Effect of Carbohydrates on the Swelling of a Lyotropic Lamellar Phase

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The effect of carbohydrates on the swelling of the DMPC L_α phase has been studied by small angle X-ray scattering (SAXS) and small angle neutron scattering (SANS). The carbohydrates are glucose, oligomers of glucose (up to $n = 5$), polymers of glucose (pullulan, $M = 45\,000$), and a hydrophobically modified polysaccharide (cholesteryl−pullulan = CHP). Three regimes of swelling have been observed: (1) Small sugars (up to $n = 3$) penetrate into the aqueous layers of the lamellar phase and cause a monotonic swelling of the lamellar phase. (2) Macromolecular carbohydrates are sterically excluded from the aqueous layers and retain some of the water in a coexisting isotropic phase; this causes an osmotic compression of the lamellar phase. (3) Hydrophobically modified carbohydrates (CHP) can be introduced into the aqueous layers. The polymer containing lipid phase is a new lamellar phase (L_p), with water thicknesses much larger than those observed in the absence of polymer.

Introduction

Lecithins are zwitterionic phospholipids which represent the most important class of lipids in biological membranes. DMPC is a synthetic lecithin which forms lamellar structures in water over a wide range of concentrations.[1] Due to the lack of strong electrostatic repulsion between bilayers, lecithins have low swelling limits corresponding to hydration levels of 40% water. At hydration levels above this value, the L_α phase at maximum swelling coexists with almost pure water in excess. In this biphasic system, the repeat distance of the lamellar phase is due to the equilibrium between two opposed contributions: the attractive van der Waals force and the hydration force originating from the interaction of water molecules with the polar head groups of the lipid.[2]

The modification of swelling by small sugars and polysaccharides in the egg lecithin/water system has been investigated by LeNeveu et al.[3−6] Increasing concentrations of sugars solubilized in the aqueous region of the lamellar phase caused an increase followed by a decrease in the maximum swelling of the lamellar phase. These observations were interpreted as successive weakening and strengthening of the attractive van der Waals force with increasing sugar concentrations. Equilibrium with aqueous solutions of dextran caused an osmotic compression of the lamellar phase.

In this work we are interested in the behavior of cholesteryl−pullulan (CHP), a hydrophobically modified polysaccharide obtained by modification of natural pullulan produced by the fungus *Aureobasidium pullulans*. This semisynthetic polymer is of interest because of its surfactant properties and associative behavior in aqueous solution.[7−9] Polymer solutions of CHP in water lead to the formation of soluble micellar type aggregates, with a hydrophobic cholesterol core surrounded by the solvated polysaccharide chains. The formation of these aggregates is hydrophobically driven and originates both from intramolecular and intermolecular interactions between cholesterol groups. The ability of hydrophobically modified soluble polymers to interact with phospholipid bilayers by anchoring of lateral hydrophobic groups has been already investigated.[10,11]

Our aim is to introduce the CHP derivatives in the aqueous layers of the DMPC lamellar phase and to examine how the anchoring and the swelling of the macromolecules affect the thickness of the water layers. We will also consider the effect of anchoring the polysaccharide on the membrane properties.

The bending of membranes by linear polymers bearing a single lipid anchor has been recently reported theoretically.[12] In our system, the polysaccharide backbone is a rather low molecular weight (50 000) flexible chain in good solvent in water with an average number of three anchors per chain. The low molecular weight of the polymer makes negligible the entropy loss arising from the confinement of the chains at the lipid−water interface compared to the anchoring energy.[12]

Materials

Chemicals. DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Glucose (α-D-glucopyrannose) and maltose (D-maltose monohydrate) were purchased from Fluka (Buchs, Switzerland). The oligosaccharides maltotriose and maltopentaose were purchased from Merck (Darmstadt, Germany).

The repeat units that built pullulan and the hydrophobically modified pullulan, cholesteryl−pullulan (CHP), are presented in Figure 1. Pullulan is an amorphous and flexible homopolymer[13] composed of maltotriose (A) repeat units with glucose subunits linked via α-1,4- and α-1,6-glycosidic bonds.[14] The polysaccharide chains are in good solvent in water.[13,15−17] Pullulan 45 (molecular weight = 45 000) was from Hayashibara Biochemical Laboratories (Okayama, Japan). We have checked the molecular weight and polydispersity of native pullulan by size exclusion chromatography−law angle laser light scattering (SEC-LALLS). The results yielded $M_n = 44\,500$, $M_w = 57\,500$, and a polydispersity $M_n/M_w = 1.29$.

The CHP derivative was a gift from Prof. J. Sunamoto and Dr. K. Akiyoshi[18] and was synthesized as described in ref 9. $CHP_{50-0.9}$ ($M_w = 50\,000$ and degree of substitution = 0.9 cholesterol group/100 glucose units) is a random copolymer of native A units (99.1 mol %) and hydrophobically modified B

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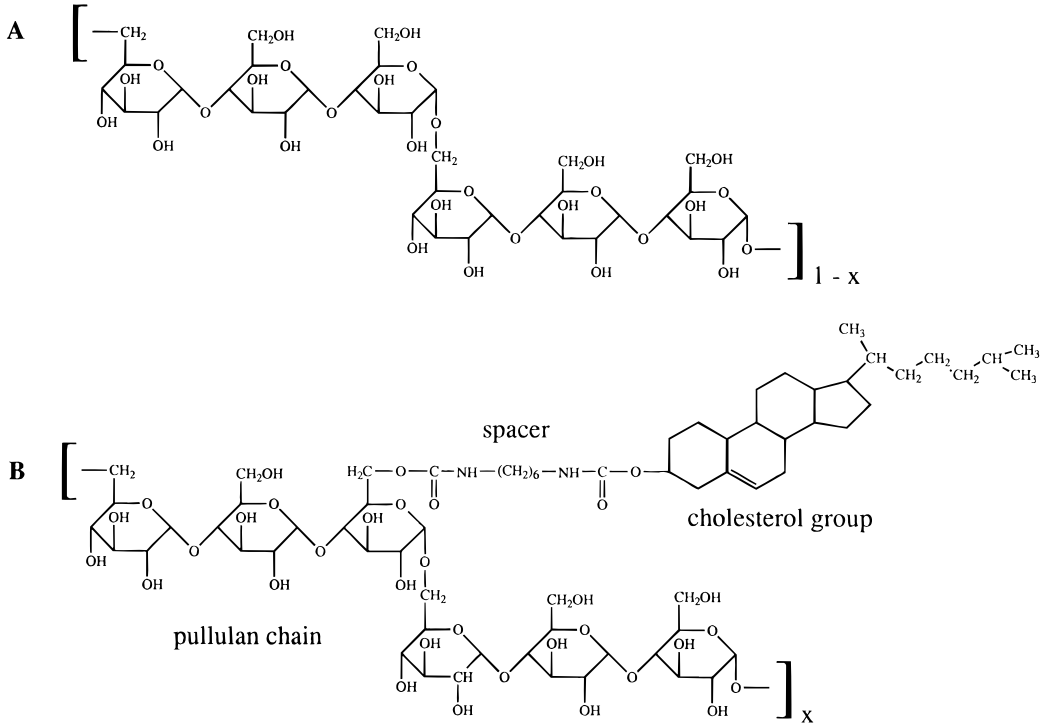

Figure 1. Molecular structure of pullulan (A units) and cholesteryl−pullulan (A/B units with a 99.1/0.9 molar ratio).

units (0.9 mol %) that bear lateral cholesterol groups attached to the polysaccharidic backbone via a flexible spacer. The substitution degree of the polysaccharide corresponds to an average number of 3 cholesterol groups/chain and a mean distance of 620 Å between the anchors on the stretched polysaccharidic backbone.

Water was produced by the Milli-Q water purification system from Millipore (Bedford, MA). All experiments were performed in glassware cleaned with a solution of $(NH_4)_2S_2O_8$ in pure sulfuric acid and abundantly rinsed with the Milli-Q water. Heavy water used in the SANS experiments (deuterium enrichment = 99.8%) was from Euriso-top (Saclay, France).

