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Steric-Induced Effects on Stabilizing a Lamellar Structure

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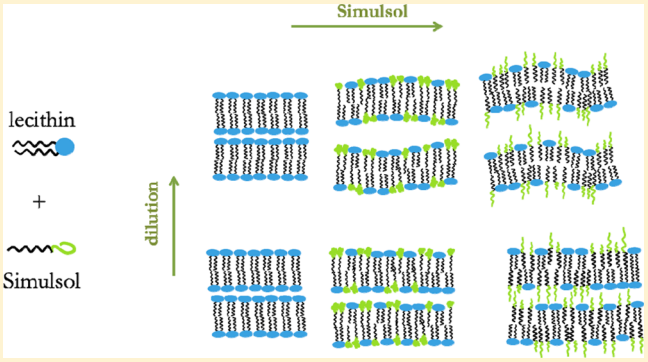
ABSTRACT: We investigate the behavior of multilamellar phases composed of lecithin and a commercial cosurfactant (Simusol), which is a mixture of ethoxylated fatty acids. Using X-ray scattering and a new procedure to fit the data, relevant parameters characterizing the lamellar structure were determined as a function of membrane composition, varying from 100% of lecithin to 100% of Simulsol. Scattering data illustrating the swelling of the lamellae for different amounts of cosurfactant are presented with the respective behavior of the Caillé parameter. With this experimental approach, we show that the incorporation of ethoxy brushes onto the lipid surface enhances repulsive interactions arising from membrane fluctuations and changes the interactions at the interface between bilayers.



INTRODUCTION

Lamellar systems composed of regularly stacked bilayers have been used to investigate several biological processes,[1−4] providing important knowledge on fundamental aspects such as interactions between membranes,[5−8] and also on many biotechnological applications.[9−12] The structure is periodic only across the direction perpendicular to the membrane surface whereas in the other two dimensions the bilayers behave as a fluid. The fluidity of the membranes comes either from the orientational and translational disordering of surfactant molecules or from the flexibility of hydrocarbon chains.[13−15] Thermal fluctuations in membranes give rise to repulsive interactions as a result of the mutual steric hindrance when they are in close proximity. Such interaction plays a fundamental role for stabilizing the lamellar structure since its range and strength competes with van der Waals attraction for flexible enough bilayers.[16−19]

X-ray and neutron scattering techniques have proved to be powerful tools to obtain a detailed picture of the lamellar structure.[20−28] Thermal fluctuations deeply affect the (quasi) long-range positional order, resulting in an enhanced diffuse scattering. The appropriate analysis of the scattering data brings information not only about the structure of the lamellar phase but also on its elastic properties. In this context, a major variable is the so-called Caillé parameter, which carries coupled information on both the bending constant of membranes and the compression modulus of the stack.[29]

In previous reports, some of us demonstrated the ability of mixed lecithin/Simulsol phase to host DNA fragments and form lipoplexes from neutral lipids.[30−34] Our findings have shown that steric forces in lamellar system play a major role for complexation with DNA and hydration of the system (and consequently, its smectic periodicity) is able to drive confinement of nucleotides in-between bilayers. The confinement induces a rich polymorphism of supramolecular ordering of DNA-double-chains embedded in lamellar stacks. In the current work, we further investigate on steric interplay in these lecithin/Simulsol phases by using a recently developed model to analyze X-ray scattering data.[23]

We investigate the stability of lamellar structures, when the range and strength of steric repulsive interactions are modulated by varying the amount of (short) amphiphilic polymers playing the role of a cosurfactant grafted at the membrane surface. The experimental approach consists in a systematic variation of the membrane composition, by adding the cosurfactant to the bilayers composed, initially, of lecithin, a common lipid. The cosurfactant, commercially known as Simusol, is a mixture of ethoxylated fatty acids with, typically, about 10 ethoxy groups. Because of its nonionic nature, the overall charge of the membrane remains neutral since lecithin is a zwitterionic molecule. The incorporation of single chain molecules to the bilayer is expected to increase its flexibility; however a second effect related to the hydrophilic part of the cosurfactant molecules is found: an extra repulsion of steric origin between membranes with an extended range depending on both grafting density and chain length, arises because water at room temperature is a good solvent for the ethoxylated part of the cosurfactant. Different domains of interactions between membranes can be experimentally accessed, by varying two experimental parameters. The first one is the hydration of the

