Rheological Properties of Lipopolymer−Phospholipid Mixtures at the Air−Water Interface: A Novel Form of Two-Dimensional Physical Gelation

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ABSTRACT: Recent surface rheology and film balance experiments on monolayers of PEG lipopolymers at the air−water interface showed that the PEG chains are able to form a quasi-two-dimensional physical polymer network if forced into a highly stretched brushlike configuration. To obtain a deeper understanding of the complex film balance and rheological transition behavior of lipopolymers, we performed surface rheology and film balance experiments on phospholipid (DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine)/PEG lipopolymer (DSPE-PEG2000: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000]) mixtures at the air−water interface. We found that the high-film-pressure transition observed between 40 and 100 mol % lipopolymer at about 20 mN/m, which is related to a first-order-like alkyl chain condensation, is a necessary requirement for the existence of a rheological transition. While the rheological transition appeared at a specific area per lipopolymer of 165 Å^2, thereby being independent of the amount of phospholipids incorporated, the area per lipopolymer at the high-film-pressure transition clearly depends on the lipopolymer−phospholipid molar concentration. Our data clearly support the Flory model of physical gelation, which predicts no thermodynamic transition at the gel point, because the isothermal compressibility and its derivative show no discontinuity at this point. The $\pi-A$ isothermal behavior at the high-film-pressure transition of the phospholipid/lipopolymer mixtures can be interpreted if we assume that microphase separation occurs between phospholipids and lipopolymers. Our data indicate that the two-dimensional physical network of lipopolymers is formed by two different kinds of associative interactions: (1) microcondensation of alkyl chains of lipopolymers to small clusters; (2) water molecule mediation of the interaction between adjacent PEG clusters via hydrogen bonding.

Introduction

One class of lipopolymers consists of molecules in which one terminus of a single polymer chain is covalently attached to the headgroup of a phospholipid molecule. Because they can tether a phospholipid bilayer onto a hydrophilic polymer layer, the lipopolymers can be used for the engineering of complex biomembranes on solid substrates. Here, the polymer layer acts as cushion between a stabilized biomembrane and a solid substrate.[1−3] Furthermore, experiments using these amphiphilic molecules at the air−water interface allow the graft density of the polymer moieties to be varied over a wide range in a very controlled manner. This allows studies of how the "grafted" polymer chains interact in different polymer configurations.[4,5] For example, because of their ability to stabilize vesicles, PEG lipopolymers are of considerable interest to researchers developing drug delivery systems.[6] The biological inertness of lipopolymer-modified vesicles is supposed to be linked to the unusual behavior of PEG.[7−9] Unlike other polyethers, PEG is water-soluble as a result of a specific structuring of water molecules along the polymer chains.[10] PEG has also been suggested to form H-bonds with surrounding water molecules via its ether oxygens.[10,11] Therefore, the observed behavior of PEG is very likely to be caused by a complex interplay among hydrophobic interactions, H-bonding between water and PEG, and PEG−PEG interactions.

Pressure−area isotherms of monolayers of PEG lipopolymers at the air−water interface show two phase transitions: a desorption of the PEG chains from the air−water interface at about 10 mN/m, π_{low}, and a high-film-pressure transition at 20−40 mN/m, π_{high}.[4] Although lipopolymers might be considered to be phospholipids with very bulky headgroups, they show properties not found for phospholipids at the air−water interface. For example, IR experiments of lipopolymers with deuterated alkyl chains verified a first-order transition in their alkyl chains at the high-film-pressure transition.[12] Note that this occurs at an area per lipopolymer of about 200−250 Å^2, which is 2−3 times the accepted value for the first-order transition of phospholipids between the liquid-expanded phase and liquid-condensed phase. The presence of a lipidlike anchor seems to be necessary for a high-film-pressure transition to exist since it was not observed with measurements on diblock copolymers.[13] Therefore, these lipopolymers should be considered to be distinct entities with novel physical properties rather than, for example, modified phospholipids or polymers.

