Thermodynamics of membrane lipid hydration*

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Abstract: Zwitterionic or polar lipids that form lamellar phases swell in the presence of water to take up between 10 and 30 water molecules per lipid. The degree of the water uptake depends both on the state of the alkyl chains, liquid or solid, and on the nature of the polar group. The swelling behavior has been extensively characterized on the free-energy level through measuring the relation between the chemical potential of the water and the degree of swelling. In spite of the extensive studies of this type, consensus is still lacking concerning the molecular mechanism causing the swelling. The two main ideas that explain the existence of the effectively repulsive force between two opposing bilayers are (i) a water structure effect and (ii) thermal excitations of the lipid molecules. The first is from a thermodynamic perspective caused by a negative partial molar enthalpy of the water, whereas for the second, the repulsion is caused by positive entropy.

A further insight into the swelling behavior is obtained by simultaneously measuring the partial molar free energy and the partial molar enthalpy using a twin double calorimeter. Such measurements show for binary lipid–water and for ternary lipid–cholesterol–water systems that the first four water molecules enter the bilayer driven by a favorable enthalpic interaction, whereas for higher water contents, the partial free energy and the partial enthalpy have opposite signs. In spite of the fact that the partial free energy varies with water content in a similar way, the partial enthalpies depend strongly on the nature of the lipid sample. Thus, it is obvious that a molecular interpretation of the enthalpy values has to involve the lipid degrees of freedom. This is a strong indication that the interpretation of the free energies should also include the same degrees of freedom. This gives experimental evidence in favor of an interpretation of the molecular mechanism causing the swelling that involves changes in the thermal excitation of the lipid degrees of freedom.

INTRODUCTION

When the lipids extracted from biological membranes are rehydrated, they typically form a lamellar liquid-crystalline phase. In equilibrium with a large excess of pure water, the extent of the uptake into the lamellar structure depends on the nature of the polar head-group of the lipid. Among the extracted lipids of the order of 10 to 20 % are charged and, when exposed to pure water, such a fraction of charged groups is more than sufficient to cause a strong and, in practice unlimited, swelling. However, the main fractions of the extracted lipids are zwitterionic of the phosphatidyl choline or phosphatidyl ethanolamine type, or they carry a polar sugar head-group. In pure form, these lipids only show a limited uptake of water in excess water, provided they form a lamellar phase. Depending on the head-group, the maximum swelling is in the range 10 to 30 water molecules per lipid. These numbers are

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substantially reduced when the temperature is decreased so that one enters the gel phase with frozen lipid alkyl chains [1].

For the charged systems, the extensive swelling is clearly due to the repulsive double layer force [2]. The entropy increase of the counterion distribution caused by an increase in available volume substantially exceeds attractive van der Waals force and, in practice, this leads to a water uptake until the lamellar structure is so dilute that it breaks up into finite vesicles. Direct force measurements verify that the changes in free energy associated with this swelling can be accurately described using the Poisson–Boltzmann description of the electrostatic effects [3]. The exception to this general rule is that for highly charged systems, and with divalent counterions like Ca^{2+} and Mg^{2+}, ion–ion correlation effects can turn the picture upside down [4,5], but this is a separate story [6]. The Poisson–Boltzmann description also provides a prediction of the enthalpy of hydration in addition to the free energy. Owing to the anomalous temperature dependence of the dielectric permittivity of water, even the direct ion–ion Coulomb interaction is dominantly of an entropic nature and the enthalpy contribution has, in fact, the opposite sign to the free energy [7]. There are a number of direct calorimetric measurements that support this qualitative prediction [8]. However, the enthalpies are small and difficult to measure at the high swelling limit, where one expects the continuum description, implicit in the Poisson–Boltzmann theory, to be most accurate.

