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# Coexistence of two lyotropic lamellar phases induced by a polymer in a phospholipid–water system

Bruno Demé [a,*], Monique Dubois [a], Thomas Zemb [a], Bernard Cabane [a,b]

[a] *Service de Chimie Moléculaire, CEA-Centre d'Études de Saclay, 91191 Gif sur Yvette, Cédex, France*
[b] *Unité Mixte CEA-RP des Matériaux Dispersés, Rhône-Poulenc, 93308 Aubervilliers, France*

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**Abstract**

The effect of a hydrophobically modified polysaccharide, cholesteryl–pullulan (CHP), on the swelling of the DMPC $L_\alpha$ lamellar phase has been investigated by small angle neutron scattering. The CHP derivative can be introduced in the aqueous layers of the lamellar phase by anchoring lateral cholesterol groups into the bilayers. The resulting lamellar phase ($L_p$) is stabilized at large membrane separations by the introduction of a new repulsive and long range contribution in the force balance of the system.

We emphasize here the temperature dependence of two coexisting lamellar phases ($L_\alpha + L_p$) differing in their polymer content and in their periodicities. At low polymer content (DMPC:CHP=99:1 by weight), the two lamellar phases at thermodynamic equilibrium change into a single phase on heating from room temperature to 50°C. The new phase ($L_p'$) is characterized by a very large correlation peak whose position is consistent with a lamellar structure following an ideal dilution law. The transition $L_\alpha + L_p \rightarrow L_p'$ is reversible on cooling, indicating that the observed coexistence of the two lamellar phases at room temperature is a true thermodynamic equilibrium. At higher polymer content (DMPC:CHP=95:5 by weight) the critical behaviour has not been observed. The periodicity of the $L_p$ phase slightly decreases on heating indicating a reduction in the miscibility gap and a possible critical point at temperatures higher than 50°C. However, in the investigated temperature range, the thermodynamic coexistence of the two lamellar phases is not affected in this case. © 1997 Elsevier Science B.V.

*Keywords:* Anchoring; Lamellar phase; Membrane; Phase equilibrium; Phospholipid; Polysaccharide

## 1. Introduction

The effects of polymers on the swelling of lyotropic lamellar phases are of interest since they represent a way to introduce a long-range repulsive contribution in systems dominated by the van der Waals attraction. Indeed, in non-ionic or zwitterionic systems such as lecithins, the only repulsive contribution is the hydration force [1,2]. This force is short range and overcomes the van der Waals attraction only at distances in the range 0–25 Å. At larger bilayer separations, the membranes are in an attractive regime dominated by van der Waals forces. These systems are very sensitive to the introduction of a few surface charges and stability at larger membrane separations can be obtained by solubilization of a few per cent of ionic surfactant in the membranes [3]. However, in biological applications, high ionic strength (150 mM NaCl) results in a strong screening of this electrostatic contribution.

* Corresponding author. Present address: Institut LAUE-LANGEVIN, BP 156, F-38042 Grenoble, France.

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Solubilization of a polymer in a collapsed stack of membranes is difficult to achieve because of the size of the swollen chain relative to the bilayer separation at maximum swelling. The confinement of the polymer coil in a thin slit with a dimension smaller than the gyration radius of the polymer results in a configurational entropy loss associated with the transition from a three-dimensional solution (unconfined coil) to a two-dimensional system. If the entropy loss is not counterbalanced by the adsorption energy of the chains (non-adsorbing polymer), the solubility may be strongly reduced and may result in a polymer-induced phase separation. This leads to the coexistence of a polymer-rich phase where the configurational entropy of the chains is maximized in equilibrium with a surfactant-rich phase. In some cases, the polymer may be completely excluded from the lamellar phase resulting in an osmotic compression which further reduces the bilayer separation. This behaviour is typical of lecithin–polysaccharide mixed systems and has been widely used in osmotic stress experiments for the measurements of short-range repulsive hydration forces between polar lipidic surfaces in water [1]. The size exclusion of soluble polymers by lecithin lamellar phases has been described using non-adsorbing polymers such as dextran [4–8], pullulan [9,10] and poly(ethylene glycol) [11] as osmotically active polymer solutions.

When adsorbing or anchoring chains are used, the situation is different since the entropy loss of the confined polymer chains may be overcome by the adsorption or anchoring energy of hydrophobic groups in the bilayers [12,13]. Then, if this condition is satisfied, the polymer chains may be kept in the lamellar lattice and stabilization at large dilutions may be observed [8,10]. The stabilization is due to the introduction of a non-screenable steric interaction of excluded volume type.

