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Quantification of phase transitions of lipid mixtures from bilayer to non-bilayer structures: Model, experimental validation and implication on membrane fusion

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ABSTRACT

Lipid bilayers provide a solute-proof barrier that is widely used in living systems. It has long been recognized that the structural changes of lipids during the phase transition from bilayer to non-bilayer have striking similarities with those accompanying membrane fusion processes. In spite of this resemblance, the numerous quantitative studies on pure lipid bilayers are difficult to apply to real membranes. One reason is that in living matter, instead of pure lipids, lipid mixtures are involved and there is currently no model that establishes the connection between pure lipids and lipid mixtures. Here, we make this connection by showing how to obtain (i) the short-range repulsion between bilayers made of lipid mixtures and, (ii) the pressure at which transition from bilayer phase to non-bilayer phases occur. We validated our models by fitting the experimental data of several lipid mixtures to the theoretical data calculated based on our model. These results provide a useful tool to quantitatively predict the behavior of complex membranes at low hydration.

1. Introduction

Lipid bilayer interactions have been extensively studied from the mid-70s to the early 90s and are now very well characterized (Rand and Parsegian, 1989; Marra and Israelachvili, 1985; Horn et al., 1988; Helfrich and Servuss, 1984; Evans, 1991; Pincet et al., 1994; Lis et al., 1982). Experimentally, the Surface Force Apparatus (Marra and Israelachvili, 1985; Israelachvili and Adams, 1978), the Osmotic Stress (Rand and Parsegian, 1989; LeNeveu et al., 1976; Parsegian et al., 1979; Rand et al., 1988) and the vesicle adhesion techniques (Evans and Metcalfe, 1984; Evans, 1980, 1992; Gourier et al., 2004) are the three main complementary approaches that have been used. Theoretically, many groups have been working on various types of interbilayer interactions. The results were summarized by Evans (1991) where the major interactions were unified under a simple formalism.

When lipid bilayers are forced in close proximity, an extremely large repulsion, known as short-range repulsion (hereinafter noted SR), is generated (Rand and Parsegian, 1989; Horn et al., 1988). Even though the exact origin of SR remains somewhat controversial, it is believed that it comes from hydration and/or protrusion effects. SR expresses the resistance of the bilayers to dehydration. Empirically, SR decays exponentially with the interbilayer distance. The characteristic decay length is always on the order of 0.1 nm. SR dominates the interbilayer interactions when the distance between the bilayers is from 1–3 nm to 0.2–1 nm. These upper and lower limits depend on the type of lipid considered. When the bilayers are further compressed (interbilayer distance <0.2–1 nm), they become too dehydrated and no longer stable, leading to a transition to a non-bilayer phase where the lipids of the former bilayers are mixed. For instance, when many bilayers are stacked, the lipids are said to be in a lamellar phase, called the Lα-phase for fluid lipids. When the lamellar phase is dehydrated, a new phase appears (Luzzati et al., 1968; Tardieu et al., 1973), the most common of which is the inverted hexagonal phase (Luzzati et al., 1968; Tardieu et al., 1973; Gruner et al., 1986). Other phases, such as the rhombohedral phase (Luzzati et al., 1968; Leaver et al., 2001; Yang and Huang, 2003), have also been found.

Early on, the connection between this lipid phase transition and membrane fusion has been recognized (Rand and Parsegian, 1986). The similarity between both processes is striking: when two membranes are fused and their lipids are mixed, their phase and structure also change. Due to experimental limitations, this connection had been hypothesized but never concretely established until a few years ago. In 2002–2003, everything changed when it was experimentally determined that a fusion intermediate, the fusion stalk (Kozlovsky et al., 2002; Kozlovsky and Kozlov, 2002), exists in rhombohedral phases (Yang and Huang, 2003, 2002; Yang et al., 2003; Hyde and Schroder, 2003). Rhombohedral phases have been known for 40 years (Luzzati et al., 1968; Tardieu et al., 1973; Leaver

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et al., 2001) but the presence of fusion stalks within the rhombohedral phases had not been observed until then. From this result, the connection between lipid phase transitions and membrane fusion became clear.