Recent surface rheology and film balance experiments of PEG lipopolymers at the air−water interface revealed a remarkable change of the rheological properties within the range of the high-film-pressure transition, when the

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polymer chains are forced into highly stretched polymer configurations.[5] The monolayer was fluid below the rheological transition, with the surface storage modulus, G_s', being smaller than the surface loss modulus, G_s'', but became elastic above the transition, with $G_s' > G_s''$. The experiments indicated a strong resemblance between the reversible, high-film-pressure transition at π_{high}, which is a first-order transition, and the formation of a physical gel. The formation of the physical network was explained by water intercalates mediating the interaction between adjacent PEG chains via hydrogen bonding.

Keeping the above results in mind, we were interested in expanding our surface rheology experiments from pure lipopolymers, which can be seen as a model system for the study of two-dimensional physical networks, to lipopolymer−phospholipid mixtures. The idea is that phospholipid molecules incorporated into a lipopolymer monolayer may simply act as spacer molecules, thereby changing packing and stress conditions between adjacent PEG chains, which are sensitive parameters for the formation of physical networks. We will address several questions with our experiments: (1) Can we learn more about the correlation between the high-film-pressure transition, which is due to the alkyl chain condensation, and the rheological transition, which results from the physical gelation of the PEG chains? (2) What is the nature of the rheological transition? (3) Can we obtain a better structural understanding of the quasi-two-dimensional network formation? (4) How does the presence of phospholipid molecules affect the rheological transition behavior?

Combined interfacial stress rheometer and film balance experiments provide a relationship between configurational and mechanical properties of amphiphiles, as one might expect. Significantly, they may also lead to new insights into the structure−function relationship of these unique molecules at the molecular level, as measurements on pure PEG lipopolymers have shown.[5] There are a variety of methods used to measure the rheological properties of amphiphiles at the air−water interface, such as deep-canal devices, channel-flow devices, and rotating disks or rings.[14,15] In our recent study on pure PEG lipopolymers and in the present study, we used a recently developed interfacial stress rheometer that is based on an oscillating rod at the air−water interface.[16]

Materials and Methods

Materials. The phospholipid studied was 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC). The lipopolymer investigated was 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000] (DSPE-PEG2000). Both amphiphiles were purchased from Avanti Polar Lipids (Alabaster, AL). Their chemical structures are given in Figure 1. Chloroform was used as a spreading solvent for preparing the monolayers at the air−water interface. Milli-Q Water (pH = 5.5, 18 MΩ·cm resistivity) was used as a subphase material for all film balance experiments.

Interfacial Stress Rheometer Apparatus. To investigate the rheological properties of the mixed lipopolymer−phospholipid monolayer at the air−water interface, we used an interfacial stress rheometer. Figure 2 shows a schematic of the experimental setup. With this device, a magnetized rod, which was kept at the air−liquid interface due to the surface tension, was subjected to an oscillatory force generated by a pair of Helmholtz coils in a well-defined flow geometry between two glass walls. A mini-Langmuir trough (KSV Instruments) was used to change the surface concentration and detect the

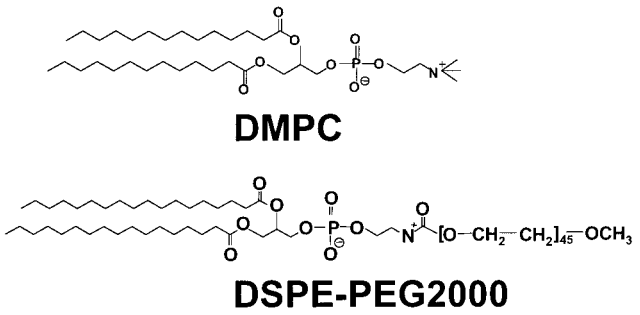

Figure 1. Molecular structures of the phospholipid, DMPC, and the lipopolymer, DSPE-PEG2000, investigated.