At low water content, the free energy of the confined chain corresponds to the work required to compress a spherical blob into a pancake [14,15]. On dilution of the lamellar phase, the chains revert to a more spherical configuration and repel the membranes at larger distances satisfying the configurational entropy of the chains. If the grafting density is high enough the stretching of the chains induces a repulsion at distances higher than the gyration radius of the chains anchored or adsorbed at the lipid–water interface.

Cholesteryl–pullulan (CHP) derivatives belong to this type of hydrophobically modified water soluble polymer (Fig. 1) which interacts with membrane structures by anchoring hydrophobic groups in bilayers [16,17]. Their solubilization in the DMPC $L_\alpha$ lamellar phase has been reported recently [9,10]. The phase diagram of the ternary system and the dilution law of the mixed DMPC–CHP lamellar phase are given in Fig. 2. The highly swollen polymer rich lamellar phase ($L_p$) has been observed either in coexistence with the pure DMPC $L_\alpha$ phase or as a single lamellar phase in a large monophasic domain. At high bilayer separations (220–570 Å), this new lamellar phase exhibits a strong deviation from ideal swelling. In a range corresponding to smaller separations (25–220 Å), an ideal behaviour is observed. It follows the ideal swelling of DMPC in the regime dominated by hydration forces (0–25 Å). Deviation from ideality at large separations is attributed to membrane bending induced by the anchored polysaccharide chains. The transition between regimes II and III, observed at 220 Å, corresponds to the critical overlapping concentration $C^*$ of the confined polymer solution [9].

Evidence of the possible coexistence of two lamellar phases with different water contents was given by Vincent and Skoulios [18,19] and by Khan et al. [20]. The thermodynamic coexistence of lyotropic lamellar phases has been discussed by Wennerström [21], and more recently the critical behaviour of two coexisting lyotropic lamellar phases has been reported in the DDAB–water binary system by Zemb et al. [22]. A polymer-induced phase separation has been reported by Ligoure et al. in an electrostatically stabilized lamellar phase [23].

We emphasize here the two phase behaviour in the DMPC–CHP–water ternary system and the critical behaviour of the two coexisting lamellar phases ($L_\alpha + L_p$) at low polymer content. These two lamellar phases strongly differ in their polymer content and in their periodicities. In coexistence with the $L_\alpha$ phase, the $L_p$ phase has been observed

