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Phospholipidic Monolayers on Formamide

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Résumé. — Nous présentons le premier diagramme de phase d'un film de Langmuir à l'interface air-formamide. On observe, sur des isothermes et par microscopie de fluorescence, des transitions de phase dans des films stables de phospholipides comme le DPPC ou le DSPC. Cinq phases bidimensionnelles sont mises en évidence : gaz, liquide, solide, ainsi que deux mésophases ; ces deux dernières coexistent avec le liquide sur un plateau très marqué de transition du premier ordre. Nous comparons ces observations avec les résultats connus pour les films sur l'eau.

Abstract. — We report the first phase diagram of a Langmuir film at the air-formamide interface. Stable films of phospholipids such as DPPC or DSPC undergo phase transitions observed on isotherms or by fluorescence microscopy. They display bidimensional gas, liquid and solid phases, as well as two mesophases, the latter coexist with liquid on a sharp first-order transition plateau. We compare these observations with known results on films on water.

1. Introduction

Monolayers of amphiphilic insoluble molecules deposited at the air-water interface often serve as experimental tests for two-dimensional phase transition models. However, such a simplification relies on the assumption that the system is purely two-dimensional, i.e. that water plays no role other than that of a passive substrate and a thermal reservoir.

To investigate the role of the substrate in the microscopic structure of a Langmuir film, numerous non-aqueous solvents have been used as subphase: e.g. ethylene glycol, glycerol, oleic acid, tricresyl phosphate, liquid mercury, and more recently formamide [1-3]. The advantages of the latter are its high polar moment and dielectric constant, 3.4 Debye and 109.5, respectively, at 20 °C vs. 1.85 and 80.2 for water). Its solvation properties, hydrogen bonds, surface tension (58.2 mN.m⁻¹ at 20 °C, vs. 73 mN.m⁻¹ for water), and molecular stability make it as suitable

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as water for spreading a Langmuir film. Moreover, its viscosity (less than 4 times higher than water) and wetting properties do not constitute any inconvenient for manipulation.

We investigated the differences between water and formamide for a film of phospholipids [4]. As shown in this paper, we discovered that a film on formamide has a phase diagram as rich as on water, and deserves a thorough study of both its equilibrium and dynamics of phase transitions. We present a characterization of the phases observed between 5 and 50 °C, and the exploration of high-pressure, low surface tension properties.

2. Experimental Methods

Our home-made teflon Langmuir trough undergoes a symmetrical compression by two teflon barriers, varying the area from 260 cm$^2$ to 45 cm$^2$ (accuracy 0.1 cm$^2$) with a typical compression rate of 4 to 10 Å$^2$/molecule per minute. It is covered with glass against evaporation and dust, except for a hole where the Wilhelmy balance (Riegler & Kirstein, resolution 0.1 mN.m$^{-1}$) dips into the formamide. The microscope is equipped with epifluorescence and an intensified video camera; its plate is removed and, instead, the trough is placed under 10 mm working distance dry objectives. For safety and cleanliness reasons, the whole set-up is placed in a glove box, with all controls outside: trough displacements, compression, area and pressure recordings, thermal regulation, video monitor and tape recorder.

The trough is filled with formamide for analysis, ACS (> 99.5%), Merck 9684, used without further purification. Its temperature is regulated, with fluctuations less than ±0.1 °C (or ±0.3 °C above 45 °C), at a chosen value $T$ limited by freezing (2.3 °C) and evaporation of formamide. After cleaning its surface by aspiration, we deposit with a microsyringe a chloroform solution of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), a phospholipid with two 18-carbons chains purchased from Avanti Polar Lipids and also used without further purification. Alternatively, we use its 16-carbons analog, the DPPC. Successive compressions and decompressions yield recordings of isotherms, i.e. pressure $P$ versus total area (Fig. 1). The concentration and amount of solution deposited yield the area per molecule $A$; its precision is ±10%, as assayed from the reproducibility of isotherms.