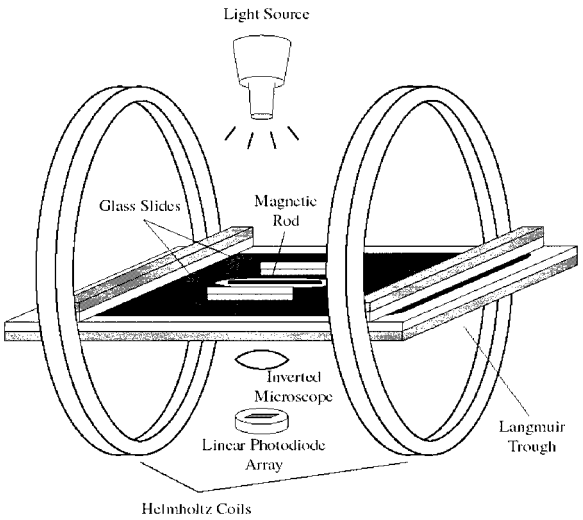

Figure 2. Schematic of the interfacial stress rheometer (ISR) used to measure the mechanical shear properties of Langmuir monolayers. With this device, a magnetized rod is subjected to an oscillatory force generated by a pair of Helmholtz coils, and the resulting motion is detected using a microscope and photodiode array. From measurements of the amplitude ratio and phase of the applied force to the resulting displacement, the dynamic surface moduli ($G_s^* = G_s' + G_s''$) can be detected. The storage modulus, G_s', is a measure of the elasticity of the monolayer, and the loss modulus, G_s'', is a measure of the viscous nature of the film.

film pressure of the monolayer. The resulting motion of the needle was detected using an optical microscope and a photodiode array that detects the shadow of the needle behind the light source. The oscillating magnetic field induced a sinusoidal motion of the rod at the same frequency but different in phase. From the rod's position (surface strain, γ_s) in relation to the applied magnetic field (surface stress, σ_s), one is able to determine the phase lag, δ, between the strain and the stress and the ratio of their amplitudes, AR. The relation between stress and strain

$$\sigma_s(\omega) = G_s^*(\omega)\,\gamma_s(\omega) \tag{1}$$

leads to a material property, the dynamic surface modulus, G_s^*. In general, G_s^* is a complex number

$$G_s^* = G_s' + iG_s'' \tag{2}$$

where the real part, the storage modulus, G_s', represents the elastic properties, while the imaginary part, the loss modulus, G_s'', describes the viscous behavior. From measurements of the amplitude ratio and phase lag of the applied force to the resulting displacement of the rod, the dynamic surface moduli ($G_s^* = G_s' + iG_s''$) can be obtained using

$$G_s^* = \frac{1}{AR} e^{i\delta} \frac{1}{AR} \cos\delta + i \frac{1}{AR} \sin\delta \tag{3}$$

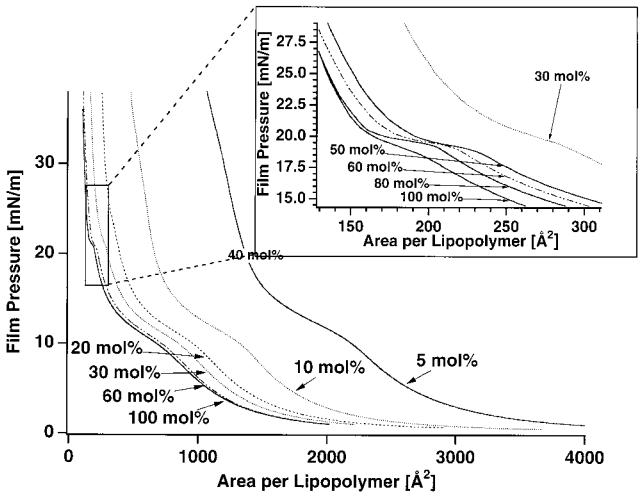

Figure 3. $\pi-A$ isotherms of different DMPC−DSPE-PEG2000 mixtures for lipopolymer molar concentration of 5−100 mol %. Below 30 mol % lipopolymer, the $\pi-A$ isotherms only show a desorption transition of the PEG chains ($\pi_{low} \sim 8$ mN/m). Both the desorption and the high-film-pressure transitions ($\pi_{high} \sim 19$ mN/m) can be found for lipopolymer concentrations ≥30 mol % (see also inset of Figure 3). The latter transition is related to the alkyl chain condensation among lipopolymers.