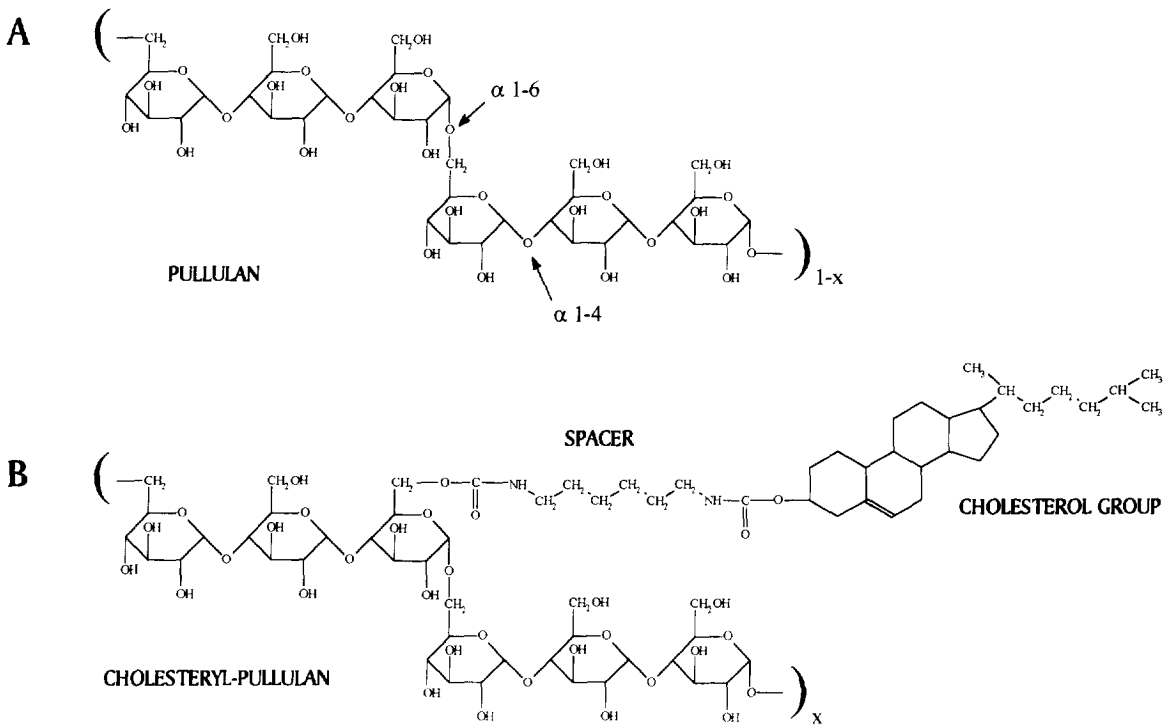



Fig. 1. Molecular structure of pullulan (A units) and $CHP_{50-0.9}$ (pullulan of molecular weight 50 000 and degree of substitution $x$ = B:(A + B) = 0.9%).

only at spacings that correspond to the ideal behaviour of the $L_p$ phase (regime II, Fig. 2).

## 2. Materials

### 2.1. Chemicals

DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL).

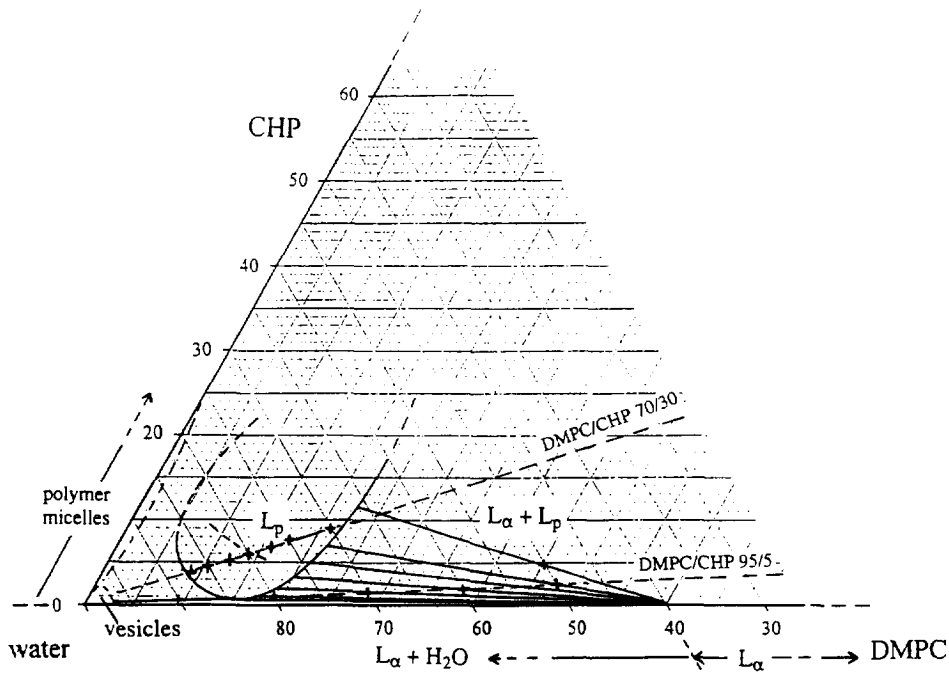
The repeat units of pullulan and hydrophobically modified pullulan (CHP) are presented in Fig. 1. Pullulan is a flexible, amorphous, and water-soluble homopolymer composed of maltotriose (A) repeat units with glucose subunits linked by α-1,4 and α-1,6 glycosidic bonds. The polysaccharidic chains dissolve well in water [24–27].