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lamellar phase, which affects the distance between bilayers. The second is the membrane composition with consequences on both the flexibility and the range and strength of the extra steric repulsions.[28,35]

We determine relevant parameters of the lamellar structure by varying the membrane composition from 100% of lecithin to 100% of Simulsol and scanning a dilution line for each composition. In the analysis of X-ray scattering data, the electron density profile is modeled by a set of symmetrical Gaussian functions.[23] The fitting is performed directly to the scattered intensity, using a partial constrained least-squares routine, which allows the simultaneous determination of the form and structure factors and several other parameters that describe the model, such as bilayer thickness and Caillé parameter. The systematic variation of composition combined with the analysis method reveals a new regime of intermembrane interactions that is not accessible for membranes composed of pure lecithin.

THEORETICAL BACKGROUND

The stability of lamellar phases is usually described as the interplay between attractive van der Waals interactions and repulsive forces that can be of different natures.[16−19,36−38] Experimentally, for highly dehydrated compositions where bilayers are almost in contact a strong short-range repulsive interaction has been observed, which is known as hydration force.[39,40] A second repulsive mechanism, conceptually a subtle consequence of the previous one, was proposed by Helfrich,[16] considering the fluid aspects of the membranes, which can present undulations. It gives rise to a repulsive force that for flexible enough bilayers is comparable in order of magnitude and range to the (attractive) van der Waals forces. One of the simplest possible approaches is to assume that the various interaction potentials can be added. In this case, the free energy density of a multilayered structure composed of noncharged flexible bilayers could be tentatively written as[36,41]

$$V = V_{\mathrm{hyd}} + V_{\mathrm{vdW}} + V_{\mathrm{und}} \tag{1}$$

with V_{hyd}, V_{vdW}, and V_{und} referring to hydration, van der Waals, and undulation forces, respectively.

When bilayers are brought closer together, there is a loss of configurational entropy associated to bilayer undulations. This is accompanied by an increase in free energy and, according to Helfrich, this contribution is expressed as[16]

$$V_{\mathrm{und}} = \frac{3\pi^2}{128}\left(\frac{k_{\mathrm{B}}T}{\kappa a^2}\right) \tag{2}$$

where κ is the bending rigidity of the bilayer, a is the membrane separation, and T is the temperature. The free energy density can then be written as

$$V = -\frac{H}{12\pi}\left[\frac{1}{a^2} - \frac{2}{(a+\delta_{\mathrm{M}})^2} + \frac{1}{(a+2\delta_{\mathrm{M}})^2}\right] + V_{\mathrm{hyd}}e^{-a/\lambda_{\mathrm{H}}} + \frac{3\pi^2}{128}\left(\frac{k_{\mathrm{B}}T}{\kappa a^2}\right) \tag{3}$$

with H equal to the Hamaker constant of the bilayer—solvent system; δ_{M} the membrane thickness, and λ_{H} is the decaying length of the hydration force. Other attempts to describe theoretically the potential between membranes have been done, introducing modifications in the above expressions. Such trials have resulted in predictions about the stability of the lamellar phase and the nature of the transition from bounded to nonbounded states, depending on the strength of interactions.[17−19,42]