A more detailed description of the experimental setup can be found in Brooks et al.[16]

To determine the dynamic moduli of the mixed lipopolymer−phospholipid monolayers at the air−water interface, we used the same experimental strategy described previously for analogous experiments on pure lipopolymer monolayers.[5] All the films investigated showed strain-independent response (over the strain range 0.005−0.07), indicating linear viscoelastic behavior. In the case of frequency-dependent experiments, the frequency was varied from 0.5 to 10 rad/s. Most of the surface rheological experiments were, however, conducted as a function of the film pressure (or, equivalently, area per molecule), where the frequency was kept constant at a frequency of 0.92 rad/s. At least five measurements were performed at each film pressure to achieve statistical confidence. Because the compression range of the ISR setup is limited, we used a separate KSV5000 Langmuir trough for measurements of the complete $\pi-A$ isotherms.

Results

Figure 3 shows $\pi-A$ diagrams of the DMPC/DSPE-PEG2000 mixed monolayer at the air−water interface for six different molar concentrations of lipopolymer: 5, 10, 20, 30, 60, and 100 mol %. The isotherms represent the relationship between film pressure and the area per lipopolymer molecule, A_{lipo}. All isotherms show the characteristic low film pressure transition, π_{low}, at about 9 mN/m, which is related to the desorption of the PEG chains from the air−water interface into the subphase.[4,13] The inset in Figure 3 shows the corresponding behavior of the high-film-pressure transition, π_{high}, for 30, 40, 50, 60, 80, and 100 mol % lipopolymer. This transition can be observed for ≥30 mol % DSPE-PEG2000 but disappears for lower lipopolymer concentrations of 5, 10, and 20 mol % DSPE-PEG2000. Additional fluorescence microscopy measurements, where 2 mol % NBD−DMPE was added to each DMPC/DSPE-PEG2000 mixture, provided no experimental evidence for the existence of macroscopic phase separation within these binary phospholipid−lipopolymer mixtures at the air−water interface (data not shown).

The corresponding surface rheological behavior for DMPC/DSPE-PEG2000 monolayers of lipopolymer molar concentrations of 40, 50, 60, 80, and 100 mol % is shown in Figure 4A,B. Figure 4A represents the relationship between the loss modulus, G_s'', and the average area per DSPE-PEG2000 molecule, A_{lipo}, at a constant frequency of 0.92 rad/s. Different lipopolymer molar ratios lead to changes in the rheological transition behavior. Three different situations can be found: (1) For lipopolymer molar concentrations of ≤30 mol %, there is no rheological transition observed (data for lipopolymer molar ratios <30 mol % are not shown). (2) For intermediate lipopolymer concentrations of 40−50 mol %, we observe a rheological transition at about $A_{rheo} = 165$ Å^2, with G_s'' first increasing by up to 1.5 orders of magnitude, followed by decreasing values of G_s'' if A_{lipo} is further lowered beyond A_{rheo}. (3) For lipopolymer molar concentrations of ≥60 mol %, we observe a rheological transition behavior similar to that found for the pure DSPE-PEG2000 monolayer.[5] The rheological transition can again be found at $A_{rheo} = 165$ Å^2, with G_s'' now increasing by more than 2 orders of magnitude reaching a saturation value of $G_s'' \approx 0.45$ mN/m for $A_{lipo} < A_{rheo}$.

Figure 4B shows the corresponding relationship between the storage modulus, G_s', and the average area per DSPE-PEG2000 molecule, A_{lipo}, at a constant frequency of 0.92 rad/s (solid lines with markers). Again, we can distinguish three different situations: (1) For lipopolymer molar concentrations of ≤40 mol %, we observe no rheological transition (data for lipopolymer molar concentrations of <40 mol % not shown). (2) There is an intermediate range at 50 mol %, where a rheological transition can be observed at about $A_{rheo} = 160$ Å^2, with G_s' increasing by up to 1.5 orders of magnitude with decreasing values of G_s' if A_{lipo} is further lowered beyond A_{rheo}. (3) For a lipopolymer molar concentration of ≥60 mol %, we observe a rheological transition similar to that already found for the pure DSPE-PEG2000 monolayer.[5] The rheological transition is again located at $A_{rheo} = 160$ Å^2, with G_s' now being changed by up to more than 3 orders of magnitude. Below A_{rheo}, it either reaches a saturation value of $G_s' \approx 0.45$ mN/m at 60 mol % DSPE-PEG2000 or follows a power-law-like behavior for even higher lipopolymer molar concentrations.