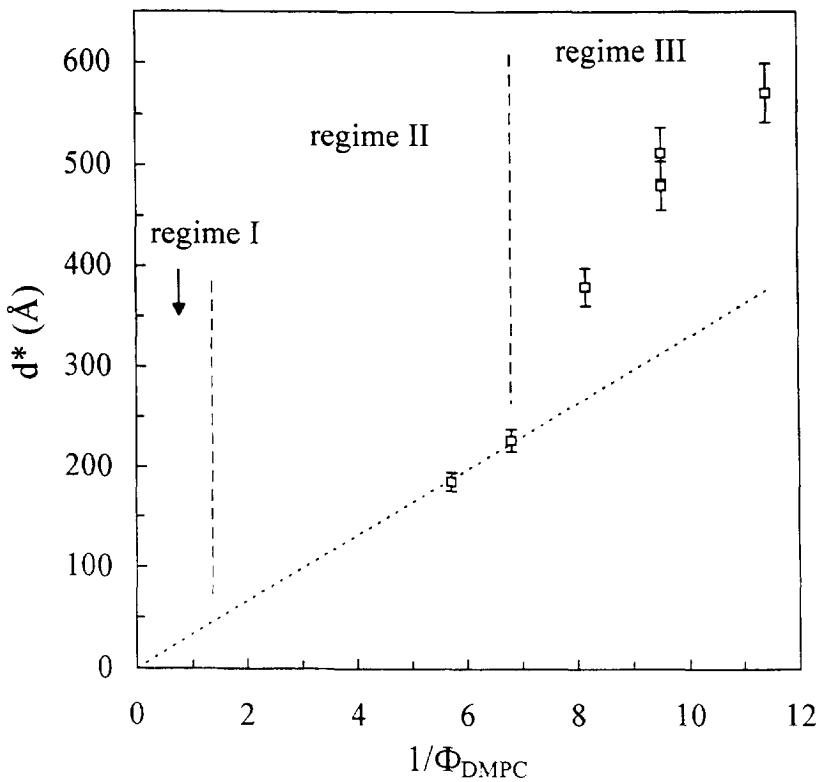
The CHP derivative was a gift from J. Sunamoto and K. Akiyoshi and was synthesized as described in Ref. [28]. $CHP_{50-0.9}$ (molecular weight, 50 000; degree of substitution, 0.9 cholesteryl group per 100 glucose units) is a random copolymer of native A pullulan units ($1-x = 99.1$ mol%) and hydrophobically modified B units ($x = 0.9$ mol%) that bear lateral cholesterol groups attached to the polysaccharidic backbone by a flexible spacer. The substitution degree of the polysaccharide corresponds to an average number of three hydrophobic groups per chain.

All manipulations of the lipid were performed in water produced by the Milli-Q purification system from Millipore (Bedford, MA), except the final hydration step which was performed in heavy water according to a procedure previously described [9,10]. Heavy water (deuterium enrichment 99.9%) was from Euriso-top (Saclay, France).

### 2.2. Neutron scattering experiments

The neutron scattering experiments were performed at the PAXE spectrometer located at the


CHP
60
50
40
30
20
0
polymer micelles
DMPC/CHP 70/30
DMPC/CHP 95/5
$L_p$
$L_\alpha + L_p$
vesicles
water
80
70
60
50
40
30
$L_\alpha + H_2O$
$L_\alpha$
DMPC


regime I
regime II
regime III
d* (Å)
600
500
400
300
200
100
0
0
2
4
6
8
10
12
$1/\Phi_{DMPC}$

end of the G5 cold neutron guide of the Orphée reactor (Laboratoire Léon Brillouin, CEN Saclay, France). 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 $\Delta\lambda/\lambda = 10\%$. With this configuration of the spectrometer, the $Q$ values range from 0.007 to 0.11 $Å^{-1}$ in a single run (detector shifted).

The samples were held in 1 mm thick quartz cells thermostatted at a controlled temperature of at least 30°C, so that the aliphatic chains of DMPC are in the fluid $L_\alpha$ state [29–31].

(SANS) spectra are represented by the $I(Q) = f(Q)$ function where $I(Q)$ is the radially averaged scattered intensity of the 2D pattern recorded on a 2D 64 × 64 channel gas detector, and $Q$ the scattering vector. The periodicity $d^*$ of the lamellar phase is calculated according to the relation using the position observed for $n$ Bragg peaks: $d_n^* = 2\pi n/Q_n$.

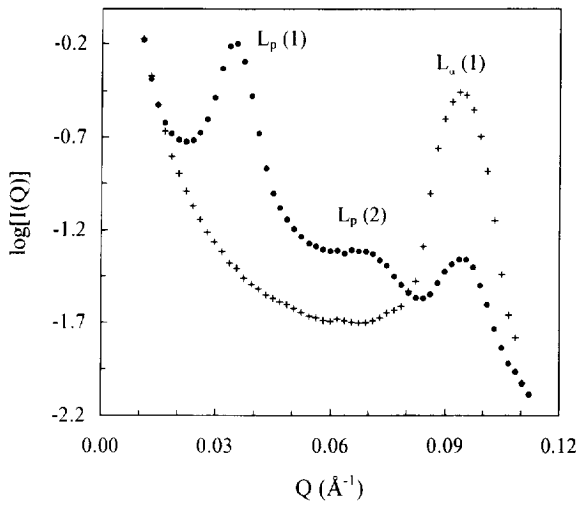



Fig. 3. SANS spectra of pure DMPC in excess water (70%) (+) and of a sample with a DMPC:CHP ratio of 95:5 (by weight) at the same hydration (●). This sample was prepared in the biphasic $L_p$–$L_\alpha$ domain of the phase diagram Fig. 2. The $L_p$ phase is swollen at 182 Å and the $L_\alpha$ phase at 64 Å.