However, there remained a lack of predictive modeling for SR and phase transitions of lipid mixtures (Kozlovsky et al., 2004). Hence, the purposes of this paper are: first, to model SR for lipid mixtures; second, to show how the resulting SR can be used to predict the pressure (or, equivalently, the hydration) at which the phase transition of lipids from bilayer to non-bilayer structures occurs. Both models will be validated by experimental data using lipid mixtures of dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), stearoyloleoylphosphatidylcholine (SOPC), palmitoyloleoylphosphatidylethanolamine (POPC) and digalactosyldiacylglycerol (DGDG).

2. Short-range repulsion of lipid mixtures: model

In order to understand what phases and structures occur during dehydration of bilayers, it is convenient to focus on lamellar phases in which the presence of a large number of bilayers allows measurements on a large scale, usually by X-ray diffraction.

In a single lipid system, when pressure, P, is applied to lipid bilayers in their lamellar phase, water is squeezed out of the space between the bilayers. Once equilibrium is reached, a water layer with an average thickness of d_w separates the bilayers. It has been well documented that SR of a single lipid system decays exponentially with d_w (Rand and Parsegian, 1989). Thus, for any given lipid i:

$$d_w = \frac{n_i v_w}{a_i} \tag{1a}$$

$$P(d_w) = P_{0_i} \exp\left(-\frac{d_w}{\lambda_i}\right) \tag{1b}$$

where n_i is the number of water molecules per lipid polar head; a_i, is the mean cross-sectional area projected onto the average plane of the bilayer; λ_i, is the decay distance of SR; and v_w is the volume of a water molecule ($v_w = 0.03\,\mathrm{nm}^3$). λ_i can only be experimentally obtained and is of the order of 0.1 nm. It should be noted that a_i slightly varies with n_i (less than 10%, Rand and Parsegian, 1989). Here, this variation will be neglected.

The model attempts to estimate SR in a lamellar phase made of several lipid types that freely mix. First, consider the case of a binary system with a fraction α (molar fraction) of component 1 and $(1-\alpha)$ of component 2. In the simplest model, the lipids are not affected by their neighbors. This means that n_i only depends on the applied pressure. At equilibrium, this pressure is uniform. Therefore, n_i can be directly obtained from Eq. (1):

$$n_i = \frac{a_i \lambda_i}{v_w} \ln\left(\frac{P_{0_i}}{P}\right) \tag{2}$$

d_w can easily be deduced from the average hydration and molecular area, a_t, of the lipids:

$$d_w = \frac{n_t v_w}{a_t} \tag{3}$$

where n_t is the weighted average number of water molecules per lipid polar head. n_t and a_t are expressed as:

$$n_t = \alpha n_1 + (1-\alpha) n_2 \tag{4a}$$

$$a_t = \alpha a_1 + (1-\alpha) a_2 \tag{4b}$$

Define $\kappa = \alpha a_1 / a_t$, then

$$d_w = \kappa\lambda_1 \ln\left(\frac{P_{0_1}}{P}\right) + (1-\kappa)\lambda_2 \ln\left(\frac{P_{0_2}}{P}\right) \tag{5a}$$

It can be rearranged as:

$$\ln(P) = \frac{\kappa\lambda_1 \ln(P_{0_1}) + (1-\kappa)\lambda_2 \ln(P_{0_2}) - d_w}{\kappa\lambda_1 + (1-\kappa)\lambda_2} \tag{5b}$$

Hence, SR of the mixture also decays exponentially.