X-ray and Neutron Scattering Experiments. The X-ray scattering experiments were performed on a Guinier-Méring type camera operating in vacuum with a linear 1024 channel detector and a high-flux Huxley-Holmes type camera equipped with a 2D 256 × 256 channels detector. The sample to detector distances are respectively 490 and 2100 mm and the $Q = 4\pi(\sin\theta)/\lambda$ ranges respectively 0.03−0.75 and 0.02−0.35 Å^{-1}. The Huxley-Holmes camera is described in detail in refs 19 and 20.

The neutron scattering experiments were performed on the PAXE spectrometer located at the end of the G5 cold neutron guide of the Orphée reactor (Laboratoire Léon Brillouin, CEN Saclay, France). The spectrometer is equipped with a 2D 64 × 64 channels detector. In this work, most experiments were performed with a sample to detector distance of 5100 mm and a wavelength of 7 Å with a spread in $\Delta\lambda/\lambda = 10\%$. With this configuration the Q values range from 0.007 to 0.11 Å^{-1}. Some additional experiments were carried out with a wavelength of 15 Å to investigate lower Q values (0.003 Å^{-1}) and to enhance the resolution in this Q range.

The samples were held in 1 mm thick cells covered with kapton windows for the X-ray scattering experiments and in 1 mm thick quartz cells for the neutron scattering experiments. In both cases, the cells are thermostated at a controlled temperature of at least 30 °C.

The SAXS and SANS spectra are represented by the $I(Q) = f(Q)$ function, where $I(Q)$ is the radially averaged scattered intensity of the two-dimensional pattern and Q the scattering vector. The periodicity of the lamellar phase (d^*) is calculated according to the relation using the position observed for n Bragg peaks $d_n^* = 2\pi n/Q_n$.

Osmotic Pressure Experiments. The osmotic pressure of the pullulan solutions was measured on a thermostated A0278 Knauer membrane osmometer (Berlin, Germany). The experiments were performed at 30 °C.

Preparation of the Samples

The samples were prepared in Milli-Q water in SAXS experiments. In SANS experiments the best contrast in scattering length density is obtained with a completely protonated lipid (Nb = 0.28 × 10^{10} cm^{-2}) in pure heavy water (Nb = 6.37 × 10^{10} cm^{-2}).

Due to problems with the homogeneity of polymer concentration in concentrated lipid samples, great care was taken in the preparation procedures.

Procedure 1. The carbohydrates are dissolved in water at the final water content of the samples. Weighted amounts of DMPC are directly hydrated with the carbohydrate solutions. The samples are sonicated and incubated for 14 days before the small angle scattering experiments. All steps involving the lipid are performed at 40 °C, a temperature higher than the P_β'/L_α transition of the pure lipid in water (23 °C).[21] At that temperature, the aliphatic chains of the lipid are molten (fluid L_α state). As small sugars and pullulan are very soluble in water, it is possible to prepare samples in a large range of concentrations through this procedure.

Procedure 2. Due to the low solubility of CHP in water, it is not possible to hydrate the lipid with concentrated solutions of polymer. In addition, the differences in polarities between phospholipids and carbohydrates make it impossible to prepare a binary mixture of the two compounds in organic solution. For instance, DMPC is soluble in alcohols, chloroform, and other chlorinated solvents, while CHP derivatives are only soluble in water and dimethyl sulfoxide (DMSO), which are both nonsolvents of DMPC. Consequently, the commonly used method

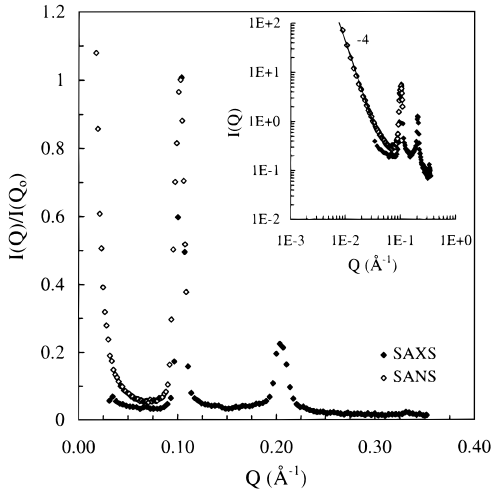

Figure 2. SAXS and SANS spectra of pure DMPC hydrated with 70% water. The spectra are normalized to the first Bragg peak intensity for the comparison. Insert: log/log plot showing the Q^{-4} power law of the scattering at low Q.

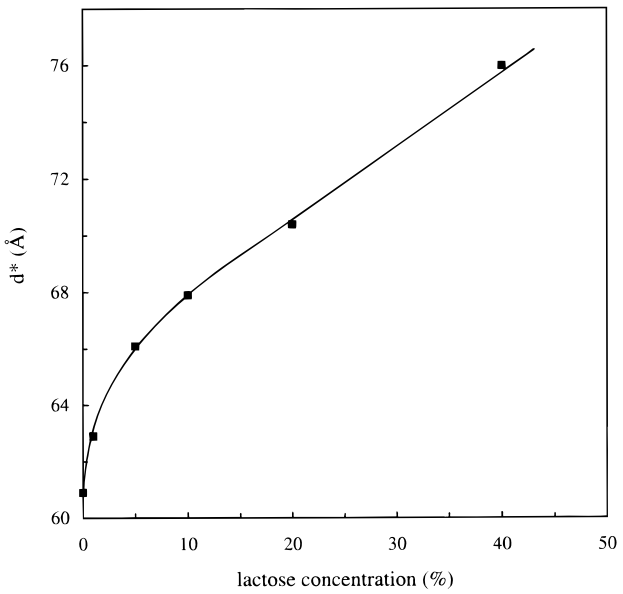

Figure 3. Lamellar repeat distance of DMPC determined by SAXS, as a function of lactose concentration at a lipid hydration of 70%.

of hydration of a mixed film obtained by evaporation of the solvent is not efficient in this case.

Therefore, we first solubilize DMPC in chloroform (2 mg/mL). The solvent is removed by evaporation in a rotating evaporator, and the obtained lipid film is hydrated by a dilute solution of polymer with a large excess of water. Typically, the lipid concentration is 0.5% (w/w), and the concentration of the polymer solution is 0.1% (w/w). The suspension is sonicated and extruded at 40 °C on polycarbonate membranes (porosity 200 nm) to form small unilamellar vesicles (SUV). This step allows non-sterically excluded interactions between the lipidic surface and the polymer. Anchoring of the hydrophobic cholesterol groups in the bilayers is made possible. After incubation of the dilute ternary system, the suspension is freezed by immersion in liquid nitrogen to avoid any phase separation and then freeze-dried. The resulting powder is weighted and rehydrated at fixed water contents. The samples are incubated at 40 °C during 14 days before the small angle scattering experiments. As in procedure 1, all steps are performed at a temperature largely higher than 23 °C. We have controlled the chemical integrity of DMPC by comparing the SANS spectra of pure DMPC hydrated by procedure 1 and with this method. The treatment has no effect on the lamellar spacing and on the shape of the Bragg peaks. This is not a true chemical control, but it is well-known that hydrolysis of diacylphospholipids leads to the production of lysophospholipids and charged fatty acids. We also know from the work of Rydhag and Gabran[22] that the hydration/van der Waals forces equilibrium is very sensitive to the introduction of a few surface charges in the system and that dramatic effects on the periodicity of the lamellar phase are observed at molar ratios of the order of 1%. Any degradation of DMPC during the preparation and incubation period would result in a swelling due to the introduction of an electrostatic contribution in the force balance. We can then conclude that no degradation of the lipid occurs during the preparation and incubation of the samples.