## 3. Results

### 3.1. Thermodynamic coexistence of two lamellar phases

The coexistence of two lamellar phases in a mixed DMPC–CHP sample (95:5 by weight) hydrated by 70% water by weight is illustrated by the SANS spectrum shown in Fig. 3. On this spectrum two main Bragg peaks are observed at 0.0975 $Å^{-1}$ (64 Å) and 0.0345 $Å^{-1}$ (182 Å). Comparison with the spectrum of pure DMPC at the same hydration shows that the peak at 0.0975 $Å^{-1}$ is that of DMPC in the $L_\alpha$ phase in coexistence with the swollen phase at 0.0345 $Å^{-1}$. This phase is also lamellar as evidenced by a second-order Bragg peak at $2Q$.

### 3.2. Effect of temperature

#### 3.2.1. Low polymer content

In Fig. 4 one can see the effect of heating from 30°C to 50°C on the coexistence of the two lamellar phases in samples hydrated at 70% containing small amounts of polymer (DMPC:CHP = 99.5:0.5 and 99:1 by weight). These samples correspond to compositions very close to the pure DMPC–water line. At 30°C, for the two compositions, we observe the first Bragg peak of the $L_p$ phase at $Q_1 = 0.0267$ $Å^{-1}$ ($L_p(1)$, 235 Å) and the second at 0.0540 $Å^{-1}$ ($L_p(2)$ at $2Q_1$). The $L_\alpha$ phase is observed at 0.0832 $Å^{-1}$ ($L_\alpha(1)$, 75 Å).

On heating, we observe a transition from the biphasic $L_p$–$L_\alpha$ system to a monophasic system with a very large correlation peak at 0.053 $Å^{-1}$

Fig. 2. Phase diagram of the water-rich region of the DMPC–CHP–water ternary system and swelling behaviour ($d^*$ vs $1/\Phi_{DMPC}$) of the $L_p$ phase....., ideal swelling calculated with a bilayer thickness of 33 Å. The dilution line is represented on the phase diagram and corresponds to a DMPC:CHP ratio of 70:30 by weight. Regime I corresponds to the swelling of the $L_\alpha$ phase by hydration forces ($d^*_{max} = 61$ Å), regime II to the ideal swelling of the $L_p$ phase by anchored polysaccharide chains ($d^*_{max} = 220$ Å) and regime III to the surface excess observed in the $L_p$ phase on dilution of the polysaccharide beneath its critical overlapping concentration. This surface excess is attributed to the membrane bending by anchored chains. (From ref. [10].)

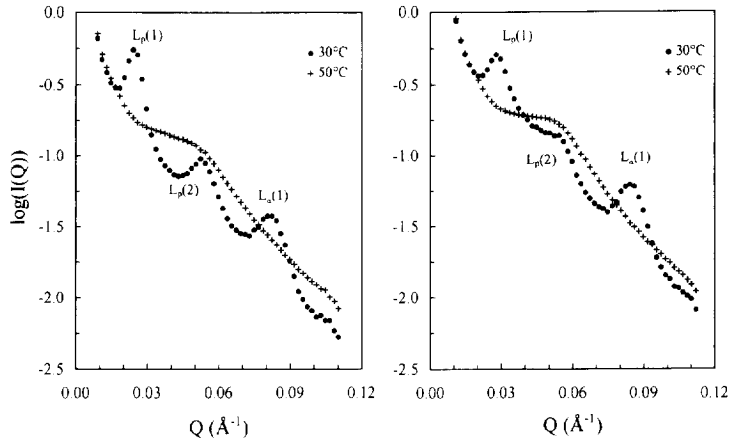



Fig. 4. SANS spectra showing the transition between the biphasic $L_p$–$L_\alpha$ system and the monophasic $L'_p$ domain on heating from room temperature to 50°C. The hydration of the sample is 70%, and the lipid:polymer ratio is (left) 99.5:0.5 by weight and (right) 99:1 by weight.