It has turned out much more problematic to establish the molecular mechanism leading to the water uptake for zwitterionic and sugar lipids. In the absence of a repulsive electrical double-layer force, the standard paradigm of colloidal stability would lead to the conclusion that the dominating interaction is an attractive van der Waals force. This operates in any symmetrical system and, if unopposed, it would simply collapse the lipid bilayers, leaving no water in between. More than 25 years ago, Parsegian and coworkers [9,10] started to conduct a pioneering set of experiments measuring the relation between the water content of zwitterionic lamellar systems and osmotic pressure, i.e., the water chemical potential. These measurements demonstrated that a short-range strongly repulsive interaction is operating in these systems. They concluded that this was a qualitatively new type of effect, and they called it the "hydration force". A somewhat unfortunate consequence of this terminology is a widespread belief that the name also carries some explanatory power. In fact, the term is purely operational and it is far from satisfactory to mechanistically explain that force causing the hydration is the hydration force.

In spite of extensive efforts, there is at present no consensus about the molecular mechanism behind the short-range repulsive forces that causes the swelling of zwitterionic lipid lamellar phases. A number of suggestions have been put forward, and one can identify two basic approaches toward explaining the effect. One choice is to focus on the properties of the water and identify a characteristic decay length determined by the properties of liquid water. Already in 1976, Marçelja and Radic [11] developed a theory along these lines, and it was later further elaborated by Gruen and Marçelja [12]. The basic idea is that the surface induces a polarization of the water, and this polarization decays into the solution with an intrinsic decay length. As the polarization from two opposing surfaces meet, there appears a frustration that can be relieved by introducing more water between the surfaces, and this amounts to a repulsive force. Since, in this model, the repulsion is caused by a structure imposed by interactions, the mechanism is basically of an energetic origin, and one should expect that the enthalpy contribution dominates the free energy.

The basic alternative to the "water structure" concept is to focus on the properties of the lipid bilayers. In the liquid-crystalline state, the lipid molecules have the conformational freedom as in a liquid, although they are confined to remain in the lamellae by basically hydrophobic interactions. In this approach, it becomes particularly important to consider the molecular nature of the head-group, which is the group that interacts directly with the water, and that is also facing the other bilayer of a lamellar stack. There are a substantial number of molecular degrees of freedom in the head-group that are highly thermally excited. When a direct molecular contact is established between molecules of opposing bilayers, this leads to a confinement that reduces the conformational freedom. This mechanism is well es-

tablished for causing repulsion in systems with polymer-covered surfaces [13]. Israelachvili and Wennerström [14] proposed that basically the same mechanism explains the observed short-range repulsive forces also in the lipid systems.

This paper is not the right place to go through the pros and cons of the "water structure" and the "lipid conformation" points of view. However, it is clear that one road toward resolving the controversy is to acquire new experimental data that will shed light on this scientific issue. The discussion of the mechanism has exclusively been based on force data, which in a thermodynamic language means free-energy data. It is obvious that a more complete thermodynamic characterization of the phenomenon is obtained if one also measures the enthalpy of the hydration.

THE CALORIMETRIC METHOD

Based on an original design by Wadsö and Wadsö [15], Wadsö and Markova [16] developed a sorption calorimetric method that allows for a simultaneous measurement of both enthalpy and free energy of hydration. The basic principle is to have two sample cells that are thermally isolated relative to one another, but with a diffusional water vapor contact between them. One of the sample cells contains pure liquid water, while the other cell contains the sample of interest where there is a gradual sorption of water provided by the reservoir of the other cell. Under the assumption that the rate-limiting step is the diffusional transport of water between the two samples, one can use the two measured heat effects in the two samples cells to determine changes in both enthalpy and free energy during the sorption process. At steady state, the rate of evaporation in the cell containing pure water is the same as the rate of sorption into the sample. From the measured heat of evaporation, one readily determines the amount of water transferred to the sample at each instant. Since the diffusion between the two cells is driven by the difference in water chemical potential, one can, after calibration, deduce the instantaneous water chemical potential of the sample from the measured transport rate. Additionally, the difference of the heat effects in the two cells, divided by the sorption rate, is a direct measurement of the partial molar enthalpy of the water in the sample counted relative to pure water.