Results

The X-ray and neutron scattering spectra of DMPC samples containing 70% water (w/w) are presented in Figure 2. On SAXS spectra the first and second Bragg peaks are observed, while, in SANS experiments, the Q range has been adjusted in a such way that the first Bragg peak corresponds to the upper limit of the studied Q range. This makes it possible to examine both the pure L_α phase and highly swollen lamellar phases in a single dynamic Q range. According to SAXS and SANS, the lamellar spacings of the L_α phase at 30 °C are respectively 60.9 Å in water and 61.4 Å in D_2O. The scattering increase seen at low angle in SANS data is due to the biphasic nature of the sample. The insert of Figure 2 shows the Q^{-4} power law of this scattering, indicating the phase separation of compact L_α microdomains in suspension in excess water.

1. DMPC/Lactose/Water System. Samples of DMPC have been hydrated with lactose solutions according to procedure 1 at a fixed water/DMPC ratio (70/30 (w/w)); consequently the samples contain the lamellar phase in equilibrium with excess hydrating solution. The lamellar repeat distance of the lamellar phase was measured through SAXS experiments. It is presented in Figure 3 as a function of lactose concentration in the hydrating solution. This curve shows the swelling effect by a small sugar whose size allows its diffusion between the lamellae. Note that the deswelling observed at high sugar concentration by LeNeveu and co-workers[5] has not been reproduced.

2. DMPC/Pullulan/Water System. The influence of pullulan on the swelling of the DMPC L_α phase has been studied in a large range of concentrations (0−40 wt %). The samples have been prepared according to procedure 1. The plot of the lamellar repeat distance as a function of the polysaccharide concentration is shown in Figure 4. From this curve, we can observe the decrease in lamellar spacing upon the addition of the macromolecular sugar. This curve is typical of an osmotic stress experiment. The osmotic compression originates from the equilibrium of the chemical potential of water molecules in the two coexisting phases, the L_α and the solution in excess. This equilibrium is obtained by diffusion of water molecules from the solution to the lamellar phase, while the polysaccharide remains sterically excluded from the interbilayer aqueous region. The consequence of this exchange is to decrease the water layer thickness in the lamellar phase. The insert represents the osmotic pressure of the pullulan solutions as a function of concentration. From these experiments it is possible to determine the relation between the water layer thickness and the pressure in the coexisting isotropic solution of pullulan. This relation will be used to determine the hydration force which competes with the van der Waals attraction. This part is treated in the Discussion.

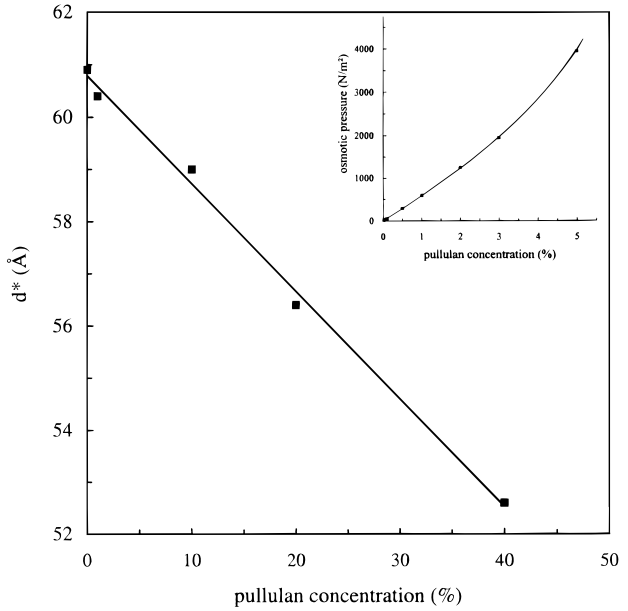

Figure 4. Lamellar repeat distance of DMPC determined by SAXS as a function of pullulan concentration at a lipid hydration of 70%. Insert: osmotic pressure of pullulan solutions as a function of concentration.

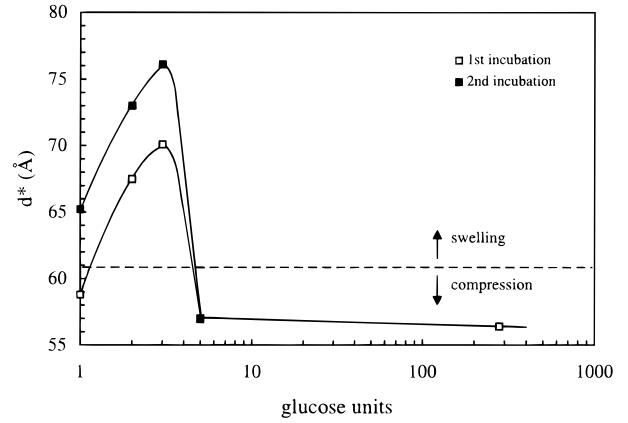

Figure 5. Lamellar repeat distance of DMPC hydrated with 20% sugar solutions determined by SAXS as a function of *n*, from glucose ($n = 1$) to pullulan 45 000 ($n = 280$) and at constant hydration (70%).

3. DMPC/Oligosaccharides/Water System. The effect of oligomers of glucose has been studied in order to determine the size exclusion limit of sugars by the lamellar phase at maximum swelling (biphasic system). The samples have been prepared according to procedure 1. The oligomers correspond to $n = 1$ (glucose), $n = 2$ (maltose), $n = 3$ (maltotriose), and $n = 5$ (maltopentaose). Figure 5 shows the lamellar repeat distance of the DMPC L_α phase as a function of *n*. The sugar concentration in the aqueous phase is constant (20% (w/w)) and the water/lipid ratio is 70/30 (w/w). The samples have been studied after two incubation periods: 14 and 28 days. The experiment was stopped after the second incubation. This experiment shows the influence of the sugar size on the swelling of the lamellar phase. From glucose to maltotriose the lamellar phase is swollen with the following efficiency: maltotriose > maltose > glucose. An opposite behavior is observed with maltopentaose which compresses the lamellar phase by osmotic stress. The point corresponding to pullulan ($n = 280$) has been added to the oligomer series to show the similarity between the polysaccharide and maltopentaose. For the three sugars solubilized in the lipid phase the second incubation period results in higher swellings, showing the importance of the diffusion kinetic of the sugars from the aqueous solution in excess to the lamellar phase. For maltopentaose and pullulan, no kinetic effects are observed. The equilibrium of the system is obtained at the end of the water diffusion step when the sugar remains excluded from the lamellar phase.

TABLE 1: Experimental Compositions Studied in the DMPC/CHP/Water Ternary System

line	DMPC CHP (w/w)	hydration (water %)	line	DMPC CHP (w/w)	hydration (water %)
dilution 1	95/5	50	dilution 2	70/30	82.5
	95/5	60		70/30	85
	95/5	70		70/30	87.5
	95/5	80		70/30	90
	95/5	82.5		70/30	92.5
	95/5	85		70/30	95
	95/5	87.5	line 3	100/0	70
	95/5	90		99.5/0.5	70
	95/5	92.5		99/1	70
	95/5	95		95/5	70
dilution 2	70/30	75		90/10	70
	70/30	80			

4. DMPC/CHP/Water System. Two dilution lines corresponding to fixed DMPC/CHP ratios of 95/5 (w/w) (line 1) and 70/30 (w/w) (line 2) have been investigated. A third line with fixed water/DMPC ratio 70/30 (w/w) and increasing concentration of CHP has also been investigated (line 3). These lines are drawn in Figure 6 (left). Additional samples were prepared to determine more precisely the limits of the observed phases. In this system, all samples have been prepared according to procedure 2. The three steps of the preparation detailed previously are represented on the phase diagram of Figure 6 (right). The compositions investigated by this procedure are given in Table 1.