$$P(d_w) = P_{0_{\mathrm{total}}} \exp\left(-\frac{d_w}{\lambda_{\mathrm{total}}}\right) \tag{6a}$$

with

$$\lambda_{\mathrm{total}} = \kappa\lambda_1 + (1-\kappa)\lambda_2 \tag{6b}$$

$$P_{0_{\mathrm{total}}} = {P_{0_1}}^{(\kappa\lambda_1/\lambda_{\mathrm{total}})} \cdot {P_{0_2}}^{((1-\kappa)\lambda_2/\lambda_{\mathrm{total}})} \tag{6c}$$

Above results for a binary system can be extended to a mixture containing m components of lipids.

$$a_{\mathrm{total}} = \sum_{i=1}^{m} \alpha_i a_i \tag{7a}$$

$$\kappa_i = \frac{\alpha_i a_i}{a_t} \tag{7b}$$

$$\lambda_{\mathrm{total}} = \sum_{i=1}^{m} \kappa_i \lambda_i \tag{7c}$$

$$P_{0_{\mathrm{total}}} = \prod_{i=1}^{m} P_{0_i}^{(\kappa_i\lambda_i/\lambda_{\mathrm{total}})} \tag{7d}$$

The set of Eq. (7) provides a complete description of SR for lipid mixture. However, several assumptions have been made to reach this result. Because of these assumptions, the model will not always be valid. The limit of validity of the model is discussed in the next section.

3. Justification and limitations of the model

The major assumption behind above model (Eq. (7)) is that contributions of each component are independent of any other one and must be additive. However, under the following circumstances, our model needs to be applied with caution.

A first difficulty comes from the cis-interactions between lipids within a membrane which may affect the trans-interactions between the membranes. For instance, if one of the components tends to aggregate or to force (cis-interactions) other lipids to form domains, the trans-interactions between the membranes will strongly depend on these aggregates or domains. A typical example of such a component is cholesterol (Rand and Parsegian, 1989; Hung et al., 2007). As mentioned in above section, the average molecular area of each component within a membrane could be calculated from Eq. (4b). However, when Eq. (4b) is applied to a 1:1 mixture of dipalmitoylphosphatidylcholine (DPPC)/cholesterol, the calculated molecular area for cholesterol is $1.27\,\mathrm{nm}^2$, but if we do the same calculation to a 8:1 mixture of DPPC/cholesterol, $0.147\,\mathrm{nm}^2$ is obtained. This enormous discrepancy is due to some local reorganization of the bilayer, which makes predictive modeling difficult. This reorganization may be due to two reasons. First, cholesterol is known to be involved in the formation of lipid domains, "rafts" (Simons and Ikonen, 1997; Rawicz et al., 2008; Brown and London, 1998, 2000). These domains make the lipid distribution in the membrane inhomogeneous, which makes the interactions very dependent on the organization of the domains in the contact area of the membranes. Second, at 25 °C, DPPC is known to be in a solid phase (Leonenko et al., 2004; Biltonen and Lichtenberg, 1993), meaning the membranes are not fluid. This lack of fluidity can also strongly alter the homogeneity of the membrane (no global tension, Needham and Evans, 1988; Needham

Table 1
Parameters of SR, P_0, λ and molecular area, a.

	λ (nm)	Log(P_0) (Pa)	a (nm^2)	Corresponding figures
DOPC experimental	0.29	8.6	0.72	Figs. 1, 3–5, 6a
DOPE/DOPC (3/1) experimental	0.209	8.75	0.64	Fig. 1
DOPE predicted	0.177	8.85	0.61	Figs. 1, 3–5, 6b
POPE experimental	0.125	9.8	0.57	Fig. 2a and b
SOPC experimental	0.235	9.1	0.64	Fig. 2a–c
DGDG experimental	0.17	9.3	0.80	Fig. 2c
POPE/SOPC (9/1) experimental	0.12	10.2	0.57	Fig. 2a and b
POPE/SOPC (9/1) predicted	0.14	9.7	0.57	Fig. 2a and b
POPE/SOPC (3/1) experimental	0.21	9.0	0.59	Fig. 2a and b
POPE/SOPC (3/1) predicted	0.17	9.5	0.59	Fig. 2a and b
DGDG/SOPC (55/45) experimental	0.18	9.6	0.73	Fig. 2c
DGDG/SOPC (55/45) predicted	0.2	9.2	0.71	Fig. 2c
POPC experimental	0.24	8.85	0.655	Fig. 6a and b

All values are deduced from experiments (from Rand and Parsegian, 1989 and Klose et al., 1992) except for the four predicted ones that are deduced from Eq. (6). The last column indicates in which figures the data have been used or displayed.

et al., 1988). Probably, both phenomena are adding up making it extremely difficult to predict the behavior of the membranes.