For the lipid systems, there are in practice mainly four factors limiting the accuracy of the measurements. First, the assumption of fast equilibration within the samples relative to the diffusional transport can break down. This is particularly important at low water contents, where the transport is fastest due to the maximal difference in water chemical potential and, more importantly, where there are occurring major rearrangements in the sample. In the measurements, we find a certain lack of reproducibility in this stage of the sorption process. An additional experimental difficulty affecting the result in this concentration regime is that, when starting from a perfectly dry sample, this is often not at the true equilibrium represented by a perfect crystalline lipid phase [17]. The other two limitations appear when one approaches conditions corresponding to the fully saturated system. Then the diffusion rate decreases, which results in reduced heat effects toward the detection limit, and the measured partial molar enthalpy therefore contains substantial uncertainties. It is, furthermore, difficult to reach accuracy for chemical potentials exceeding relative humidities of 98 % in the sample. The force measurements based on the osmotic stress technique are typically performed for osmotic pressures, giving relative humidities higher than 98 %, but there is a region where the calorimetric data overlap with the osmotic stress data.

EXPERIMENTAL OBSERVATIONS

We have performed simultaneous measurements of the partial molar Gibbs free energy and the partial molar enthalpy for a number of lipid systems [18,19]. In Figs. 1 and 2, we show a representative set of these calorimetric data. These measurements provide information not only on the hydration process as such, but also on phase transitions and phase equilibria. In particular, for the mixed system dimyristoyl phosphatidyl choline–cholesterol, the sorption studies are providing detailed information about the
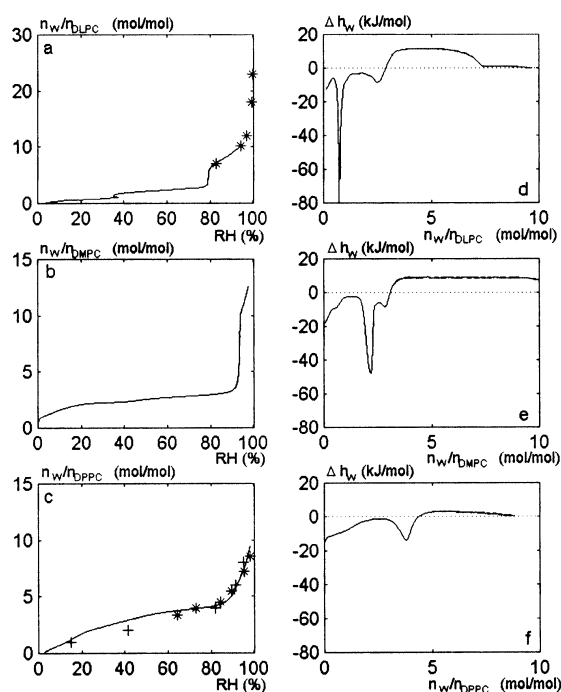
Fig. 1 Microcalorimetric data for binary systems phosphatidyl choline–water [18]. (a–c) Sorption data for (a) dilauroyl phosphatidyl choline at 25 °C, (b) dimyristoyl phosphatidyl choline at 27 °C, (c) dipalmitoyl phosphatidyl choline at 25 °C; water content [mol(water)/mol(lipid)] vs. relative humidity. For comparison; (a,c) Osmotic stress data (∗) from Lis et al. [10], (c) Results of climatic chamber technique for DPPC at 22 °C (+) from Jendrasiak et al. [20]. (d–f) Simultaneously obtained enthalpy curves for (d) dilauroyl phosphatidyl choline at 25 °C, (e) dimyristoyl phosphatidyl choline at 27 °C, (f) dipalmitoyl phosphatidyl choline at 25 °C; partial molar enthalpy of water in the sorption process as a function of water content [mol(water)/mol(lipid)].

phase behavior [19]. In Fig. 1, force data from the literature are also included [10,20]. It is very gratifying that the calorimetric method reproduces well within the experimental uncertainty the previous force data.

In relation to the problem of resolving the mechanism of the short-range repulsive forces of lipid hydration, the set of data of Fig. 1 demonstrates a few qualitative points. The force per unit area between bilayers is directly related to the chemical potential of water. Expressed in this way, it is tempting to think about the force in terms of the molecular properties of the water. However, after reflecting over the implications of the Gibbs–Duhem equation it is clear that, from a molecular point of view, we must think in terms of the combined lipid–water system. We could equally well express the data in terms of the chemical potential of the lipid after a transformation according to the Gibbs–Duhem recipe. A second qualitative feature is that, as the sorption progresses, the chemical potential must increase, by thermodynamic necessity. For the enthalpy, on the other hand, there is no such requirement on monotonic behavior, and the enthalpy curves in Fig. 1 d–f indeed show regions with both negative and positive partial molar enthalpies.