Dilution line 1: DMPC/CHP = 95/5 (w/w). SANS spectra corresponding to the 95/5 DMPC/CHP ratio at hydration ranging from 50 to 90% are shown in Figure 7 (spectra A−E). On spectrum A, one observes two main diffraction peaks at 0.0397

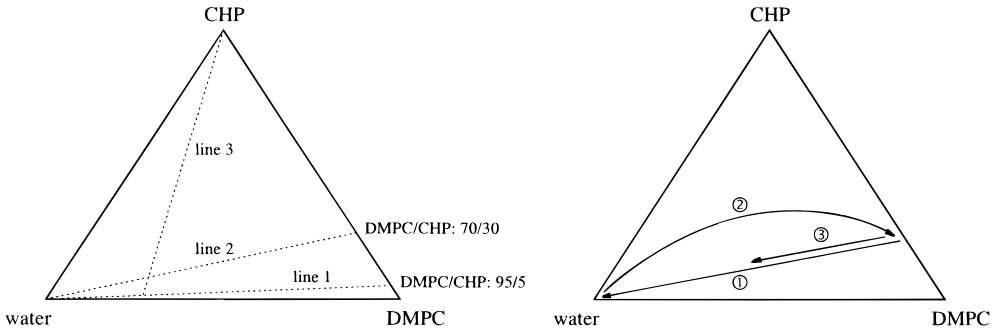

Figure 6. (Left) Experimental lines studied by SANS in the DMPC/CHP/water ternary system. (Right) Schematic representation of the three steps in the preparation of mixed DMPC/CHP/water samples using procedure 2 (see text for details). Line 1: dilution at fixed DMPC/CHP ratio. Line 2: freeze-drying. Line 3: rehydration at fixed water content.

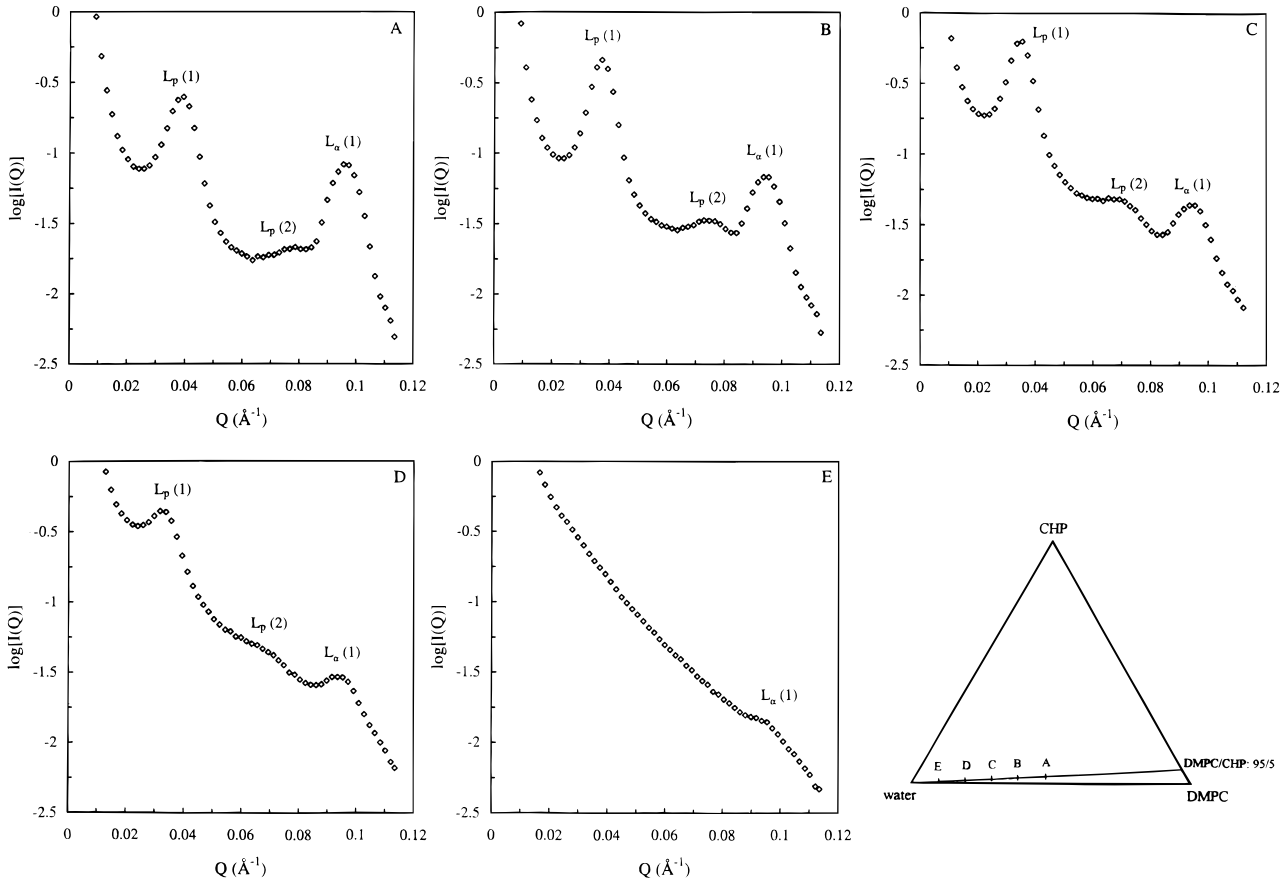

Figure 7. SANS spectra $\log[I(Q)] = f(Q)$ corresponding to the dilution line 1. The samples are prepared with a fixed DMPC/CHP ratio: 95/5 (w/w) in pure D_2O. The hydration is (A) 50, (B) 60, (C) 70, (D) 80, and (E) 90%. Spectra A−D correspond to the biphasic L_p/L_α system.

and 0.0972 Å^{-1} and a secondary peak overlapping with the peak at 0.0972 Å^{-1}. The peak at 0.0972 Å^{-1} corresponds to the first order of the pure L_α phase (Figure 2). The peak at 0.0397 Å^{-1} corresponds to another phase with a much larger swelling. The secondary peak at 0.0787 Å^{-1} corresponds to the second order of the swollen phase. The position of these two peaks corresponds to the characteristic sequence of a lamellar phase (Q, $2Q$, $3Q$, ...) with a repeat distance of 158 Å.

Increasing the hydration of the samples from 50 to 80% (A−D), the observed sequence is conserved, indicating that the phase is still lamellar. The second order of the swollen phase moves away from the first order of the L_α phase, and its position relative to the first order may be determined more precisely (B, C). The position of the first order of the L_α phase is not affected by the dilution of the samples. Therefore, in this biphasic system composed of two coexisting lamellar phases in thermodynamic equilibrium, the L_α phase is at maximum swelling while the L_p phase continues to swell from 0.0397 Å^{-1} (158 Å) at 50% water to 0.0329 Å^{-1} (191 Å) at 80% water. The dilution of the samples causes a decrease of the intensity of the Bragg peaks, but their shape is not affected and no broadening is observed. At 90% water, a residual signal corresponding to the first Bragg peak of the L_α phase is observed. The Bragg peaks of the L_p phase are either out of the investigated Q range or have a signal too low to be detected.

The effect of hydration is shown in Figure 8, where the lamellar repeat distance has been plotted as a function of the inverse of the total lipid fraction of the sample (Φ_{DMPC}) in the domain of coexistence of the two lamellar phases. One can see that the L_p phase swells linearly with $1/\Phi_{DMPC}$, showing that it behaves ideally like a separated monophasic lamellar phase under the effect of hydration. All the water added to the sample is absorbed by the swollen L_p phase. This phase follows the ideal dilution law of lamellar lyotropic phases:

$$d^* = d_b/\Phi_{lipid} \quad (1)$$

but here in the particular case of a coexistence with a lamellar phase at maximum swelling (L_α). The d^* vs $1/\Phi_{DMPC}$ plot does not cross the origin since part of the water of the sample remains in the L_α phase. In this equation, d_b is the bilayer thickness. The fact that the swollen L_p phase coexists with the L_α phase at maximum swelling, indicates that the pressure in the two coexisting phases is close to zero since the lamellar spacing of the L_α phase in this system is the same as in the L_α/water biphasic system where the pressure is zero.