The storage modulus is directly related to the average density of network-forming physical junction points, ν, via

$$G_s' = kT\nu \tag{4}$$

By assuming a value of one junction point per PEG chain, we simply can put ν equal to ρ, the area density value, which is done in Figure 4B (solid line).

Figure 5 shows the frequency dependence of the magnitude of the dynamic modulus $|G_s^*(\omega)|$, for a DMPC/DSPE-PEG2000 monolayer at 50 mol % DSPE-PEG2000, parametrized for different values of A_{lipo}. Three different situations can be distinguished: (1) At areas well above the rheological transition ($A_{lipo} \geq 175$ Å^2), the behavior of $|G_s^*(\omega)|$ agrees very well with the response of the needle at the clean air−water interface. (2) Both magnitude and slope of the curves are significantly changed if A_{lipo} reaches a value of $A_{lipo} = 170$ Å^2. This is an area representative of the transition region of the rheological transition around $A_{rheo} = 165$ Å^2 (compare to Figure 4A,B). (3) For areas representing the post-gel regime ($A_{lipo} < 165$ Å^2), we observe no further

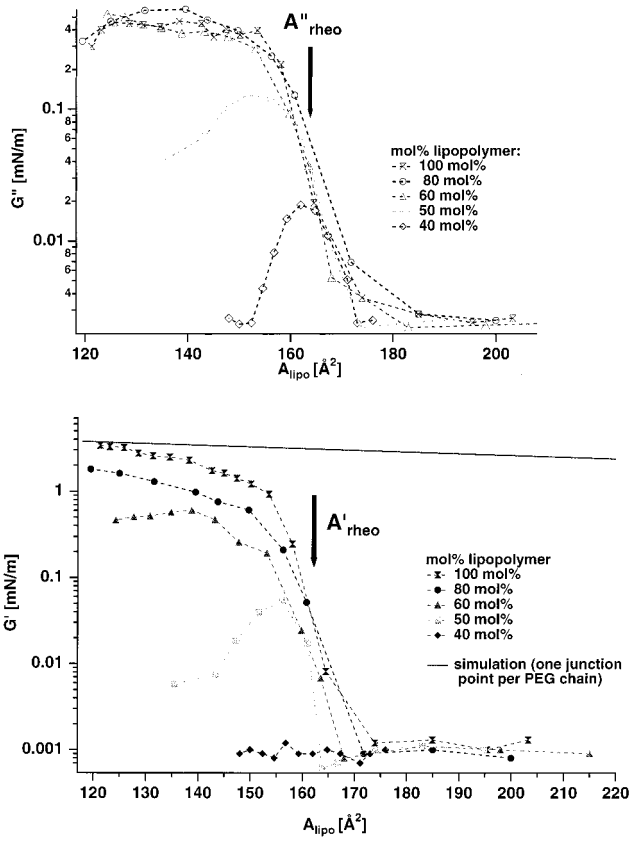

Figure 4. Rheological response of the lipopolymer (DSPE-PEG2000) monolayer shown as a function of the amount of phospholipid (DMPC) incorporated. The loss modulus G'' (A, top) and the storage modulus G' (B, bottom) are shown as a function of the average area per lipopolymer A_{lipo}. The change in the rheological properties at A'_{rheo} and A''_{rheo} is found to be related to a specific value of A_{lipo} of about 165 Å^2, thereby being independent of the amount of phospholipids incorporated. There is no rheological transition if the lipopolymer molar concentration is less than 40 mol %. The solid line in (B) represents the storage modulus predicted if there is one junction point (physical cross-link) per lipopolymer based on the equation $G' = kT\rho$, where k describes the Boltzmann factor, T is the temperature, and ρ is the area density.