(118 Å) intermediate between those of the $L_p$ and $L_\alpha$ phases observed at 30°C. If the temperature is brought back to 30°C, this lamellar phase ($L'_p$) returns to the biphasic $L_p$–$L_\alpha$ system. The observed transition is thus reversible on cooling. This is evidence of the thermodynamic equilibrium in the $L_p$–$L_\alpha$ system at room temperature.

We consider here that because of the low polymer content of the samples and the low substitution degree of the polysaccharide (0.9 cholesterol group per 100 glucose units), the bilayer content is close to 100% DMPC and the cholesterol concentration negligible (considering all cholesterol groups anchored in the membranes, the calculated cholesterol:DMPC molar ratio would be 99.98:0.02). This allows us to consider that the thickness of the bilayers is close to that of pure DMPC. We can then replace the bilayer thickness $d_b$ by 35.5 Å [30] in the following equation which describes the ideal behaviour of lyotropic lamellar phases on dilution:

$$d^* = \frac{d_b}{\Phi_{DMPC}} \quad (1)$$

The repeat distances calculated taking $d_b = 35.5$ Å and the experimental lipid volume fractions $\Phi_{DMPC} = 0.2985$ (sample at 0.5 wt.% CHP) and $\Phi_{DMPC} = 0.297$ (1 wt.% CHP) are respectively 118.9 Å and 119.5 Å. The experimental periodicities of the $L'_p$ phase (118 Å) are consistent with a lamellar structure that follows an ideal dilution law.

### 3.2.2. *High polymer content*

Fig. 5 shows the temperature dependence of the swelling of the $L_p$ and $L_\alpha$ phases in the domain of coexistence of the two phases in a sample containing 60% water and a lipid:polymer ratio of 95:5 by weight. The corresponding $d^*$ values of the $L_p$ and $L_\alpha$ phases are reported in Table 1. Increasing the temperature results in a slight decrease in the lamellar spacing of the $L_p$ phase from 169 Å at 30°C to 157 Å at 50°C. These changes in repeat distance of the $L_p$ phase with temperature are reversible and the values obtained on cooling perfectly reproduce those obtained before the heating sequence. The lamellar repeat distance of the $L_\alpha$ phase is not affected by the increase in temperature.

The slight temperature dependence is consistent with the fact that hydrophobically modified polysaccharides are less solvated in water when the temperature is increased. A decrease in the swelling of the polysaccharide is then expected until the upper critical temperature at which water becomes a θ solvent of the polymer. The decrease in $d^*$ in the $L_p$ phase evidences a deswelling of the polymer chains in the aqueous layers of the lamellar phase

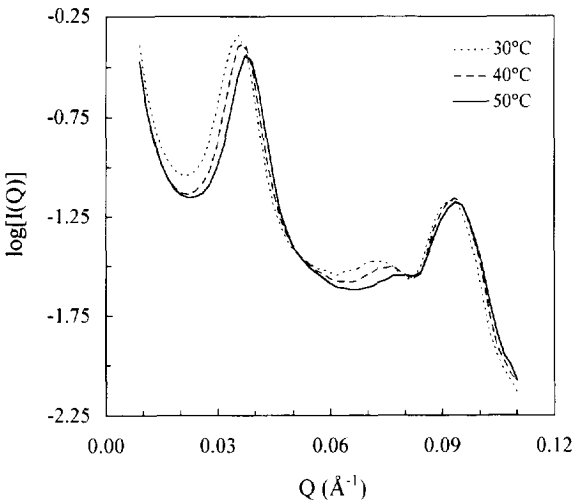



Fig. 5. Effect of temperature on the coexistence of the two lamellar phases. The SANS spectra of biphasic ($L_p+L_\alpha$) samples reveal the stability of the equilibrium between the two phases. The hydration of the sample is 70% and the lipid:polymer ratio 95:5. The changes in Bragg peak positions of the $L_p$ phase are reversible on cooling indicating that the system is at thermodynamic equilibrium. The periodicities of the two coexisting lamellar phases are reported in Table 1.

Table 1
Temperature dependence of $d^*$ in the domain of coexistence of the two lamellar phases $L_\alpha$ and $L_p$

| Temperature (°C) | $d^*(L_\alpha)$ (Å) | $d^*$ ($L_p$) (Å) |
|---|---|---|
| 30 | 65 | 169 |
| 40 | 65 | 163 |
| 50 | 64 | 157 |

The composition of the sample is DMPC:CHP = 95:5 by weight, and the hydration 60%.

and a reduction in the miscibility gap between the two coexisting phases. This observation also shows that here (ideal swelling, regime II) the origin of the swelling of the $L_p$ phase does not originate from membrane fluctuations which are known to be thermally induced (varying with $(kT)^2$) but only from the confined polymer.