For the data of binary systems lipid–water shown in Fig. 1, there is a qualitative similarity between the different lipid systems. At the lowest water contents, the partial molar enthalpy is negative, which is consistent with the notion that the hydration is caused by a strong direct interaction between the first water molecules entering the bilayer and the highly polar phosphate in the head-group. This interpretation is also consistent with direct spectroscopic observations [21]. When approximately four water molecules per lipid have entered, there is a distinct switch from an exothermic to an endothermic

behavior, and the partial molar enthalpy remains positive for the remaining range of observation. For the cases of dilauroyl and dimyristoyl compounds, the swelling induces a transition from the gel to the liquid-crystalline arrangement of the lipid chains, and, in this region, the enthalpy effect can dominantly be attributed to the melting of the hydrocarbon chains (Fig. 1, d,e). However, at higher water contents where only the liquid-crystalline phase is present, the partial molar enthalpy is still positive. Also, for the dipalmitoyl derivative, where one is below both the main- and the pretransition at all water contents, there is still a substantial positive partial molar enthalpy at high water contents (Fig. 1, f). In these cases, the challenge is to identify whether the main changes occur in the alkyl chains, or if instead are due to rearrangements in the head-group region. In any case, the swelling is clearly caused by the lowering of the free energy due to an increase in entropy, while the enthalpy is in fact increasing, giving a positive contribution to the free energy change. Figure 3, a,b show the partial molar enthalpy and the chemical potential for the swelling of the gel and the liquid-crystalline phases. It is observed that the partial molar enthalpy is higher in the gel phase than in the liquid-crystalline phase. Furthermore, the magnitude of the enthalpy is considerably larger than the magnitude of the partial free energy of opposite sign, demonstrating that, also in this case, one has a large entropy–enthalpy compensation, so often observed for aqueous systems [22].

For the ternary system dimyristoyl phosphatidyl choline–cholesterol–water, the trend is that the change in the partial molar free energies becomes more gradual the higher the cholesterol contents (Fig. 2, a,b). In contrast to this, there is a qualitative change in the behavior of the partial molar enthalpies when the cholesterol content is increased from 25 to 37 % (mol/mol) (Fig. 2, c,d). At the lower cholesterol content, the enthalpy curve shows the same qualitative features as for the pure lipid systems. For the 37 % (mol/mol) sample, on the other hand, the partial molar enthalpy is less negative at low water contents, presumably reflecting the fact that the cholesterol head-group already provides an OH group that can coordinate to the phosphate of the phospholipid. At water contents exceeding four waters per lipid, there is no longer the abrupt change to positive values, but rather a more gradual decrease in magnitude of the enthalpy. Ultimately, it turns positive at the highest water contents studied, but with values that just about exceed the detection limit.
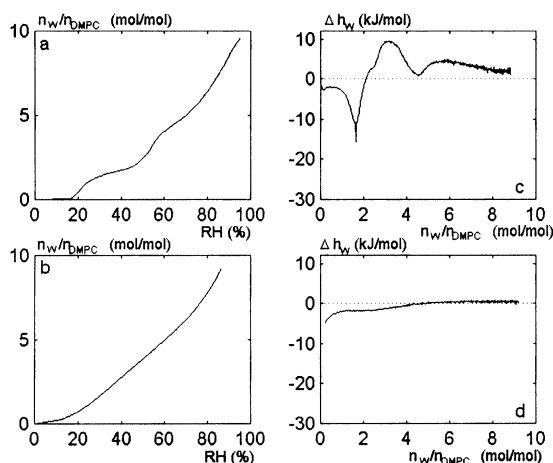

Fig. 2 Microcalorimetric data for dimyristoyl phosphatidyl choline–cholesterol mixtures at 27 °C [19]. (a–b) Sorption data for (a) X_{chol} = 25 % (mol/mol) (b) X_{chol} = 37 % (mol/mol); water content [mol(water)/mol(lipid)] vs. relative humidity; (c–d) Simultaneously obtained enthalpy curves for (c) X_{chol} = 25 % (mol/mol) (d) X_{chol} = 37 % (mol/mol); partial molar enthalpy of water in the sorption process as a function of water content [mol(water)/mol(lipid)].