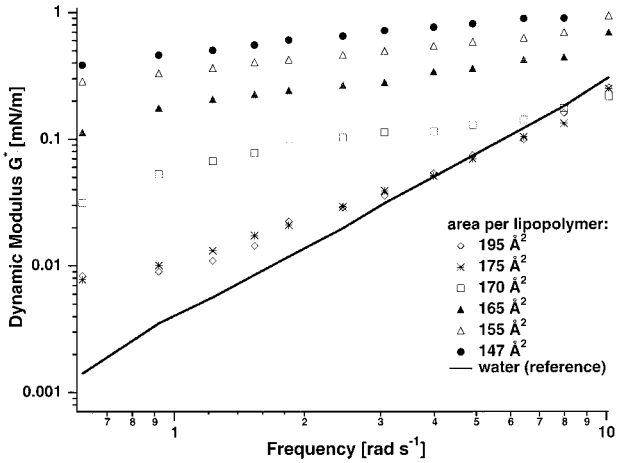

Figure 5. Frequency dependence of the magnitude of the dynamic modulus $|G^*(\omega)|$ for a DMPC/DSPE-PEG2000 mixture at a lipopolymer molar concentration of 50 mol %, compared for different values of A_{lipo}. Above $A_{lipo} = 175$ Å^2, the behavior of $|G^*(\omega)|$ corresponds well to the response of the needle at the clean air−water interface (solid line). For areas $\leq A_{rheo}$, we observe both a decrease in the slope and an increase in the magnitude of $|G^*(\omega)|$, which is related to the formation of a physical network.

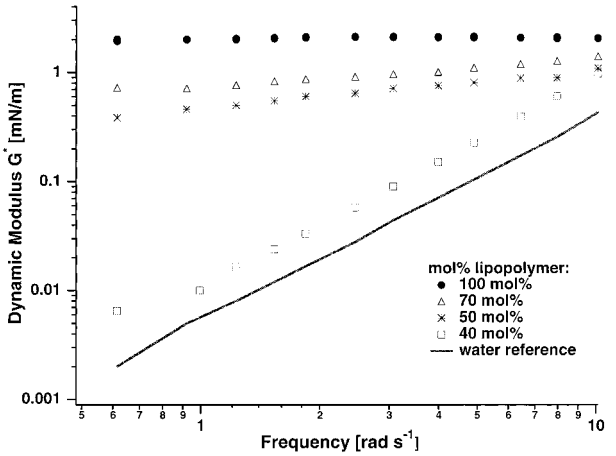

Figure 6. Frequency dependence of the magnitude of the dynamic modulus $|G^*(\omega)|$ compared for different DMPC/DSPE-PEG2000 molar concentrations of 40/50/70/100 mol % at a fixed value of $A_{lipo} = 150$ Å^2. While the data indicate the formation of a physical network between 50 and 100 mol % lipopolymer, there is only a slight increase of $|G^*(\omega)|$ if compared to the response of the needle at the clean water surface (solid line).

change in slope but an increase of the magnitude of $|G_s^*(\omega)|$ with smaller values of A_{lipo}.

Figure 6 compares $|G_s^*(\omega)|$ curves of different DMPC/DSPE-PEG2000 mixtures of 40, 50, 70, and 100 mol % lipopolymer after the area per lipopolymer is kept constant at $A_{lipo} = 150$ Å^2. At 40 mol % lipopolymer no significant difference is found if compared to the response of the needle without a monolayer (solid line). At 50 mol % lipopolymer, we find both a significantly increased magnitude of $|G_s^*(\omega)|$ and a decreased slope, indicating the formation of a physical network. At ≥70 mol % lipopolymer, we find no significant change in slope but an increase of the magnitude of $|G_s^*(\omega)|$ with smaller amounts of phospholipid incorporated.

Discussion

Correlation between High Film Pressure and Rheological Transitions. One of the main results from recent surface rheology and film balance experiments on pure PEG lipopolymers at the air−water interface from Naumann et al. was the observation of a rheological transition in the range of the high-film-pressure transition.[5] The experimental data on pure PEG lipopolymers provided strong support for the conclusion that the rheological transition involved a physical gelation within the PEG chains. The formation of a physical gel can be understood if we assume the existence of polymer−water complexes. The formation of physical gels via polymer−solvent complexes was first proposed by Guenet and co-workers based on their findings on atactic polystyrene−carbon disulfide systems.[17,18] Following their assumption that solvent molecules may intercalate between adjacent polymer chains, we proposed that water molecules act in a similar way as intercalates between neighboring PEG chains.[5] A necessary requirement for the gelation phenomenon to occur is the submerging of water molecules out of the hydrated polymer layer due to the compression of the monolayer. Such a "squeezing-out" effect within Langmuir films of hydrated amphiphilic molecules is a well-known phenomenon, as, for example, neutron reflectivity experiments on a phospholipid monolayer have shown.[19] The high-film-pressure transition, on the other