We have represented on Fig. 6 the domains of the different lamellar phases as a function of temperature and lipid content $1/\Phi_{DMPC}$ of the lamellar phases for the samples with 1 and 5 wt.% CHP. We can see the reversible transition $L_\alpha+L_p \rightarrow L_p'$ at low polymer content and the stability of the equilibrium between the two phases at higher polymer content.

## 4. Discussion

The osmotic pressure vs. distance relation in the DMPC $L_\alpha$ phase clearly indicates that there is no possibility of coexistence of two lamellar phases in this binary system [10]. The equilibrium distance of the bilayers originates from the sum of the

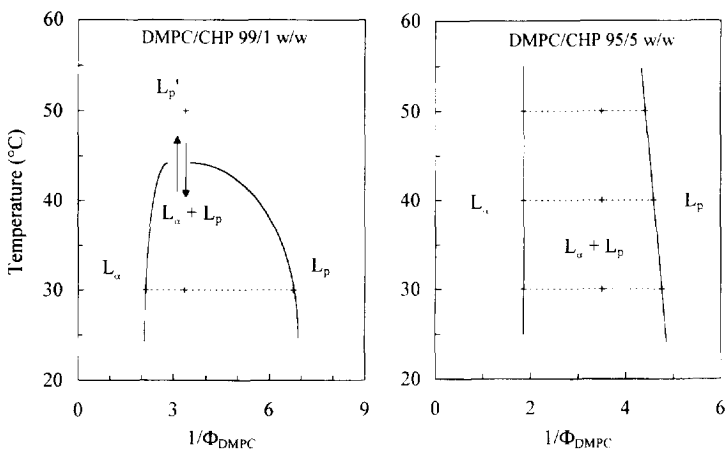



Fig. 6. Domains of existence of $L_\alpha$, $L_p$ and $L_p'$ phases as a function of temperature and lipid content at two lipid:polymer ratios. The diagrams are constructed from the lipid volume fractions of each phase determined according to the position of Bragg peaks.

hydration and van der Waals contributions which both decrease monotonically. The swelling produces a monotonic decrease in the pressure until the phospholipid coexists with excess pure water when the disjoining pressure vanishes [6,10].

The coexistence of the two lamellar phases requires the introduction of an additional repulsive contribution which may induce a non-monotonic pressure-distance relation, with an attractive regime separating two net repulsive force regimes [21]. In such cases, a Maxwell construction yields the periodicity of the two coexisting phases. If no entropic contribution and no variation in lateral interactions are involved, equality of the free energy of the two coexisting phases with finite periodicity requires

$$\int_{x_1}^{x_2} [\Pi(x) - \Pi_p]\, dx = 0$$

where $x_i$ is the thickness of water layers observed in the two coexisting phases, $\pi(x)$ the pressure at a bilayer separation $x$, and $\pi_p$ the plateau pressure. This integral is called the Maxwell construction; it is easily constructed [21,32].

In our system, the two-phase behaviour may be predicted since the repulsive contribution required for a two-phase equilibrium is induced by the anchored polysaccharide chains. This long-range repulsive interaction decreases monotonically with distance [33,34]. However, we do not know the distance dependence of this steric force so that the Maxwell construction cannot be drawn quantitatively with present available data. The knowledge of the plateau pressure $\pi_p\alpha$ is crucial in determining the strength of the added repulsive mechanism.

We have observed a critical behaviour at low polymer content, but the coexistence of the two lamellar phases at higher polymer concentration seems to be rather stable. The deswelling of the $L_p$ phase on heating (Fig. 5) slightly decreases the coexistence domain of the two lamellar phases, i.e. the miscibility gap (Fig. 6), indicating the possible existence of a critical point at higher temperatures.

In the biphasic system ($L_\alpha + L_p$), the CHP derivative is mainly contained in the polymer-rich $L_p$ phase. It is possible that at high polymer content (DMPC:CHP = 95:5 by weight) the transition from the $L_p$ to the $L_p'$ phase involves a higher entropy loss than at low polymer concentration, since the periodicity of the $L_p'$ phase is smaller than that of $L_p$ phase and results in a stronger confinement of the chains. This could explain the higher stability of the two coexisting phases at high polymer content.

The importance of this long-range repulsive interaction is demonstrated by the stability (months) of mixed DMPC–CHP vesicles obtained by dilution and sonication of this swollen lamellar phase [9].