To apply the model it is also important to discuss the additivity of the interactions in view of protrusion and hydration forces that are mainly responsible for SR.

Individual lipids can more or less protrude from the membrane. When two membranes are getting in tight contact, protrusion of lipids is restrained, which generates a repulsive force, defined as protrusion force. Clearly, if two lipids with very different chain length are mixed, protrusion will be different from that of pure lipids and protrusion forces are unlikely to be additive. However, if the chain lengths of the various components of the lipid mixture are similar, protrusion of the lipids is unlikely to be much different from that of pure lipids and is additive as predicted by current models (Aniansson et al., 1976; Aniansson, 1978; Israelachvili, 1985).

Lipid polar heads usually bind to water molecules in solution. When membranes come into contact, the organization of these bound water is disrupted, which induces a repulsive force, defined as hydration force. Pressure is needed to expel the bound water. Similar to the protrusion force, chain length will be of primary importance. If lipids of very different chain lengths are mixed, the shortest one will be shielded by the longest ones and will not contribute to hydration forces. Therefore, there is no additivity. However, for lipids with similar chain lengths, hydration forces are likely to be additive (Israelachvili, 1985).

As a summary of above discussions, our model is expected to be valid mainly for lipids having similar chain length in a fluid phase and that do not form aggregates/domains. This may seem very restrictive but in most biophysical models currently used, membranes are indeed composed of lipids in a fluid state with a chain length of 16–20 carbons. Again, when cholesterol is involved, this model will not be valid. The examples given below show that the model can be safely applied to fluid bilayer made of the most commonly used lipids: DOPC, SOPC, DOPE and POPE. It also shows it can be valid for glyceroglycolipids such as DGDG.

4. Short-range repulsion of lipid mixtures: experimental validation on common fluid lipids: DOPC, SOPC, DOPE, POPE and DGDG

Eq. (7) can be validated using experimental measurements reported by Parsegian and Rand who performed extensive studies on SR of lipid lamellar phases for 20 years between the mid-70s and the mid-90s (Rand and Parsegian, 1989; Parsegian et al., 1979; Rand et al., 1988). Their invaluable data is a unique source that can be used here to validate the model. The experimental procedures by which pressure can be applied to a lamellar phase are described in detail in their publications.

First, the example of DOPE/DOPC mixture was chosen because it is well described in the literature. It is interesting to note that DOPE cannot form lamellar phase at 25 °C (Gawrisch et al., 1992). However, when mixed with DOPC, DOPE can be incorporated into lamellar phases. Eq. (7) can be used to obtain the theoretical SR of DOPE lamellar phase from that of the DOPC/DOPE mixture. The molecular area, a, and the force parameters, P_0 and λ are directly obtained from the experimental data for pure DOPC and a DOPE/DOPC (3/1) mixture at 25 °C (cf. Table 1). Eq. (7) predicts a, P_0 and λ for pure DOPE. The corresponding results are given in Table 1. It is known that SR does not vary significantly between 14 °C and 23 °C (Kozlov et al., 1994). Above 14 °C, the range of accessible pressures becomes small and van der Waals forces plays an important role over this range which make the measurement of SR very difficult. Hence, it is better to use data measured at 14 °C and compare it to the predicted one (Fig. 1) (Gawrisch et al., 1992). They are in good agreement. Also, the molecular area can be compared: 0.61 nm^2 and 0.62 nm^2 for the predicted and measured values respectively. Again, these values are in perfect agreement. The model can similarly be applied to other lipids. Data are also available in the literature for other common lipids such as POPE, SOPC and DGDG. Fig. 2 and Table 1 show that applying the model to pure POPE, SOPC and DGDG provides a satisfactorily prediction of SR of mixtures of these lipids.