To assay the purity of DSPC, we first check the pressure value of the first order transition plateau: it is stable and reproducible for hours (not shown). It is sensitive to impurities: e.g., in the range 22 °C-35 °C, adding 0.25% (molar) DiO-C18 makes no detectable difference, but 1.8% (molar) NBD-DPPC increases the value of $P$ by 50%. Second, we check the transition's sharpness, quantified by the plateau's slope (-$A$ d$P$/d$A$)$_T$: it is surprisingly good for a fresh monolayer (Figs. 1 and 2), denoting a good purity of both DSPC and formamide substrate. After 4 hours around 30 °C, or after 1/2 hour around 50 °C, the isotherm slope (-$A$ d$P$/d$A$)$_T$ on the plateau doubles, and the slope in the liquid phase increases by about 20%, probably indicating a degradation of the monolayer. Dyes degradation markedly decreases these durations and even prevents fluorescence observations above 40 °C.

For fluorescence microscopy, we mix pure DSPC with a small amount of fluorescent molecule, e.g. an 18-carbons double-chained carbocyanine (DiO-C18, Molecular Probes), an 18-carbons lipid grafted with a NBD dye (NBD-C18, Molecular Probes) or a DPPC grafted with a NBD dye (NBD-DPPC). These dyes provide visual contrast between the different phases: observation under microscope, during isotherm recording, thus detects the coexistence between two contrasted phases, and therefore the beginning and the end of phase transitions (Figs. 3a, b). Contrast lasts for a few hours, until the focalisation is prevented by out-of-plane fluorescence, probably denoting partial dissolution of probes in formamide. DiOC18 is more stable and contrasted than NBD, which furthermore photo-bleaches.
Fig. 1. — Isotherms of DSPC (see inset) on formamide. The plateau separates: i) gas and solid phases at 15 °C; ii) liquid and mesophase 1 at 22.5 °C and 25 °C; iii) liquid and mesophase 2 at 32.5 °C, 38 °C, 46 °C and 50 °C. Molecular areas are indicative, due to the incertitude on the number of molecules deposited.

Fig. 2. — Slope of isotherms of fresh DSPC monolayers, measured on both sides of the change of incline (right end of the plateau). Circles: compression modulus of the liquid at the transition. Squares: slope of the plateau, indicating the sharpness of the transition.
Fig. 3. — a) Transition from mesophase 1 (bright) to mesophase 2 (dark), showing cellular patterns; liquid-mesophase 2 coexistence region is similar. b) Transition from gas to mesophase 1, showing growing irregular mesophase domains (bright); gas-solid coexistence region is similar. c) Transition from solid to gas, showing drift (arrows) of undeformed bright solid plates. d) Transition from mesophase 1 to gas, showing fraying mesophase domains (bright). Field size: a) 220 x 220 μm², b) 300 x 220 μm², c) 500 x 270 μm², d) 270 x 270 μm².
3. Results

Langmuir films are characterized by three thermodynamical variables: the temperature $T$ of the liquid substrate, the average area $A$ per molecule, and the two-dimensional pressure $\Pi = \gamma_0 - \gamma$, equal to the difference between the surface tension $\gamma_0$ of pure liquid substrate and its surface tension in the presence of the film. We built the phase diagram by identifying the phase transitions.

3.1. First Order Transition: Liquid-Mesophase Transition. — The most prominent feature of isotherms is a first order transition (Fig. 1). Its wide and well defined plateau has a low slope (Fig. 2); on a fresh monolayer at low temperatures, the slope $(d\Pi/dA)_T$ is virtually zero, at least less than our precision on $\Pi$ ($0.1 \text{ mN.m}^{-1}$) over a range of $50 \text{ A}^2/\text{molec}$, i.e. $2 \times 10^{-3} \text{ mN.m}^{-1}\text{.A}^{-2}$. Increasing the compression velocity, up to $100 \text{ A}^2/\text{molecule per minute}$, does not yield any slope increase or nucleation bump: equilibrium is quickly reached.