Dilution line 2: DMPC/CHP = 70/30 (w/w). SANS spectra corresponding to the 70/30 DMPC/CHP ratio at hydration levels

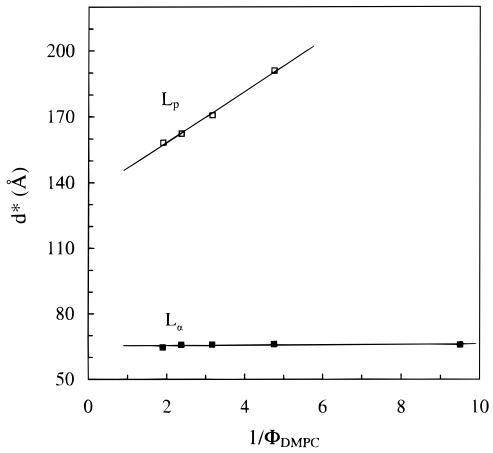

Figure 8. d^* vs $1/\Phi_{DMPC}$ in the domain of coexistence of L_α and L_p phases (Φ_{DMPC} is the total lipid fraction of the samples).

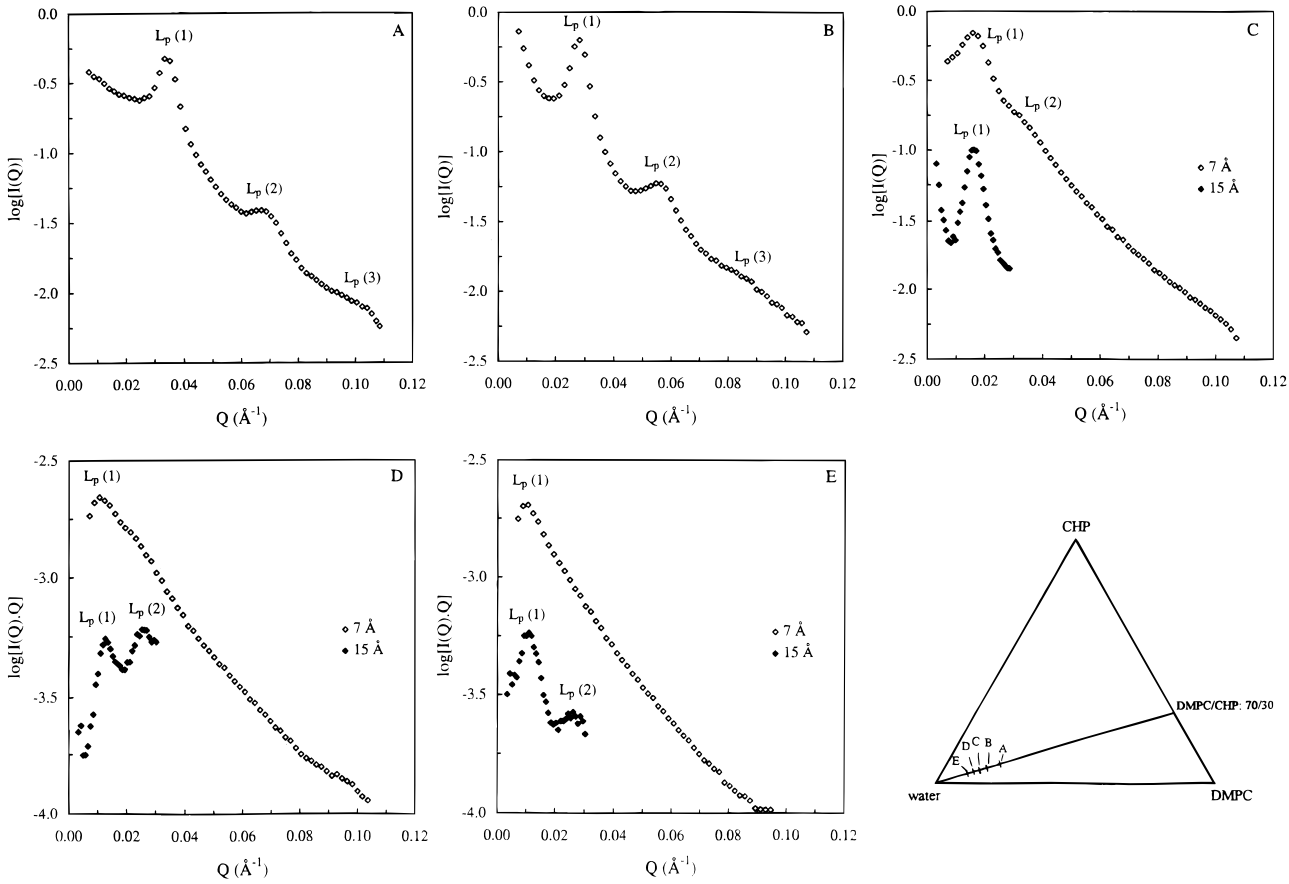

Figure 9. SANS spectra $\log[I(Q)] = f(Q)$ corresponding to the dilution line 2. The samples are prepared with a fixed DMPC/CHP ratio: 70/30 (w/w) in pure D_2O. The hydration is (A) 75, (B) 80, (C) 82.5, (D) 85, and (E) 87.5%. In D and E we have plotted $\log[I(Q)Q] = f(Q)$. These spectra correspond to the monophasic polymer rich L_p domain.

ranging from 75 to 87.5% are shown in Figure 9 (spectra A−E). On spectrum A, three diffraction orders of the L_p phase are observed with a lamellar sequence and a corresponding repeat distance of 186 Å. From 75 to 87.5% water the L_p phase continues to swell and reaches a spacing of 570 Å at 87.5% water. In order to observe such large periods and to enhance the resolution at low Q, the samples have been observed at a wavelength of 15 Å. The spectra obtained at 7 and 15 Å are given for the samples at 82.5, 85, and 87.5% water (spectra C−E). At 85 and 87.5% water, the intensity of the Bragg peaks is very low, and the best representation to determine the Bragg peaks sequence is obtained by plotting $\log[I(Q)Q]$ as a function of Q.

The lamellar repeat distance of the L_p phase is plotted as a function of $1/\Phi_{DMPC}$ in Figure 10. We have calculated the ideal swelling of the lamellar phase with a bilayer thickness of 33 Å. The dashed line represents this ideal swelling and shows that, below 80% water ($d^* < 220$ Å), the L_p phase swells ideally. Note that the adjusted bilayer thickness of 33 Å is close to that of pure DMPC at maximum swelling (35.5 Å).[1] At hydrations higher than 80 % water, the experimental lamellar spacing is higher than the ideal one. The three regimes indicated in Figure 10 are approached in the Discussion.

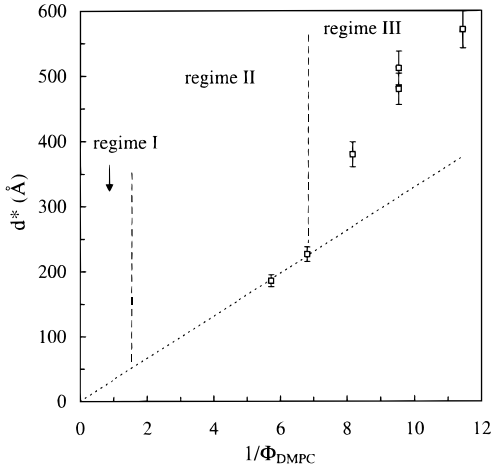

Figure 10. d^* vs $1/\Phi_{DMPC}$ in the monophasic L_p domain. The dotted line represents the ideal swelling calculated according to eq 1 with a bilayer thickness of 33 Å. Regime I corresponds to the swelling of the L_α phase by the hydration force, regime II to the ideal swelling of the L_p phase by the steric interaction of anchored polymer chains, and regime III to the swollen L_p phase with excess area.

Discussion

1. Balance between Hydration and van der Waals Forces. In the biphasic region corresponding to the coexistence of the lamellar phase with almost pure water in excess, the repeat distance corresponds to the equilibrium between the attractive van der Waals force and the repulsive hydration force. In the case of DMPC which is a zwitterionic lipid, electrostatic forces may be neglected. We also do not consider undulation forces because of the rigidity of DMPC bilayers ($\kappa \gg kT$). The hydration force has been discussed in detail by Rand and Parsegian[2] and by Israelachvili and Wennerström.[23] It originates from the orientation of water molecules by the electric fields surrounding the lipid head groups. It differs from the short range (<5 Å) steric interaction between phospholipid head groups[24,25] and from fluctuation pressures due to thermally induced undulations.[26,27] It decreases exponentially with distance in the

high-pressure range (10−25 Å) with a typical exponential decay length of ≈2 Å.