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## References

[1] R.P. Rand and V.A. Parsegian, Biophys. Biochim. Acta, 988 (1989) 351.
[2] J.N. Israelachvili and H. Wennerström, Langmuir, 6 (1990) 873.
[3] L. Rydhag and T. Gabran, Chem. Phys. Lipids, 30 (1982) 309.
[4] D.M. LeNeveu, R.P. Rand, D. Gingell and V.A. Parsegian, Science, 191 (1975) 399.
[5] D.M. LeNeveu, R.P. Rand and V.A. Parsegian, Nature (London), 259 (1976) 601.
[6] D.M. LeNeveu, R.P. Rand, V.A. Parsegian and D. Gingell, Biophys. J., 18 (1977) 209.
[7] V.A. Parsegian, N. Fuller and R.P. Rand, Proc. Natl. Acad. Sci. USA, 76 (1979) 2750.
[8] E. Evans and D. Needham, in J. Meunier, D. Langevin and N. Boccara (eds.), Physics of Amphiphilic Layers, Proceedings in Physics Series 21, Springer, Berlin, 1987, pp. 178–198.
[9] B. Demé, Ph.D. Thesis, Université Paris XI, November 1995.
[10] B. Demé, M. Dubois, Th. Zemb and B. Cabane, J. Phys. Chem., 100 (1996) 3828.
[11] K. Gawrich, D. Ruston, J. Zimmerberg, V.A. Parsegian, R.P. Rand and N. Fuller, Biophys. J., 61 (1992) 1213.
[12] G. Cevc and D. Marsh, in Phospholipid Bilayers: Principles and models, Wiley, New York 1987.

[13] R. Lipowsky, Europhys. Lett., 30 (1995) 97.
[14] M. Daoud and P.-G. de Gennes, J. Phys. France, 38 (1977) 85.
[15] M. Singh, R. Ober and M. Kleman, J. Phys. Chem., 97 (1993) 11108.
[16] H. Ringsdorf, J. Venzmer and M. Winnik, Angew. Chem., Int. Edn. Engl., 30 (1991) 315.
[17] J.C. van de Pas, Th.M. Olsthoorn, F.J. Schepers, C.H.E. de Vries and C.J. Buytenhek, Colloids Surf., 85 (1994) 221.
[18] A.M. Vincent and A. Skoulios, Acta Crystallogr., 20 (1966) 432.
[19] A.M. Vincent and A. Skoulios, Acta Crystallogr., 20 (1966) 447.
[20] A. Khan, B. Jönsson and H. Wennerström, J. Phys. Chem., 89 (1985) 5180.
[21] H. Wennerström, in J. Meunier, D. Langevin and N. Boccara (eds.), Physics of Amphiphilic Layers, Proceedings in Physics Series 21, Springer, Berlin, 1987, pp. 171–176.
[22] Th. Zemb, D. Gazeau, M. Dubois and T. Gulik-Krzywicki, Europhys. Lett., 21 (1993) 759.
[23] C. Ligoure, G. Bouglet and G. Porte, Phys. Rev. Lett., 71 (1993) 3600.
[24] T. Kato, T. Okamoto, T. Tokuya and A. Takahashi, Biopolymers, 21 (1982) 1623.
[25] T. Kato, T. Tokuya and A. Takahashi, J. Chromatogr., 256 (1983) 61.
[26] T. Kato, T. Tokuya and A. Takahashi, Macromolecules, 17 (1984) 1726.
[27] E. Nordmeier, J. Phys. Chem., 97 (1993) 5770.
[28] K. Akiyoshi, S. Yamaguchi and J. Sunamoto, Chem. Lett. Jpn., (1991) 1263.
[29] M.J. Janiak, D.M. Small and G.G. Shipley, Biochemistry, 15 (1976) 4575.
[30] M.J. Janiak, D.M. Small and G.G. Shipley, J. Biol. Chem., 254 (1979) 6068.
[31] G.S. Smith, E.B. Sirota, C.R. Safinya and N.A. Clark, Phys. Rev. Lett., 60 (1988) 813.
[32] Th. Zemb, L. Belloni, M. Dubois and S. Marcelja, Prog. Colloid Polym. Sci., 89 (1992) 33.
[33] P.-G. de Gennes, Adv. Colloid Interface Sci., 27 (1987) 189.
[34] K. Hristova and D. Needham, J. Colloid Interface Sci., 168 (1994) 302.