It is worth mentioning again that even though the model works extremely well for these common fluid mixtures composed of lipids with similar chain length, care should be taken when it is applied to predict SR of other lipid mixtures with significantly different chain length (see above section).

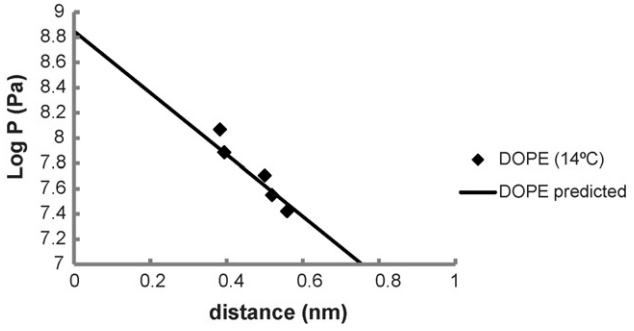

Fig. 1. SR for DOPE bilayers. The line represents the repulsion predicted in Table 1; diamonds are the data points for DOPE Lα-phase at 14 °C (Gawrisch et al., 1992).

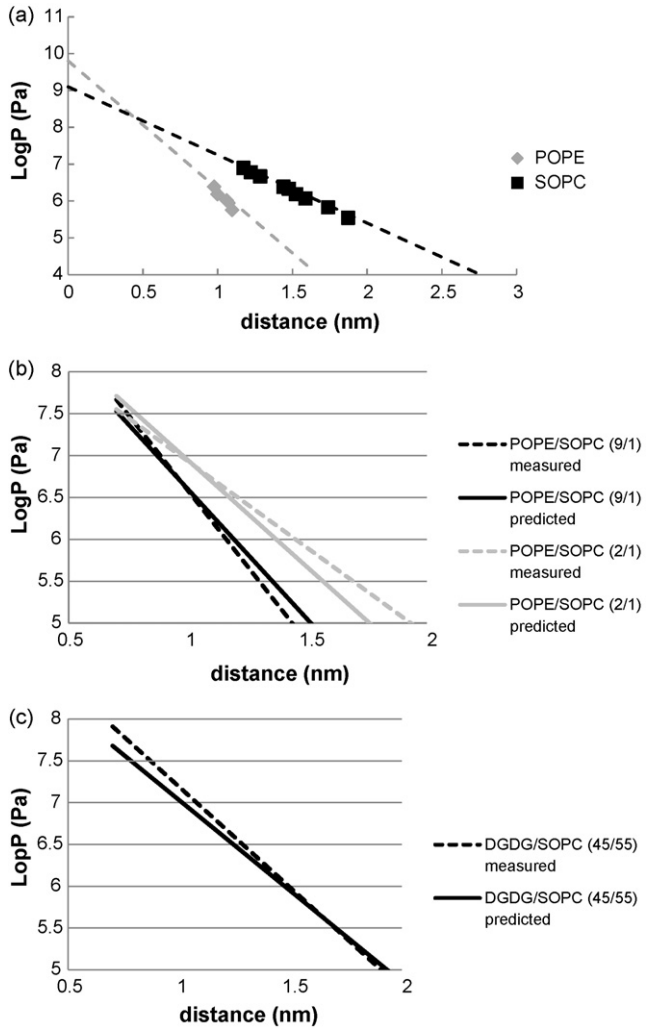

Fig. 2. (a) Experimental data from POPE and SOPC taken from Rand and Parsegian (1989). The solid lines represent the exponential approximation of SR that we have given in Table 1. (b) Measurements (1) and predictions (from Eq. (6)) of SR of two POPE/SOPC mixtures. (c) Measurements (1) and predictions (from Eq. (6)) of SR of a 45/55 DGDG/SOPC mixtures. Since the data points of the SR measurements with DGDG are not available in the literature, we chose to take the approximation given in Rand and Parsegian (1989) without checking it. All predictions in (b) and (c) are well within the experimental accuracy which is typically 0.3 for the pressure on a Log scale and 0.1 nm for the distance on a linear scale.