Isotherms and fluorescence yield complementary observations of the transition from $[22^\circ\text{C}, 0.2 \text{ mN.m}^{-1}]$ just above the gas-liquid-condensed triple point, up to the highest point we can reach $[50^\circ\text{C}, 32.6 \text{ mN.m}^{-1}]$, where we still observe this transition (see Sect. 3.4). Fluorescence observations, although unusable above $40^\circ\text{C}$, turn useful between $22^\circ\text{C}$ and $25^\circ\text{C}$, where pressures can be measured but are still too low to display a visible plateau on isotherms (Fig. 1). In addition, fluorescence confirms that the transition is first order, by proving that the plateau actually corresponds to coexistence between two phases.

Note that similar studies on DPPC, in which both chains consist of 16 instead of 18 carbons, yield analogous results, but transition temperatures are $13^\circ\text{C}$ lower (Fig. 4). This is compatible with phase correspondence between monolayers of similar molecules with different chain lengths, as already observed on water [5].
The phase at the right part of isotherms, namely the high temperature/low pressure region, is moderately dense. Its area per molecule $A$ is large and decreases with pressure (Fig. 1): at a given temperature, its reaches at the beginning of the plateau its lower limit, e.g. $A = 185 \text{ Å}^2$ at 25 °C. Its compression modulus\(^1\) $K$ is low, independent of impurities, and increases with pressure (Fig. 2): along the transition line, it is e.g. 3.4 mN.m\(^{-1}\) at 25 °C and 50 mN.m\(^{-1}\) at 50 °C. By analogy with monolayers on water, this denotes a two-dimensional liquid phase (L). As expected for a liquid, it incorporates a large amount of probes, thus appears bright on fluorescence.

On the left side of the plateau, two low temperature/high pressure phases are observed. With typical molecular area values of 50-60 Å\(^2\), and a compression modulus $K$ larger than 300-500 mN.m\(^{-1}\), they are close packed phases. They differ by their ability to incorporate probes; we note them tentatively M1 and M2, where “M” stands for “mesophase”.

The intermediate pressure phase M1 is slightly brighter than L, which is natural if probes do not segregate between both phases, since M1 is denser than L. Coexisting domains of liquid and M1 thus display a low contrast.

On the opposite, domains of the higher pressure phase M2 appear dark by expelling probes. M2 domains thus never invade the whole surface, yielding characteristic cellular patterns at liquid-M2 or M1-M2 transitions (Fig. 3a), or even honeycomb patterns as bright borders get thinner.

\(^1\) We use here the two-dimensional compression modulus $K = -(\text{d}P/\text{d}A)_T$. Recall that $K$ is the inverse of the compressibility [6] and is expressed in mN/m. It is measured by determining the ordinate of the point where the tangent to the isotherm intersects the pressure axis.
3.2. Transitions from the gas. — Deposition is done at a typical area $A = 250 \, \text{Å}^2$/molecule. Above $22 \, ^\circ\text{C}$, this is fully in the liquid phase region and no coexistence is visible. Indeed, the liquid phase is highly extensible and is stable until hundreds of $\text{Å}^2$/molecule.

On the opposite, under $22 \, ^\circ\text{C}$, such an area is in the region of coexistence between two phases. Immediately at deposition, contrasted domains are readily visible with fluorescence, denoting a first order transition. Under compression, they grow digitated, probably by diffusion-limited aggregation of sparse molecules (Fig. 3b).

The dark phase has thus a low density of molecules, and of course of probes. It exists only at pressures too weak to be detected within the precision of our Wilhelmy balance. An obvious analogy with monolayers on water helps recognizing a two-dimensional gas (G). The triple point liquid-gas-mesophase M1 should lie just below $22 \, ^\circ\text{C}.

The bright domains consist in close packed phase. At temperatures lower than $19.5 \, ^\circ\text{C}$, it is an incompressible phase; its area per molecule at $20 \, \text{mN.m}^{-1}$ is compatible with the $45 \, \text{Å}^2$ measured by Sackmann and coworkers for solid phase on water [7, 8]. Visually, its rigidity is denoted by the way it holds the plate of the Wilhelmy balance. On decompression, it is brittle: cracks separate large plates which drift like continental crusts (Fig. 3c). These plates remain undeformed for hours. Clearly, it is a solid (S) and not a fluid phase; see also Section 3.4.