The results obtained with small sugars show that they induce a significant swelling of the lamellar phase from 61 Å in pure water to 76 Å at 40% lactose concentration. Consequently, if we consider that the bilayer thickness is not modified by the addition of lactose, the increase in repeat distance (Figures 3 and 5) may be attributed to the change of the bilayer separation caused by the modification of the equilibrium between the hydration and van der Waals forces. However, it is not possible to conclude whether the hydration or the van der Waals force is modified by the solubilization of these sugars in the interbilayer region.

In the work of LeNeveu et al.,[5] the modification of the egg lecithin lamellar repeat distance by the addition of small sugars was discussed in terms of successive weakening and strengthening of the van der Waals attraction. After an increase of the lamellar spacing in the sugar concentration range between 0 and 20 wt %, a decrease was observed at higher concentrations. This effect was observed with glucose and saccharose, with a swelling effect slightly higher with the disaccharide than with the monosaccharide. Our results are not consistent with these observations. In the sugar concentration range of 0−20 wt %, we observe a swelling as found by LeNeveu et al. with an increase of 8 Å at 20% lactose (Figure 3). However, at higher concentrations, the lamellar phase continues to swell. The disaccharide used in our work is lactose and not saccharose, but recent results obtained in our group with saccharose are consistent with this result.[28] From the kinetic observations made on Figure 5 we have seen that samples in the metastable state may have a behavior opposite to that of thermodynamic equilibrium. For instance, after the first incubation period, glucose stresses the lamellar phase, while after the second one it swells it. In nonequilibrated samples, as long as the concentration of the sugar in the excess solution is higher than in the lamellar phase, the osmotic compression competes with the swelling due to the solubilization of the sugar. The observed decrease in lamellar spacing observed by LeNeveu et al. might be attributed to this kinetic effect if the samples have not been incubated until equilibrium between the coexisting phases was obtained.

2. Compression of the Lamellar Phase by Osmotic Stress. The steric exclusion of the polysaccharide from the interbilayer aqueous region may be related to the respective dimensions of the macromolecule and of the water layer thickness in the pure L_α phase at maximum swelling. The radius of gyration of the pullulan may be calculated from the empirical relation[16]

$$R_g\,(\text{Å}) = 1.47 \times 10^{-1} M^{0.58} \qquad (2)$$

where the 0.58 exponent is close to the theoretical value of 0.6 for flexible polymers in good solvents. For pullulan of molecular weight 45 000, this relation yields a diameter of gyration of 148 Å, much larger than the interbilayer spacing of the lamellar phase. Therefore, the swollen polymer is sterically excluded from the interbilayer spacing, and the amount of water in the interbilayer region is determined by an osmotic equilibrium.

In this system the lamellar spacing measured at equilibrium is governed not only by the hydration and van der Waals forces but also by the osmotic pressure of the coexisting pullulan solution. The equilibrium of the pressures between the L_α phase and the coexisting pullulan solution is

$$\Pi_{L_\alpha} = \Pi_{\text{pullulan}} \Leftrightarrow \Pi_{\text{hydration}} + \Pi_{\text{vdw}} = \Pi_{\text{osmotic}} \qquad (3)$$

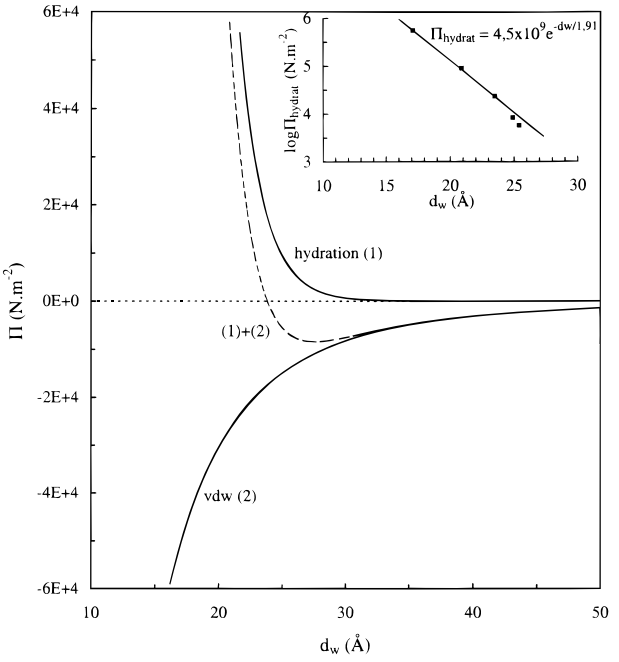

Figure 11. Force balance in the DMPC/water system as a function of the interbilayer spacing (d_w). The hydration pressure was determined according to eq 3 with the van der Waals pressure calculated according to eq 4 (d_w was varied by the osmotic stress method). Insert: semilog plot showing the short-range exponential decay of the hydration force.

In order to determine the strength of the osmotic contribution to the total pressure of the system, we have measured the osmotic pressure of the pullulan solutions using a membrane osmometer. The result is shown in the insert of Figure 4. The van der Waals pressure applied to bilayers of thickness d_b separated by a water film thickness d_w has been calculated by the relation

$$\Pi_{\text{vdw}} = -\frac{A}{6\pi}\left[\frac{1}{{d_w}^3} - \frac{2}{(d_b + d_w)^3} + \frac{1}{(2d_b + d_w)^3}\right] \qquad (4)$$

where A is the nonretarded Hamaker constant (5.1×10^{-21} J) calculated according to Israelachvili[29] for the symmetric case of two identical apolar phases interacting across water (we neglect the additional contribution of neighboring membranes).

From the osmotic pressure measurements on pullulan solutions, and according to the calculated attractive van der Waals pressure, it is possible to determine through eq 3 the repulsive hydration pressure originating from water molecules bound to the DMPC polar head groups.

The relation between the hydration pressure and the interbilayer spacing (d_w) is presented in Figure 11. The linear regression of this relation in the high-pressure range gives the exponential decay length $\lambda = 1.91$ Å and the pressure at zero separation, i.e., when bilayers are in contact. This yields the relation between the hydration pressure and d_w

$$\Pi_{\text{hydration}} = 4.5 \times 10^9 \mathrm{e}^{-d_w/1.91} \qquad (5)$$

The decay length is close to the value of 1.93 Å found by LeNeveu et al. for the egg lecithin/dextran system[5] and the value of Lis et al. (2.2 Å) for DMPC.[30] Gawrich et al. have also found a decay length of 2.2 Å for DPPC bilayers stressed by PEO solutions.[31] The magnitude of this force (prefactor in eq 5) is quite large (4.5×10^9 N/m^2). Therefore high osmotic stresses are needed to obtain a significant decrease of the lamellar spacing. This explains the high value of the pullulan concentrations indicated in Figure 4.