The membrane fluidity has been recognized as an important parameter driving long-range repulsive forces and displacing the minimum energy to larger values of membrane separation distance.[17,43−45] This problem has been theoretically[17−19,43,46] and experimentally[28,43,44,47,48] addressed using different approaches. In literature, there are experimental works investigating on the dependence of bending rigidity upon membrane composition and salt content in aqueous solutions.[35,47−51] More recently, computational simulations have been conducted to investigate the molecular origin of bending rigidity.[52−55]

From the experimental point of view, information about elastic properties of lamellar phases can be obtained using X-ray scattering techniques. Since thermal fluctuations destroy long-range positional order, Bragg peaks become broader and the intensity of high order peaks decays very fast. As above-mentioned, the analysis of full-range scattered intensities provides detailed information, not only about the global structure but also on thermal fluctuations.[20−28] The Caillé parameter describes the decaying of elastic correlations and is given by:[29]

$$\eta = \frac{q_0^2 k_{\mathrm{B}}T}{8\pi\sqrt{BK}} \tag{4}$$

where K and B are the curvature and compression modulus of the lamellar phase, respectively, and q_0 is the wave vector of the first-order Bragg peak.

Global analysis of X-ray scattering data has been used for investigating several lamellar systems dominated by undulation forces.[26,44] It has been shown that the experimental values of Caillé parameter then follow a universal expression given by

$$\eta = \frac{4}{3}\left(1 - \frac{\delta_{\mathrm{M}}}{D}\right)^2 \tag{5}$$

providing experimental evidence of the expression derived by Helfrich for the undulation force. In this expression, D is the smectic period, equal to $2\pi/q_0$, related to the previously defined geometric parameters by $D = \delta_{\mathrm{M}} + a$.

MATERIALS AND METHODS

The mixture of lipids was prepared by cosolubilizing in cyclohexane soya lecithin (Avanti Polar) and Simusol 2599 PHA (Seppic) in suitable proportions. These products (initially chosen[30−32,34] despite their complex analytical compositions because they are pharmaceutical excipients complying with the European Pharmacopoeia) were used without further purification and the solvent was evaporated in a desiccator. The major constituent of soya lecithin is dilinoleoylphosphatidylcholine and its specific mass is 1.01 g/cm^3. The major constituent of the hydrophobic part of Simulsol is oleic acid (∼72%) with also palmitic (∼11%) and stearic (3%) acid chains in noticeable amounts. The ethoxylated chain consists of polyethylene glycol (PEG) with 10 monomers on average but the longest chains may contain up to 20 monomers.[56] The average length estimated for the polymer chain is 3−4 nm fully extended but longer chains up to 7 nm can also be present. The specific mass is 1.02 g/cm^3.

The membrane composition is expressed in terms of the percentage in mass of Simulsol incorporated to lecithin membranes. The lamellar phase was prepared by adding the lipid mixture and distilled water (Milli-Q) in proper quantities. The samples were submitted to shake and centrifugation processes until homogeneity was achieved. The composition of the lamellar phase is expressed in terms of the total lipid volume fraction (φ_{lip}), that is, lecithin plus Simulsol. The

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membrane composition was varied from 100% of lecithin to 100% of Simulsol and for each composition the lipid volume fraction was varied, scanning the dilution line.

X-ray scattering experiments were performed in samples placed in glass capillaries using a laboratory equipment (Nanostar-Bruker) with resolution Δq = 0.334 nm^{-1}, is related to the beam size and detector resolution that defines the minimum distance that can be resolved in the reciprocal space by the experimental setup. This resolution also creates a peak broadening which was taken into account in the modeling procedures. The experimental data were expressed as a function of the scattering vector **q**, which is the reciprocal space momentum transfer modulus. This vector is given by $\mathbf{q} = (4\pi \sin\theta)/\lambda$, where λ is the radiation wavelength (0.154 nm) and 2θ is the scattering angle. In the standard configuration where 0.1 nm^{-1} < **q** < 3.5 nm^{-1}, up to three Bragg reflections could be observed in the concentrated regime. Experiments at higher angles with **q** values in the range 0.5−30 nm^{-1} were also performed aiming to check the nature of the lamellar phase. For some samples, experiments were performed at the Soleil Synchrotron facility, SWING beamline, with beam energy of 12 keV (λ = 0.103 nm), $\Delta\mathbf{q}$ = 0.038 nm^{-1}, and accessible **q** vectors in the range from 0.3 to 17.45 nm^{-1}. The lower the value of the $\Delta\mathbf{q}$ (higher resolution in **q** space), the sharper the Bragg peaks will appear on the scattering data. All scattering experiments were performed at room temperature (~22 °C).