At temperature higher than $20 \, ^\circ\text{C}$, the dense phase is M1. Under decompression, it is ductile and frays (Fig. 3d). Torn pieces coalesce and round slowly with time: M1 is thus probably a mesophase, intermediate between fluid and solid.

If we start on the gas-solid coexistence region and raise temperature at fixed total area, we go into the gas-M1 coexistence region. At the presumptive gas-M1-solid triple point, $19 \pm 1 \, ^\circ\text{C}$, solid domains suddenly explode. There is thus likely a finite discontinuity of molecular area $\Delta A = A_{M1} - A_S > 0$, meaning that the solid-M1 transition would be first order.

3.3. Transitions Between Close Packed Phases (Solid, M1, M2). — Transition between solid and either mesophase is difficult to characterize. Solid and M1 coexist on a very small pressure range (Fig. 5), and transition is thus better studied along the line of coexistence with gas, as reported above.

Solid-M2 transition is never crossed on isotherms: the transition line is probably vertical in $(T, \Pi)$ diagram, as is expected if the solid is barely sensitive to pressure (Fig. 5). Fluorescence is also inadapted: probes have weak diffusion coefficients in close packed phases and freeze within domains; thus no rearrangement is visible when going from solid to M2 or back. Varying the temperature $T$ at fixed area $A$ turns more instructive. During temperature cycles at a rate of $1 \, ^\circ\text{C}$ per hour, we observed a $20\%$ jump of $(d\Pi/dT)_A = (dS/dA)_T$ where $S$ is the molecular entropy (e.g. from $0.83 \, \text{mN.m}^{-1}\text{K}^{-1}$ in solid phase, to $0.65 \, \text{mN.m}^{-1}\text{K}^{-1}$ in mesophase M2). This discontinuity, barely reproducible only on fresh layers but consistently observed at $T = 20 \, ^\circ\text{C} \pm 0.5 \, ^\circ\text{C}$ at different pressures, might sign the solid-M2 transition but does not discriminate between first and second order.

What about M1-M2 transition? At the liquid-M1-M2 triple point, the three latent heats $T(S_L - S_{M1}), T(S_{M1} - S_{M2})$, and $T(S_{M2} - S_L)$ should evidently add to zero. Since we cannot see distinctly any break of slope $d\Pi/dT$ on the liquid-mesophase transition curve around $[27 \, ^\circ\text{C}, 1.8 \, \text{mN.m}^{-1} ]$, the M1-M2 latent heat is undetectable. Another hint comes from isotherms: only on very fresh layers is sometimes a small change of incline visible (e.g. at $21.5 \, ^\circ\text{C} - 1.1 \, \text{mN.m}^{-1}$). If it is not an artefact, this could sign a first order M1-M2 transition, but a very weak one, with upper limits on the discontinuity in molecular area $\Delta A < 2 \, \text{Å}^2$ and on the discontinuity in compression modulus $\Delta K < 3 \, \text{mN.m}^{-1}$. Thus the transition is either a second-order, or a weak first-order transition.

In the presence of probes, the M1-M2 transition is clearly visible. The problem is to extract,
Fig. 5. — a) Phase diagram of DSPC on formamide; b) sketch of an enlarged area. Triangles are experimental measurements; solid lines are guides for the eyes; hatched lines mark the uncertainty in determination of transitions between close packed phases; stippled regions are not accessible to our measurements. G = gas, L = liquid, M1 and M2 = mesophases, S = solid.

from this binary mixture transition, information relevant to the pure body transition. Indeed, M2 incorporates much less probes than M1; it appears that probes do not much affect the beginning of transition (nucleations of M2 within M1 phase) but drastically increase the pressure needed for disappearance of M1 phase (never fully achieved in practice). We thus estimate the transition pressure by visually detecting the first appearance of dark domains within the bright phase. M1 phase exists only close to the transition towards liquid (Fig. 5b).