At the cholesterol content of 37 % (mol/mol), the lamellar system is in the so-called liquid or-dered state [23,24], where the alkyl chains of the phospholipid adopt a near all *trans* conformation. Thus, in contrast to the binary lipid–water systems, the swelling has no major effect on the alkyl chain conformation, reducing the contribution to the enthalpy from these degrees of freedom. The combina-tion of the similarity between the partial molar free energies, and the differences between the partial molar enthalpies for the binary and ternary lipid systems, exemplifies the fact that the partial molar quantities are properties of the combined systems, and that it can be very misleading to see the partial molar quantities of the water as a property of the water per se.

MOLECULAR MECHANISM OF SWELLING

Phospholipid bilayers of a purely zwitterionic character can incorporate substantial amounts of water. However, there is a maximum uptake to the swelling limit in equilibrium with pure water. The location of this limit depends both on the polar head-group and on the state of the lipid chains [1]. Phosphatidyl choline in the liquid-crystalline state can incorporate up to 30 water molecules per lipid, while in the gel state the swelling is completed already at 15 to 20 water molecules. For the structurally similar phosphatidyl ethanolamine, the swelling is much less extensive even in the liquid-crystalline state, which can accommodate ca. 20 water molecules per lipid. The swelling is even less extensive for lipids with uncharged sugar head-groups. There is an uncertainty concerning the precise values for the swelling limit. This applies particularly to phosphatidyl choline systems, in which the liquid-crystalline phase shows a substantial swelling. One source of this uncertainty is that it is in fact very difficult to reach the true minimum of the interaction curve when it is shallow. Defects can develop in the sample and it is, for example, well established that the lamellar systems tend to form liposomal structures with many concentric bilayers [25,26]. In the swelling limit, the tensions that can develop in these liposomes are not negligible relative to the interbilayer forces, and the water uptake is furthermore affected by the packing of these liposomes.

To establish a molecular understanding of the mechanisms determining the swelling behavior, it is clearly necessary to consider the combined system of lipid and water. The experimental observations of the swelling imply that, up to the swelling limit, the water molecules prefer the environment of the aqueous space of the bilayer stack relative to that of the pure liquid water. For the first few water mol-ecules (<4), the affinity to the bilayer is very high and the enthalpy measurements show that the domi-nating contribution comes from the direct water–phosphate head-group interaction. As in crystalline hydrates, the small and polar water molecule can occupy sites between the more bulky polar groups of the lipid, in such a way that it becomes energetically favorable. Thus, in this concentration regime, it is the primary hydration of the phosphate groups that accounts for the swelling. The water molecules have a much weaker direct interaction with the quaternary ammonium group than with the phosphate. One conceivable option is then that the swelling ceases and one has an equilibrium between a crystalline lipid bilayer and excess water. For long-chain lipids, this indeed happens below the so-called subtran-sition. However, in the most-studied cases, the swelling is in fact continuous. The calorimetric meas-urements described above reveal that the swelling is essentially endothermic in this regime. This im-plies that, by introducing water, the lipid degrees of freedom are thermally excited, which increases the entropy of the system to such a degree that it compensates for the increase in enthalpy. In principle, the entropy increase could also be attributed to the water degrees of freedom, but for the water the refer-ence state is liquid water with a high entropy, in contrast to the lipid, which starts out from a solid state. Using this perspective, it is clear that the calorimetric measurements support the basic swelling mech-anism suggested by Israelachvili and Wennerström [14], which emphasizes that the entropic contribu-tion dominates the free energy. For the ternary system phospholipid–cholesterol–water of Fig. 2d, the measurements show that the swelling from zero water to full hydration is exothermic for the case of 37 % (mol/mol) cholesterol. Throughout this swelling, the alkyl chains of the phospholipid remain in a liquid ordered phase, and the observed enthalpy changes can be mainly attributed to interactions in the

polar region of the system. The fact that one also for this case observes a weakly positive enthalpy for higher water contents (Fig. 3c) is then a further indication that it is really the entropy effects that dominate the swelling.