As a complementary technique, polarizing microscopy observations were carried out to verify whether the samples presented the typical texture of lamellar phases.

EXPERIMENTAL RESULTS

The experimental scattering data were analyzed with a recently published modeling procedure,[23] where the electron density profile is described by a set of Gaussian functions and the fitting is performed directly on the scattered intensity. With this modeling method, the parameters describing the form and structure factors of the system are optimized simultaneously. The quality of the fitting is quite good (except for very low angle region) as it is shown in Figure 1 for scattering data obtained from both a laboratory equipment and synchrotron source. It is important to note that since resolution effects are included in the fitting procedure the full **q** range of the experimental data can be described for data obtained in equipments with different resolution values. The analysis method was therefore applied to the whole set of experimental data.

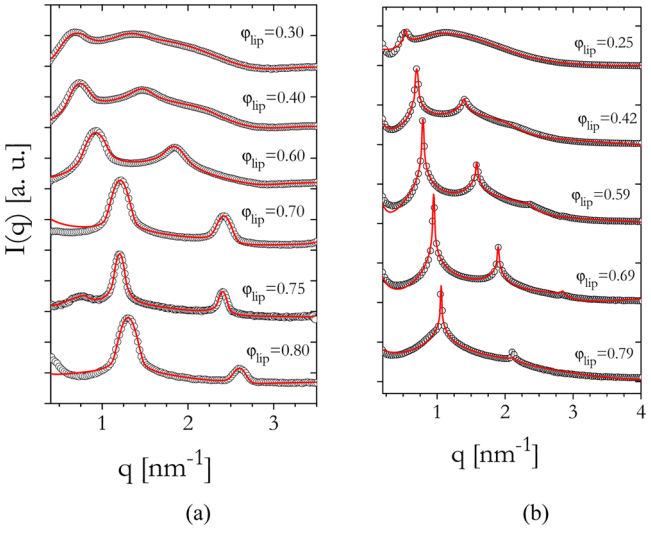

Figure 1. Scattering experimental data (symbols) and the respective theoretical fits (solid lines) for two membrane compositions (Simulsol mass fraction): 30% (a) and 90% (b). In (b), the scattering curves were obtained in a Synchrotron line.

In Figure 1, it is possible to note that as the phase is hydrated the peaks become broader and the second order Bragg reflection disappears. The scattering curves are indexed to a single smectic phase except in a narrow band where we assume that two lamellar phases coexist. The biphasic domain occurs for lipid volume fraction around 0.7 with width $\Delta\varphi_{lip} \approx 0.05$ and Simulsol content between 10 and 80% in mass. An example of coexisting phases is shown in Figure 1a for φ_{lip} = 0.75 with the respective fitting curve. The assumption of two coexisting lamellar structures is supported by optical observations in polarizing microscopy (data not shown). One phase is clearly similar to the one present at similar lipid volume fractions and the coexisting phase could be described as a phase with larger periodicities. It might be related to a phase of pure Simulsol. Since the scattered intensity from one of the lamellar phases is much lower than the other, one can conclude that this phase corresponds to a small fraction of the system as well as presenting a low number of correlated planes. In the following, the analysis of scattering curves concerning the data obtained for the single lamellar phase domain will be presented only.

