig. 11. Interactions of POPC (black) with Chol (red) and SM (white) isolated (left) and upon formation of a complex (right). The value of $\langle\omega_{AB}^{POPC}\rangle$ is the average interaction between POPC and SM/Chol in each case, using ω_{AB}=+300 cal/mol for SM/POPC, and +200 cal/mol for POPC/Chol.

experiment) between complex and gel phase is predicted at low temperatures and high Chol concentrations [19]. The ℓ_d–ℓ_o phase coexistence model actually performed quite well in describing those heat capacity functions [7]. The parameters of the microscopic interaction model correspond to $\omega_{AB} \approx \pm 1$ kcal/mol [5], which is high but closer to the values commonly found for lipid–lipid interactions (Table 1).

In the Monte Carlo simulations of Frazier et al.[46], binary mixtures of phospholipids and Chol do not show macroscopic phase separation (Figs. 5 and 7), but ternary mixtures do (Fig. 8), in agreement with experimental observations by fluorescence microscopy of GUVs [79,87]. Thus, a closed loop occurs in the ternary phase diagram (Fig. 4), which has been predicted by the condensed complex model [94,107] and observed experimentally [79,87]. The complex model stipulates that the unsaturated PC (POPC or DOPC) has a repulsive interaction with the SM/Chol complex but not with SM or Chol by themselves. What the Monte Carlo simulations show is that this extra interaction is not necessary for the observation of a closed loop in the ternary phase diagram [46]. All that is required is a relatively strong (~−350 cal/mol), favorable interaction for SM/Chol and two weaker, unfavorable interactions for POPC/Chol and SM/POPC (+200 and +300 cal/mol). In the simulations, when SM and Chol are paired they maintain the same interactions with each other and with POPC that they had in the absence of 'complex' formation. The same conclusions regarding the requirement for phase separation in ternary mixtures were reached by an approach combining Monte Carlo simulations with a mean-field treatment, using a Ginzburg–Landau free energy [110]. There too, it was found that all that was necessary were unfavorable interactions between the two phospholipids, a favorable interaction between Chol and the ordered phospholipid, and an unfavorable interaction between Chol and the disordered phospholipid. The shape of the closed-loop coexistence region in the phase diagram depends on the values of those interactions [110]. Furthermore, there is also no need to invoke multibody interactions [23] to explain the mixing behavior of SM/Chol/POPC bilayers: three pairs of binary interactions are sufficient [46].

The concept that a POPC molecule has a more repulsive interaction with a SM/Chol 'complex' than with either SM or Chol separately is entirely correct, but it is not necessary to invoke a change in the lipid–lipid interactions. This was apparent in Monte Carlo simulations of the phase transition of DPPC in a two-dimensional lattice [30]. If the lipids were represented by dimers of thermodynamically coupled acyl chains a small interaction between gel and fluid chains (ω_{AB}=175 cal/mol) was sufficient to reproduce the experimental heat capacity function in the phase transition region. When the lipid was represented by independent chains a larger interaction was necessary (ω_{AB}=300 cal/mol). Thus, when the chains are thermodynamically coupled, the effect on phase separation is larger. The formation of a SM/Chol 'complex' corresponds to coupling, though to a lesser degree, but the result is qualitatively similar. To understand its origins, consider a SM/Chol pair and its interactions with POPC nearest neighbors (Fig. 11). Using the values ω_{AB}=+200 cal/mol for POPC/Chol and +300 cal/mol for SM/POPC interactions [46], the average interaction of SM and Chol with POPC before 'complex' formation is +250 cal/mol. After SM/Chol pair formation, the average interaction between POPC and the 'complex' increases to +313 cal/mol, which is larger than the most repulsive of the two interactions (300 cal/mol). This arises because the POPC molecules that interact with both Chol and SM (marked with a star in Fig. 11) contribute +500 cal/mol to the average.

For the same reason, a lipid interacting favorably with a peripheral membrane protein is induced to cluster, by the protein, in a mixture with another lipid, if the two lipids interact unfavorably, but with an interaction that is too weak to cause domain formation in the absence of the protein [33]. In this case, the protein couples like lipids so that their interaction with unlike lipids becomes more unfavorable. In the SM/Chol/POPC case, the favorable interaction between SM and Chol couples these two lipids together, so that their repulsive interaction with POPC is enhanced [46]. The principle is the same in both cases: the combined interactions of two lipids (coupled in space through a favorable thermodynamic interaction) with another lipid species with which they interact unfavorably leads to an enhancement, on average, of this repulsive interaction and therefore to clustering in domains. If the repulsive interaction is sufficiently strong, phase separation will occur.

Acknowledgments

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