hand, should be related to an alkyl chain condensation among lipopolymers (first-order transition), as Wiesenthal and co-workers showed just recently.[12] Not answered, so far, is the question whether both transition phenomena, although being of completely different nature, are correlated to each other. In other words, should we consider the existence of a high-film-pressure transition to be a necessary requirement for the occurrence of the rheological transition?

To address this question, we compared $\pi-A$ isotherms and rheological properties of DMPC/DSPE-PEG2000 mixtures at different molar concentrations. Figure 3 shows the behavior of the $\pi-A$ isotherms of different DMPC/DSPE-PEG2000 mixtures. The $\pi-A$ isotherms show a high-film-pressure transition similar to the pure PEG-2000 monolayer if the lipopolymer molar concentration is in the range of 30−100 mol % DSPE-PEG2000 (inset in Figure 3). This transition disappears, however, if the lipopolymer molar concentration is 20 mol % DSPE-PEG2000 or less. The observed disappearance of the high-film-pressure transition should be related to the inability to force the polymer chains into a more stretched configuration. Under such circumstances, alkyl chains of neighboring lipopolymers are too far away from each other to allow their alkyl chains to condense. The corresponding rheological behavior of DMPC/DSPE-PEG2000 mixtures is shown in Figure 4A,B. Indeed, not only the pure PEG-2000 monolayer but also DMPC/DSPE-PEG2000 mixtures for ≥40 mol % DSPE-PEG2000 show a change of their rheological properties. These experimental findings strongly support the proposal that the high-film-pressure transition is a necessary requirement for the existence of a rheological transition. Nevertheless, this conclusion seems to be contradicted by our measurement at 30 mol % DSPE-PEG2000, where we were unable to detect a rheological transition, although a high-film-pressure transition was observed. Ultimately, there is a simple explanation based on an experimental limitation. We were unable to compress the film to high enough film pressures to reach the area per lipopolymer of about $A_{rheo} = 165 \pm 10$ Å^2, which was found to be a necessary threshold value to induce the rheological transition, as Figure 4A,B shows as a further interesting result.

These results lead us to the next important question: Do the high-film-pressure and rheological transitions describe either a single transition event affecting the whole lipopolymer molecule or two transitions describing correlated but different phenomena? The latter case was already discussed by Naumann et al. for pure PEG lipopolymers after they observed that the midpoints of both the high-film-pressure and the rheological transitions do not occur exactly at the same area per lipopolymer.[5] The authors were, however, unable to provide a conclusive answer because the deviation between both midpoints was still in the area range of the transition plateau of the high-film-pressure transition. Therefore, it is worth reexamining this problem again in the case of the DMPC/DSPE-PEG2000 mixtures. This is done in Figure 7, where $\pi-A$ isotherms of several phospholipid−lipopolymer mixtures (solid lines) are shown together with corresponding values of A_{fb} (squares), the area value found at the center of the plateau of the high-film-pressure transition (obtained from Figure 3), and A_{rheo} (circles), the area representative for the midpoint of the rheological transition (obtained from Figure 4A,B). Here, A_{rheo} is separately shown for the transition