A broad peak at **q** ~14 nm^{-1}, characteristic of a fluid lamellar phase, was observed for wide-angle experiments (WAXS data not shown), in the whole dilution range and membrane compositions explored in this work.

The effect of the cosurfactant amount on the swelling behavior of the phase is presented in Figure 2a, where data is obtained from the

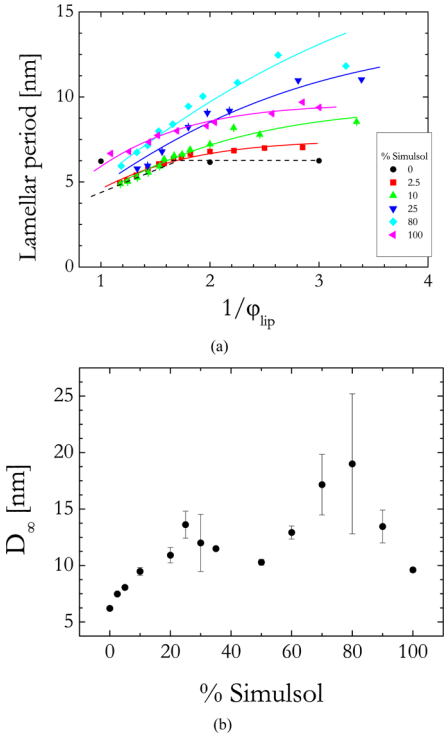

Figure 2. (a) Swelling behavior of the lamellar phase for different amounts of cosurfactant incorporated to the lamellar phase. The solid lines are fits of eq 6 to experimental data. The dashed line is a guide to the eyes and illustrates the swelling regime and the dilution limit of the pure lecithin lamellar phase. (b) Asymptotic lamellar period D_∞ obtained by extrapolating $1/\varphi_{lip} \to \infty$.

structure factor parameter $\mathbf{q}_0$. It can be noted that lamellar stacks composed of pure lecithin attain a maximum dilution limit with smectic periodicity equal to 6.3 nm. On the other hand, lamellae containing cosurfactant are able to incorporate larger amounts of water, attaining higher periodicities around 12 nm for 80% Simulsol (Figure 2a). The evolution of the smectic step as a function of the inverse lipid volume fraction cannot be described with the simple dilution law, namely $D = \delta_M/\varphi_{lip}$, which is encountered in many lamellar systems. This simple law is derived from geometric

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considerations where the area per lipid molecule at the bilayer−solvent interface is constant, and the bilayers are rigid enough to remain flat. The observed departure from the geometric swelling law being from below, it cannot be ascribed here to bilayer undulations, by the way, a rather small effect.[49,57−60] It is thus the consequence of a significant increase of the (effective) area per lipid/cosurfactant molecule as dilution proceeds, itself presumably resulting from conformational changes (or perhaps even transitions, when the lamellar−lamellar phase separation is encountered at low hydration for most membrane compositions for instance) of the Simulsol ethoxy heads, as argued below from a different perspective. As the lamellar phase is hydrated, the period tends to a limiting value and an attempt of describing the behavior of D as a function of $1/\varphi_{lip}$ can be done with the help of the parametric function

$$D = D_\infty \tanh\left[\frac{\delta_M}{D_\infty \varphi_{lip}}\right] \quad (6)$$

where D_∞ is the maximum lamellar period extrapolated for $1/\varphi_{lip} \rightarrow \infty$, that is, pure water, and δ_M is a parameter that can be interpreted as the membrane thickness in the highly dehydrated limit. As shown in Figure 2a, this phenomenological expression provides a reasonable fit to the swelling data, even though the parameter D_∞ is obviously not precisely defined when the departure from the simple swelling law is small—80% Simulsol being the most questionable sample in this respect.