5. Water thickness at the transition point: model and experimental validation

Having demonstrated that the model predicts SR for mixtures of lipids with similar chain lengths and in fluid phase, it can be shown that the model may also be employed to predict the pressure/dehydration at which the transition to a non-bilayer phase will occur. As mentioned above, it is well known that Lα-phases are not always stable. When dehydrated, or, equivalently, when pressure is applied, bilayers can convert to a non-bilayer phase, usually an inverted hexagonal phase, H_{II}, or a rhombohedral phase, *R*. There has been a reemergence of interest in these transitions during the past few years because of their relevance to membrane fusion. Since real membranes are composed of various lipid mixtures, it would be useful to be able to predict the transition for such bilayers. When the transition is documented for pure lipids, the model presented above allows the description of the transition for any mixture of these lipids. The simplest approach begins with the assumption that the water thickness at the transition for a lipid

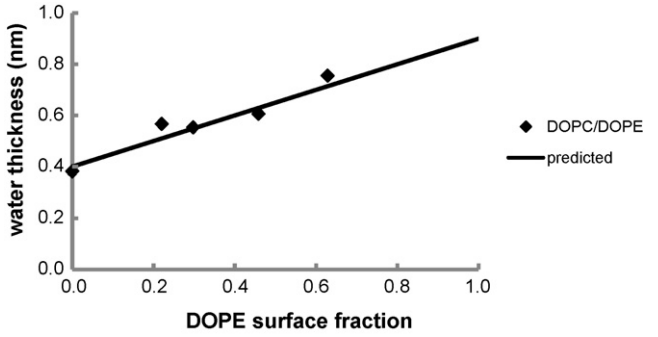

Fig. 3. Water thickness at the transition for DOPE/DOPC mixtures. Diamonds are deduced from experiments (see text for details) and predictions are made according to Eq. (8). The DOPE surface fraction is obtained by Eq. (7b).

mixture, $d_{\text{transition}}$, is the average of those of the component pure lipids, $d_{\text{trans},i}$, weighed by their surface fraction, κ_i.

$$d_{\text{transition}} = \sum_{i=1}^{m} \kappa_i d_{\text{trans},i} \tag{8}$$

Even though it will only be shown in the next section that Eq. (8) is valid on the specific example presented here, that of DOPC/DOPE mixtures, there is indeed no physical justification of this assumption. Here, the main focus will be placed on DOPC/DOPE mixtures because, to our knowledge, extensive studies have only been performed on these mixtures. However, in general, for a binary mixture, it can be expected that $d_{\text{transition}}$ will vary monotonically with the molar fraction of one component. Since $d_{\text{trans},i}$ will remain in a narrow range between 0.1 nm and 1 nm, in reality, there can only be slight deviations from this simple approach and the error resulting of Eq. (8) will be limited.

In order to verify the approach stated in Eq. (8), we compared the calculated $d_{\text{transition}}$ curve of DOPE/DOPC systems with those obtained by experimental data (Yang et al., 2003), as shown in Fig. 3.

The water thicknesses at the transition for pure DOPC and DOPE are found in the literature. Yang et al. (2003), have measured the relative humidity (RH) at which transition occurs for various DOPE/DOPC mixtures. RH is directly related to $d_{\text{transition}}$ as described by Eq. (9) which is valid for pure lipids, as well as lipid mixtures.

$$d_{\text{transition}} = \lambda_{\text{total}} \ln\left(-\frac{P_{0\text{total}} v_w}{k_B T \ln(\text{RH})}\right) \tag{9}$$

For DOPC the relative humidity at the transition is 0.45. $P_{0_{DOPC}}$ and $\lambda_{0_{DOPC}}$ are given in Table 1. Therefore $d_{\text{DOPC}} = 0.4$ nm.