3.4. High Pressure. — Films are characterised by a very high stability, not only in time, but also in pressure, possibly related to the good anchoring of polar heads by strongly polar formamide molecules. We recall that the two-dimensional pressure is \( \Pi = \gamma_0 - \gamma \); thus the surface tension \( \gamma \) of a film on formamide vanishes at a pressure \( \Pi = 58 \text{ mN.m}^{-1} \). Therefore films on formamide can display manifestations of very small \( \gamma \), such as the decrease of the capillary length \( \lambda \approx \sqrt{\gamma / \rho_{\text{form}} g} \) followed by the disappearance of the meniscus on the teflon walls when the surface tension becomes too weak to balance gravity. Given the design of our teflon trough, formamide overflows the trough when the meniscus vanishes, so that there is an upper limit to the pressures which we can reach; this limit decreases abruptly around 50 °C (Fig. 5a).

Note that, on water, a film at the same pressure \( \Pi \) would have a surface tension \( \gamma = 15 \text{ mN.m}^{-1} \) and further compression would be hindered by the collapse of the film, preventing access to low-\( \gamma \) regions. On formamide, a limited collapse is observed only at pressures where the meniscus begins to diminish, around 55 mN.m\(^{-1}\).

On formamide, the mechanical resistance of the solid phase is surprising. Under high uniaxial constraint, more than 20 mN.m\(^{-1}\), a stable and permanent undulation of the surface appears parallel to the compression barrier. It is visible under normal microscopy with no necessity of fluorescent probes: the film buckles instead of collapsing. A detailed experimental and theoretical study of this phenomenon, which had been already predicted [9] but never observed, will be published elsewhere [10].
4. Discussion and Conclusion

- It is possible to form stable films of phospholipids on formamide. DSPC displays at least five bidimensional phases differing by their density, their elastic properties and their ability to incorporate fluorescent probes: gas, liquid, solid, as well as two mesophases which we denote by M1 and M2. No optical anisotropy is visible under crossed polariser and analyser.

- Let us recall that on water [7], some global features of the phase diagram are identical: existence of gas, liquid and solid with similar molecular areas and analogous liquid-mesophase transition. But the relative positions of the phases are different. Most strikingly, the liquid-mesophase transition on water ends on a tricritical point.

- On the opposite, on formamide, the liquid-mesophase transition extends unmodified up to the highest accessible pressures. It is a first-order pure-body transition characterized by a sharp plateau whose slope dII/dA increases with time at high temperatures. Despite our investigations, there is no indication that the slope visible on 46 °C and 50 °C isotherms should reveal the existence of any tricritical point.

- More quantitatively, we note that, by comparison with Figure 11 of reference [7], the plateau pressure is higher on formamide but dII/dT is similar. This would be readily interpreted if the value II were mainly determined by interactions between polar heads, and dII/dT by entropy of aliphatic tails.

- The stability of the film against high pressures manifests itself by the access to low surface tensions and by a buckling of the film in solid phase, instead of collapsing.

- Grazing incidence X-ray diffraction experiments are in preparation. This method has recently demonstrated the existence of highly crystalline phases (correlation length over 1000 Å) for arachidamide on formamide [3]. Previous comparisons between films on formamide and on water have demonstrated that solvent molecules can affect the microscopic structure of the film, e.g. by the formation of dimers through hydrogen bonds between amide groups [2]. Here, we show that the solvent can also affect the whole phase diagram.

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Note added in proof: since we first submitted this paper, we observed unambiguously in X-ray diffraction experiments [11] that the solid phase is crystalline, with tilted chains. When the pressure is increased from 1 to 38 mN.m⁻¹, the area per molecule decreases from 46.2 Å² to 45.7 Å², with three different peaks; the structure undergoes no phase transition. Further studies, including X-ray characterization of M1 and M2 phases on formamide, are planned.
References