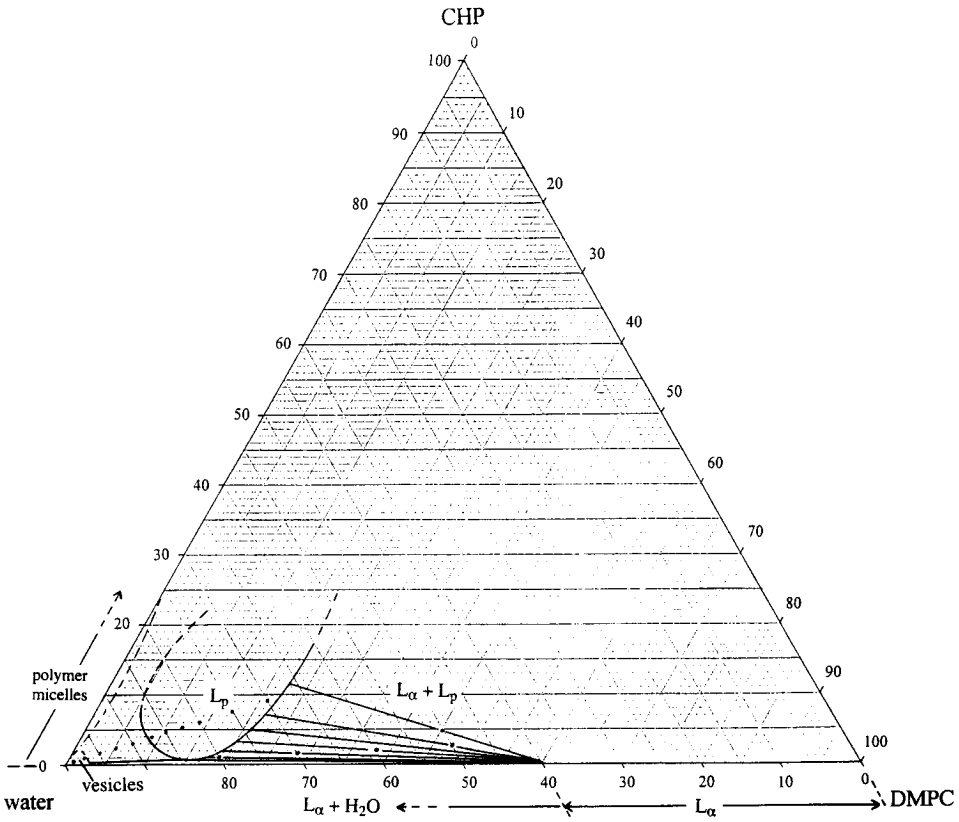

Figure 12. Phase diagram of the DMPC/CHP/water ternary system at 30 °C.

3. Crossover from Solubilization to Exclusion. Figure 5 shows that the limit between the regimes of solubilization, and steric exclusion occurs at low sugar molecular weights: At a concentration of 20%, maltotriose ($n = 3$) swells the lamellar phase from 60.9 to 76.1 Å, while maltopentaose ($n = 5$) compresses it to 57.0 Å.

Kinetic observations make it possible to understand the steps that lead to the equilibrium state of the swollen or compressed lamellar phase. In both cases the first step that follows the addition of the solution to the dry lipid is a rapid diffusion of water from the solution to the lamellar phase. When the sugar is sterically excluded, the equilibrium is reached at the end of this step and the lamellar phase is compressed. For instance, with maltopentaose and pullulan, no change in lamellar spacing is observed between two incubation periods of 14 days. In the opposite case where the sugar is sufficiently small, this first step is followed by the diffusion of the sugar from the solution to the lamellar phase. This second step is rate limiting and takes place over longer times: several weeks, compared to the hydration step (a few minutes). Thus, considering that diffusion of the sugar is negligible during the initial step of water diffusion, one may say that in all cases the hydration of the lipid by a sugar solution results first in a "kinetic stress". This has been observed with glucose: at the end of the first incubation period, the samples are not at equilibrium, and the difference in sugar concentration between the solution in excess and the interbilayer water is high enough to produce an osmotic compression of the phase. After the second incubation period, the sample is closer to the equilibrium state and we observe the swelling by the small sugar.

From these kinetic observations, one may wonder if the two cases of solubilization and steric exclusion are really opposed or if the result at infinite incubation time would be the same regardless of the size of the sugar: a partition of the solute between the two aqueous phases, the solution in excess, and the interbilayer space. Actually, we consider that when the sugar is excluded, the osmotic compression further reduces the lamellar spacing and diminishes the accessible water layer thickness, so that the equilibrium corresponds to the stressed lamellar phase.

The origin of the exclusion of small sugars by the lamellar phase is interesting. Indeed, the lamellar spacing is large enough to incorporate the sugar molecules into the aqueous layers. A plausible explanation is that the sugars are excluded from the hydration layers of the polar head groups. The accessible water thickness in the L_α phase at maximum swelling would be reduced from 24.9 to 12.3 Å (we take 13 water molecules per polar head group (from ref 5), a molecular volume of 30 Å^3 for water, and an area of 62 Å^2/DMPC head group (from ref 1)). The resulting loss in accessible water thickness is $2 \times (13 \times 30/62) = 12.6$ Å. Reduced to 12.3 Å, the thickness of accessible water is smaller than the size of maltopentaose which has a gyration radius of 9.5 Å. This is consistent with its exclusion from the aqueous layers of the lamellar phase.

In the present stage we can conclude on this point that three different regimes are possible: (1) In the case of small sugars such as glucose, no steric exclusion occurs and one may expect that the concentration in the solution in excess and in the accessible water in the lamellar phase is the same. (2) With partially excluded sugars, for instance maltopentaose, the concentration in the coexisting isotropic solution is higher than in the lamellar phase and causes an osmotic stress. The induced compression competes with the swelling due to the solubilization of some sugar in the lamellar phase. The value of d^* measures the balance of these two opposite effects. (3) With high molecular weight polysaccharides, complete exclusion of the macromolecular solute is achieved and the osmotic compression may be quantified.

4. Swelling by CHP Polysaccharides. The phase diagram of the DMPC/CHP/water system determined by SANS experiments is shown on Figure 12. The highly swollen polymer rich L_p phase has been observed in two domains of the phase

diagram, either in coexistence with the pure L_α phase or as a single phase in a large monophasic domain.

Two new regimes of swelling have been observed. Regime I shown in Figure 10 corresponds to the swelling of the L_α phase by the hydration force. In the presence of CHP, an ideal swelling has been observed in the biphasic L_p/L_α system (Figure 8) and in the monophasic L_p domain (Figure 10): at spacings below 220 Å, the L_p phase swells ideally according to eq 1 (regime II). This swelling is attributed to the steric repulsion induced by the confined polysaccharide chains anchored into the membranes. The force balance in the system may be written

$$\Pi_{L_p} = \Pi_{vdw} + \Pi_{polymer} \qquad (6)$$

where $\Pi_{polymer}$ is the osmotic pressure of the confined polymer layer anchored in the DMPC bilayers. In this regime dominated by the polymer contribution, the lamellar spacing is such that hydration forces do not contribute to the force balance.

5. Excess Area in the L_p Phase. At spacings higher than 220 Å, a third regime is observed where d^* diverges strongly from ideality (regime III, Figure 10). In this regime, the experimental lamellar spacing is higher than that calculated according to eq 1 with a bilayer thickness of 33 Å.

In order to explain the observed deviation, we have introduced an additional repulsive contribution to the steric interaction originating from anchored polymer chains. We have used the logarithmic deviation proposed by Roux et al. which accounts for the excess area in systems dominated by undulation forces in dilute lyotropic lamellar phases.[32] According to Roux et al. the dilution law is of the form

$$d^* = \frac{d_b}{\Phi_{DMPC}}\left[1 + \frac{kT}{4\pi\kappa}\ln\left(c\frac{d_b}{a}\left[\frac{\kappa}{kT}\right]^{1/2}\frac{1}{\Phi_{DMPC}}\right)\right] \qquad (7)$$

where κ is the membrane bending constant of the CHP grafted DMPC bilayer, c a constant equal to $(32/3\pi)^{1/2}$,[33] and a the transverse size of DMPC. This equation is valid at large dilutions where $d^* \gg d_b$, and a plot of $d^*\Phi_{DMPC}$ as a function of $\ln(\Phi_{DMPC})$ gives a linear relation with negative slope in systems dominated by undulations. For rigid membranes such as DMPC, we have $\kappa \gg kT$,[34] and the second term in eq 7 tends to zero. In this case, the slope of the function is zero and eq 1 is verified without correction.