For DOPE, d_{DOPE} is directly obtained from the amount of water in the H_{II} phase when the lipids are fully hydrated: $d_{\text{DOPE}} = 0.9$ nm (Gawrisch et al., 1992). Applying these values for pure component lipid to Eq. (8), the predicted curve of $d_{\text{transition}}$ is obtained as a function of DOPE surface fraction, shown as solid line in Fig. 3.

Applying Eq. (7), we can obtain $P_{0_{\text{total}}}$ and λ_{total} for the various lipid mixtures used by Yang et al. (2003). Eq. (9) can then be applied with their experimental RH data to obtain the corresponding $d_{\text{transition}}$ at different DOPE surface fractions. These experimental $d_{\text{transition}}$ values are shown as diamonds in Fig. 3.

Fig. 3 clearly shows that the simple model predicts accurately the water thickness at the transition point over the whole range of DOPE surface fractions that are experimentally achievable.

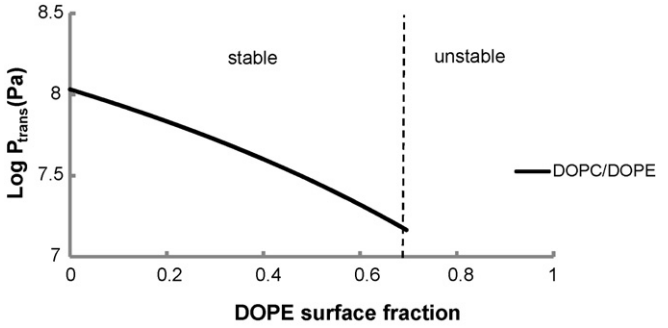

Fig. 4. The full curve of transition pressures for DOPE/DOPC mixtures as a function of the DOPE surface fraction. P_{min} is directly obtained from the interactions of DOPE lamellar phase at 14 °C.

6. Pressure for the phase transition from lamellar phase: model and experimental validation

Now that the model provides a good estimate of the water thickness at the transition point, it can be shown how to obtain the pressure that has to be applied on the lamellar phase of the membrane to induce this transition, $P_{transition}$. Once SR and $d_{transition}$ are known, the pressure can be immediately obtained by applying Eq. (6a) for $d_w = d_{transition}$.

However, there is still a complication: for some lipids, such as DOPE at 25 °C, bilayer structures are not stable. This is due to the fact that attractive forces, usually van der Waals forces, bring the apposing membranes into sufficiently close proximity to trigger the transition. In the mixtures where such lipids are involved, bilayers will not form when the fraction of this lipid is too large. Usually, this fraction is large enough for the "unstable" lipid to be the major component of the mixture. The pressure, P_{min}, at which the bilayer structure is no longer stable, can be estimated by calculating the van der Waals forces within this lipid. For instance, for DOPE, since van der Waals forces will not vary significantly with temperature, this pressure can be obtained from the interaction measurements on the Lα-phase at 14 °C (Gawrisch et al., 1992): $P_{min} \sim 10^{7.1}$ Pa. This means that as soon as the transition pressure is below P_{min}, the lamellar phase will not be stable even when the mixture is fully hydrated. From these considerations, the curve of the pressure of the transition can be calculated. The full pressure curve of DOPE/DOPC mixture is given in Fig. 4.