Larger limiting lamellar periods can be obtained with the incorporation of cosurfactant to the lecithin lamellar phase with D_∞ values around 15−18 nm for certain compositions (Figure 2b). Nevertheless, the dependence of D_∞ on the amount of Simulsol does not follow a simple monotonic function, and 50% visually marks a kind of boundary between two otherwise qualitatively similar domains, one rich in lecithin and the other rich in Simulsol.

The evolution along dilution lines of the structure factor-related Caillé parameter reveals a conspicuous behavior (Figure 3). The

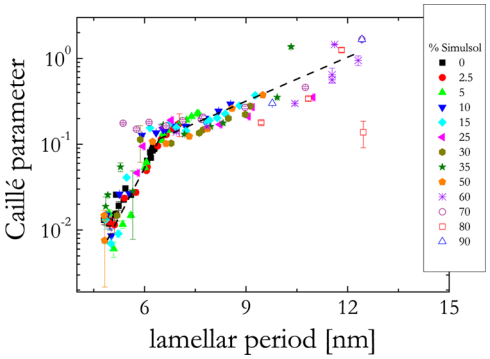

Figure 3. Caillé parameters obtained from fits to the scattering curves, varying the amount of cosurfactant incorporated to the lipid membranes and the hydration. The two dashed lines are only guides to the eyes.

presence of cosurfactant in the membrane introduces significant changes in elastic fluctuations and two distinct hydration regimes can be observed, apparently universal and independent of the amount of cosurfactant incorporated to the bilayer. Two roughly exponential dependences on hydration are observed with a change taking place for a lamellar period of approximately 6.5 nm. Taking δ_M = 4 nm as the typical value that results from fits of eq 6 to our data, such a distance corresponds to a separation between membranes equal to 2.5 nm, a value comparable to the extended length of the polymer chains of Simulsol molecules, consisting on average of 10 ethylene glycol monomers.

In the more hydrated regime, the Caillé parameter attains values close to 1, usually characterizing very flexible membranes. Considering the lamellar period in the range of 10−12 nm, the distance between bilayers is typically 2.5−3 times larger than the membrane thickness and the membranes should be weakly interacting. The behavior of the Caillé parameter shows that in fact the incorporation of single chain molecules into the lecithin bilayers contributes for increasing membrane flexibility, opening a new regime of interactions that was not present in the lamellar phase composed of lecithin only.

Another remarkable effect, related to cosurfactant polymer chains at the interfaces of bilayers, has emerged from our investigations. It is revealed by our form factor model description of the scattering intensity and can be noticed by comparing the electronic contrast of the bilayers, as shown in Figure 4a, for increasing amounts of

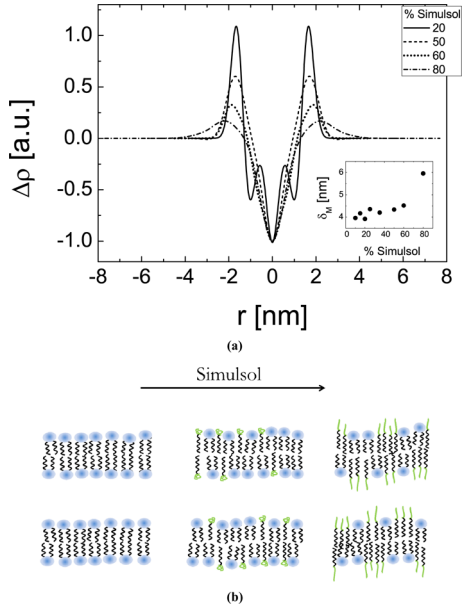

Figure 4. (a) Electronic contrast obtained from the fitting procedure for a lamellar period approximately equal to 8 nm and varying amount of Simulsol. In the figure, r = 0 represents the center of one bilayer, and the definition of negative and positive contrasts is taken by using the electron density of water as reference. Inset: Effect of the cosurfactant on the membrane thickness, as it results from the electron density profile. (b) Schematic representation at maximum swelling of lipid bilayers initially composed of lecithin with incorporation of Simulsol molecules.

cosurfactant at constant lamellar period, $D \approx 8$ nm. The contrast of the polar region becomes less pronounced and broader as the amount of Simulsol in the membrane increases. The membrane thickness, not to be confused with parameter δ_M in eq 6, being obtained from the electronic profiles considering the distance between the points where the contrast falls to the half of the maximum value,[23] its values are plotted in the inset of Figure 4a. The increase in membrane thickness with Simulsol content also appears as a two-step effect.