Layer undulations as a sterically stabilizing interaction were first reported by Helfrich.[35,36] These undulations produce a pressure that originates from the confinement of the membrane in a volume limited by its neighbors. The effect of such undulation pressure on the lamellar spacing in a nonionic system was first reported by Strey et al.[37] The membrane fluctuations induce a long-range repulsive contribution which has to be taken into account in the force balance of the system. The pressure induced by these undulations may be calculated according to

$$\Pi_{undul} = \frac{3\pi^2}{128}\frac{(kT)^2}{\kappa(d^*)^3} \qquad (8)$$

We have plotted $d^*\Phi_{DMPC}$ as a function of $\ln(\Phi_{DMPC})$ according to eq 7 for the DMPC/CHP system in Figure 13. We effectively observe the predicted negative slope in the dilution range where the excess area has been observed (regime III, Figure 10). The dilution range where no excess area was observed (regime II) yields a plateau in agreement with eq 7. Using the slope observed in regime III, we have calculated the membrane bending constant κ of the polymer grafted DMPC bilayer. We obtain a value of 0.22×10^{-21} J (0.05 kT) which is more than 2 orders of magnitude smaller than the value of κ for pure DMPC in the L_α phase (1.5×10^{-19} J, 35 kT).[34] This value of κ is excessively small, even for the case of a system dominated by membrane fluctuations. Previously reported values are in the range 0.5−2.7 kT.[32]

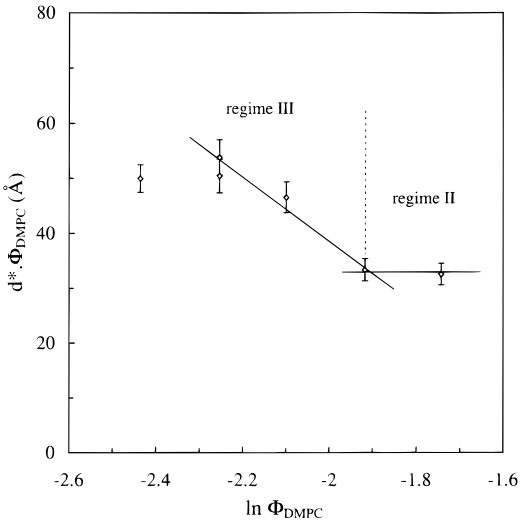

Figure 13. Plot of $d^*\Phi_{DMPC}$ as a function of $\ln(\Phi_{DMPC})$ according to eq 7.[32] See Figure 10 for the definition of regimes II and III.

Although it does not seem reasonable to consider that the undulation model applies here, it is clear that κ has to be reduced from its initial value of 35 kT to observe undulations in the lamellar phase.[38] The bending of the membranes is required for the excess area to be observed, but we cannot conclude whether this excess area results from the bending of the membranes by the anchored chains, as reported recently by Lipowsky,[12] or from a dynamic process involving undulations due to a reduction of the membrane stiffness.

The existence of a swelling limit is interesting. In the absence of any long-range attractive contribution, the L_p phase should swell indefinitely. Up to now, we have neglected the possibility of membrane bridging by polysaccahride chains. The observed swelling limit might be attributed to a residual bridging of the membranes by a few polysaccharidic chains anchored in neighboring membranes. The mean distance between two cholesterol groups on the stretched polysaccharidic backbone (620 Å) is consistent with the swelling limit observed at 570 Å. In regime II, it is quite conceivable that bridging prevents the membrane undulations and results in an ideal swelling with no excess area. This regime would be then controlled by two competing contributions:[39] steric repulsions originating from anchored polymer chains (the osmotic pressure) and bridging (the elastic restoring force). At larger bilayer separations the osmotic term still dominates (regime III), but the probability of bridging is reduced and membrane undulations are made possible.

Conclusions

The range of water thicknesses and the forces involved in the investigated systems are summarized in Figure 14.

All the observations presented here have a common origin: sugars are repelled by the polar head groups of DMPC (actually they compete for water against these polar head groups). If the aqueous layers of the lamellar phase contain water in excess of the hydration shells of the head groups, then this extra water can solubilize sugars. However, the amount of free water contained in the aqueous layers of the L_α phase is not sufficient to solubilize oligomeric sugars (beyond $n = 3$) or polysaccharides.

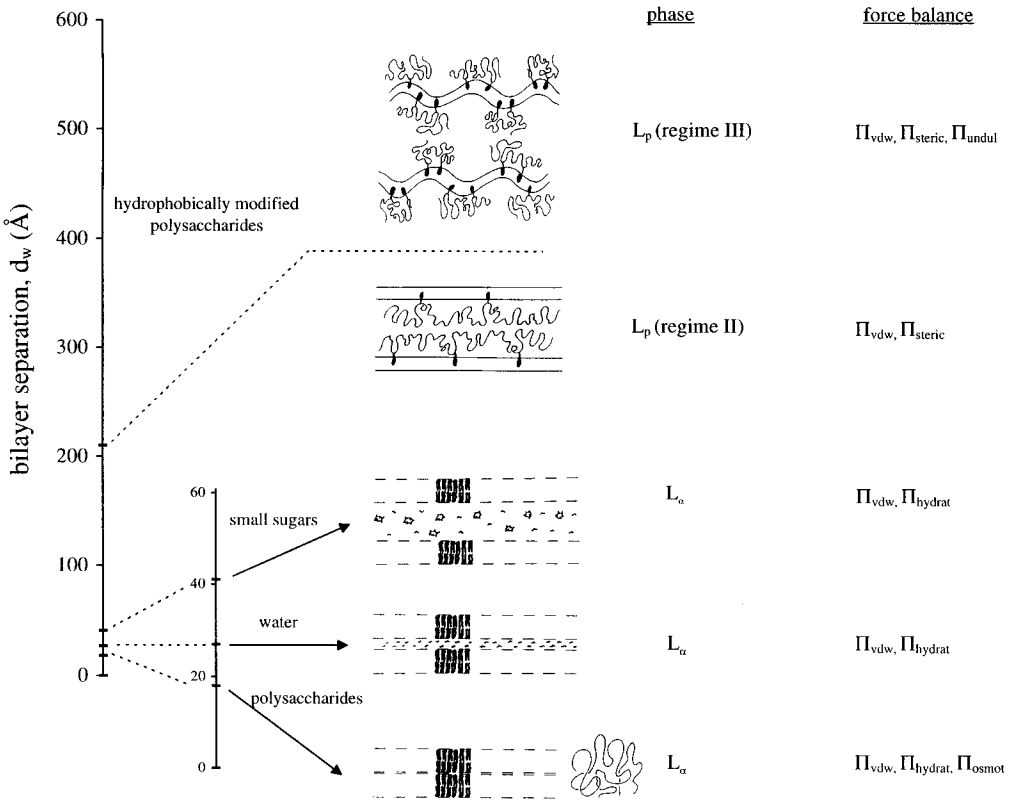

Figure 14. Schematic representation of the various regimes observed in the investigated DMPC/carbohydrates/water systems.

The swelling of the system is regulated by the force balance which may be modified either by an increase of the osmotic pressure of a coexisting aqueous phase (osmotic stress) or by the solubilization of small solutes in the aqueous interbilayer region.

We have developed a new procedure to prepare mixed lipid/polymer samples at thermodynamic equilibrium where steric exclusion and diffusion of the polymer in confined environment have been eliminated. By this procedure, and in spite of their dimension in solution, hydrophobically modified polysaccharides can be introduced in the aqueous layers of the lamellar phase. The stability of the system originates from the anchoring energy of cholesterol groups that counterbalance the entropy loss originating from the confinement of the polysaccharide in the lamellar phase.

A new polymer rich (L_p) phase has been observed, and the thermodynamic coexistence of two lyotropic lamellar phases, differing in their polymer content and in their periodicities, was also demonstrated. Since the sugar backbone of the macromolecule is repelled rather than adsorbed by the polar heads, water is driven into the aqueous layers and swells the polymer backbones. The resulting lamellar spacings ($d_w = 540$ Å) are much larger than those observed in the absence of polymer ($d_w = 26$ Å).

These observations may have some significance for biological membranes, where polysaccharide chains bound to membrane proteins play an important role in cell recognition. In this case as well, the polysaccharide chains must be repelled rather than adsorbed by the lipidic surface. Consequently, the polysaccharides are forced to extend into the surrounding medium; this configuration may be useful for specific interactions and recognition between surfaces carrying polysaccharide chains.

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