Again, the calculated value of P_{min} and the full pressure curve can be compared to experimental data. Fig. 5 shows that predicted RH values at the transition are in full agreement with the data points experimentally measured by Yang et al. (2003). It is not surprising that a good agreement occurs below P_{min}, because this is basically

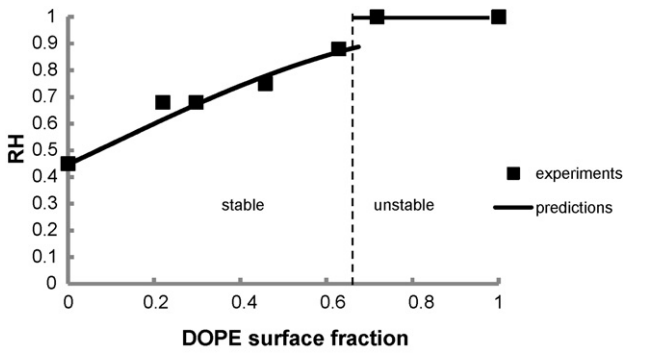

Fig. 5. The relative humidity at which the transition from Lα-phase to a H_{II}- or R-phase for DOPE/DOPC mixtures. The experimental data (square) are directly taken from Yang et al. and the predictions are made using Eqs. (6a), (8) and (9).

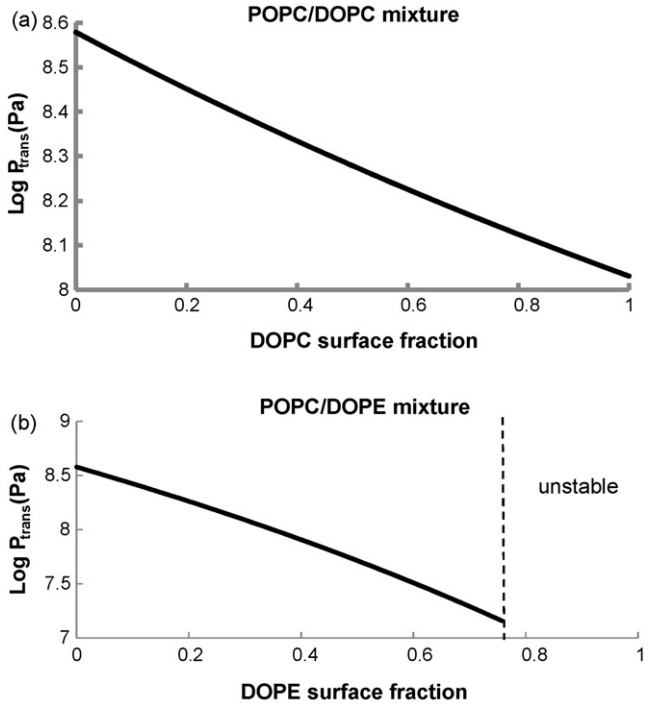

Fig. 6. Transition pressure for (a) a mixture of POPC and DOPC lipids. (b) A mixture of POPC and DOPE lipids.

the same result as in Fig. 3. What is remarkable is that P_{min} is also successfully predicted by the model. The Lα-phase is no longer stable when the transition pressure is below P_{min}, meaning that under these pressures, RH = 1.

The same procedure can be applied to other lipid mixtures. For instance, POPC (palmitoyloleoylphospatidylcholine) is another lipid for which SR has been well characterized. The water thickness at the transition for POPC is 0.15 nm (Klose et al., 1992). With these data, the transition pressures for POPC/DOPC and POPC/DOPE mixtures can be predicted. Fig. 6 shows that with these three lipids – POPC, DOPC and DOPE – it is possible to finely tune the transition pressure of the bilayers between about 100 atm and 10,000 atm.

7. Conclusion

Phase diagrams and interactions of lipid mixtures are difficult to experimentally establish because of the rapidly increasing numbers of parameters involved. The results presented here show that the knowledge of the characterization of pure lipids is sufficient to deduce the properties of any mixture containing these lipids. For lipids in fluid phase, the model presented can predict: (i) the SR, (ii) the pressure/hydration at the transition point to non-bilayer phases, and (iii) the compositions at which the bilayer structure will not be stable even at full hydration. This model will be a powerful tool when applied to quantify fusion in complicated membrane systems where many lipids can be involved.

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