We tentatively propose that a kind of transition from mushroom to brush conformation of the ethoxy chains takes place for increasing density of Simulsol grafted on the bilayer surface,[57] as shown schematically in Figure 4b. The experimental data indeed suggest that such a transition occurs when the Simulsol amount in the membrane grows from 40 to 60%; as it appears in Figure 4a, the contrast of the polar region becomes diffuse in this range of grafting density with a significant and rather sharp increase of about 2 nm in the effective membrane thickness (inset in Figure 4a) mainly due to the hydrophilic part of the chains.

DISCUSSION AND CONCLUSIONS

The results presented in this paper show that the systematic variation of the membrane composition and hydration changes both structural and elastic properties of the lamellar phase. The

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incorporation of cosurfactant modifies the swelling properties, as well as the dilution limit, of the lamellar phase, an effect already reported in the literature[35,44] It also opens a new regime of interactions between bilayers that was not present in the lamellar phase composed of lecithin only and that may explain the stability of complexes where DNA is incorporated into the lamellar structure.[30−32,34]

We have explored a wide range of membrane compositions and hydrations and we observed that the behavior of the Caillé parameter is apparently well described by a master curve, independent of the amount of cosurfactant, as a function of hydration. At low water amounts, a first regime can be identified, characterized by a strong longitudinal (i.e., along the stacking axis) confinement with the typical separation distances comparable to the length of the polymer chains. Although the bilayers are expected to become more flexible with the incorporation of ethoxylated fatty acids, the longitudinal confinement of the hydrophilic end of the cosurfactant severely restricts undulation fluctuations and elastic properties are therefore still dominated by the contribution of short-range interactions: In this limit, lateral confinement effects of the hydrophilic part of Simulsol (mushroom-to-brush transition for instance) appear irrelevant. In the second regime, the Caillé parameter increases slower than in the first one and the values may become as large as 1, indicating very flexible and rather weakly interacting bilayers. Since hydration is now significant, lateral confinement effects presumably come into play. It may then seem paradoxical that they do not affect the Caillé parameter, but the universality of the Helfrich mechanism should be remembered. Indeed, membrane flexibility does not enter eq 5. The cancellation between an increased bending modulus K and a decreased compression modulus B in the product KB that enters eq 4 apparently still operates, even though the Helfrich mechanism does obviously not dominate interbilayer interactions for our dilute lecithin-Simulsol systems.

Lateral confinement effects are rather directly observed in electron density profiles for Simulsol-rich bilayers and water content large enough. We believe that they are also indirectly suggested by the surprising shape of Figure 2b, because a Simulsol-rich behavior can be distinguished from a lecithin-rich one when the cosurfactant content reaches the value where electron density profiles start to spread. The nonmonotonous evolution of the dilution limit in each region could then be associated to the mushroom-to-brush transition through the delicate interplay between steric (repulsive) and van der Waals (attractive) interactions driving the unbinding transition[19,42,43] but the actual relevance of this qualitative argument remains to be firmly established. Work is currently in progress in this respect.

In summary, the incorporation of ethoxylated cosurfacant provides a tool for controlling the intensity of repulsive interactions between bilayers. Whereas the hydrophobic part of the molecules modifies the membrane flexibility, the density and the length of polymer chains allows a fine control for modulating the interactions between membranes and the regime of fluctuations. We believe that such mechanism could open an interesting experimental window to explore interfacial phenomena in flexible membranes found in biological structures.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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