Is it also possible to interpret the calorimetric data in the framework of water-structure effects? For the binary lipid–water systems studied, there is clearly a large contribution from the chain conformations to the observed enthalpies. It is a possibility that these changes in the lipids are of secondary importance when it comes to the free energy. One can invoke a near perfect enthalpy–entropy compensation for these degrees of freedom, and maintain that the dominant contribution to the free energy has a different origin. This involves a deviation from the simplicity principle referred to as Occam's razor, but Nature does not necessarily appear simple from our perspective.
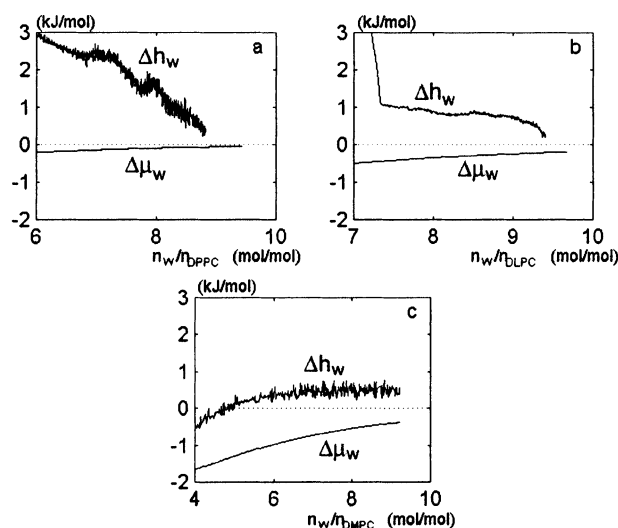

Fig. 3 The partial molar enthalpy of water, Δh_w, and the chemical potential of water, $\Delta \mu_w$ in the region of swelling of the (a) dipalmitoyl phosphatidyl choline gel phase, (b) dilauroyl phosphatidyl choline liquid-crystalline phase, (c) dimyristoyl phosphatidyl choline–cholesterol liquid ordered phase, $X_{chol} = 37$ % (mol/mol).

CONCLUSIONS

From the calorimetric measurements of lipid hydration under conditions where also the chemical potential of the water is controlled, one obtains a more complete thermodynamic characterization of the process. In contrast to the partial molar free energy, which must increase monotonically through the swelling, the partial molar enthalpy counted relative to pure water takes both negative and positive values, depending on the degree of swelling. Over the range studied, one can identify two regimes. For less than four water molecules per lipid, the swelling is enthalpy-driven, presumably owing to the direct interaction between water molecules and the highly polar phosphate group. At higher water contents, the enthalpy systematically takes a positive value, and it thus opposes the swelling. In this regime, the swelling is due to a strongly positive entropy, and this applies up to the maximum water contents that could be measured with the technique. There is typically a substantial additional swelling between 99 and 100 % relative humidity, but force measurements that include this regime do not indicate a qualitative change in swelling behavior in this range.

It is notoriously difficult to make a definite interpretation of thermodynamic data in molecular terms. For example, the calorimetric measurements demonstrate that for similar systems with similar partial molar free energies, the enthalpy values can be qualitatively different. It is a striking observation that for the sample with a high cholesterol content where spectroscopic studies show that the lipid is in

the liquid ordered state, the enthalpy effects are much smaller than in the binary lipid–water gel and liquid-crystalline phases (Fig. 3). This strongly indicates that, for the binary lipid–water systems, there is a large and positive contribution to the enthalpy from the lipid chain degrees of freedom. It then follows that one cannot ignore these degrees of freedom when interpreting also the partial molar free energy, or in other words, the force. The study provides rather compelling experimental evidence that it is essential to consider the lipid degrees of freedom when discussing the force between lipid bilayers at hydration levels up to at least 10 water molecules per lipid.

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