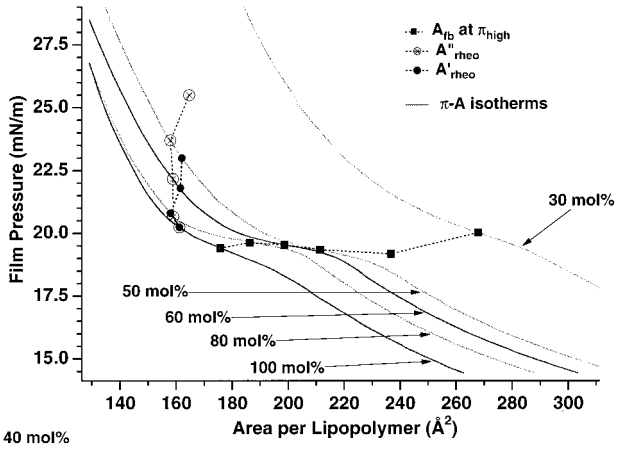

Figure 7. Comparison between high-film-pressure and rheological transitions of DMPC/DSPE-PEG2000 mixed monolayers at the air−water interface at lipopolymer molar concentrations of 30/40/50/60/80/100 mol %. The $\pi-A$ isotherms (solid lines) show the points, where the rheological transition can be observed (loss modulus: A''_{rheo}; storage modulus: A'_{rheo}), and the inflection points of the transition plateau at the high-film-pressure transition (A_{fb} at π_{high}). There is a clear correlation between the occurrence of the rheological transition and the existence of the high-film-pressure transition.

behavior of G_s' (A'_{rheo}, filled circles) and G_s'' (A''_{rheo}, open circles). Figure 7 leads to a very instructive result. High-film-pressure and rheological transitions behave quite differently and are sensitive to different parameters. The area per lipopolymer, A_{lipo}, is clearly a critical parameter for the formation of a physical network. The characteristic threshold area value at $A_{lipo} = A_{rheo} = 165 \pm 10$ Å^2 verifies the conclusion from Naumann et al. that a specific polymer configuration is necessary to form a physical gel between PEG chains.[5] The amount of phospholipids within the monolayer has, in this case, no influence on A_{rheo}. The DMPC molecules seem mainly to act as spacer molecules, thus compensating the area mismatch of the lipopolymer molecules. By considering the high-film-pressure transition at $A_{lipo} = A_{fb}$, on the other hand, we see that the critical parameter is not a specific area value, but the film pressure at the center of the high-film-pressure transition plateau, π_{high}. In contrast to earlier measurements on pure PEG lipopolymers, where A_{rheo} is still within the plateau of the high film pressure transition, we now observe a remarkable deviation between the area per lipopolymer at the high-film-pressure transition, A_{fb}, and A_{rheo}. Now several mixtures show their rheological transition significantly outside of the plateau region of the high-film-pressure transition. This leads to the interesting conclusion that high-film-pressure and rheological transitions describe, indeed, two different transition phenomena.

Nature of the Rheological Transition. So far, we have seen that the existence of a high-film-pressure transition is a necessary requirement for the occurrence of a rheological transition, although both transitions involve different critical parameters. The high-film-pressure transition was found to be a first-order-like alkyl chain condensation of lipopolymers,[12] whereas the rheological transition should be related to a physical gelation between adjacent PEG chains mediated by water molecules via hydrogen bonding.[5]

The modern theory that describes the gelation process is the percolation theory.[20] Long before the percolation theory was established, Flory and Stockmayer independently developed an analytical theory, which is based

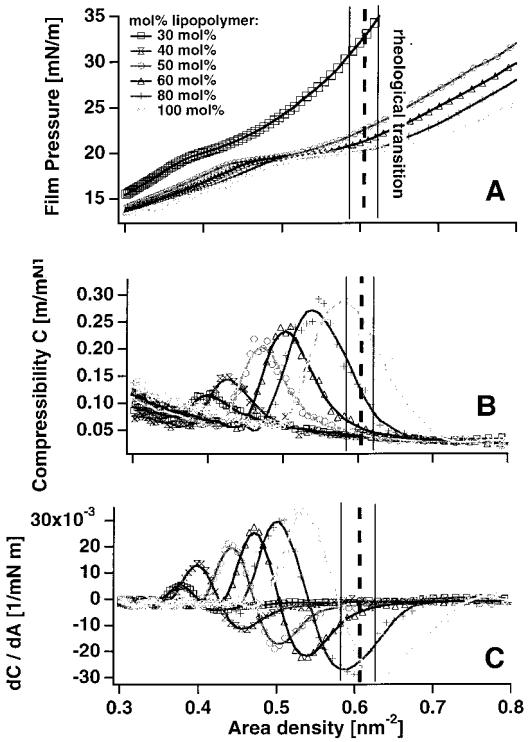

Figure 8. Film pressure (A), compressibility (B), and derivative of the compressibility (C) shown as a function of the area density for DMPC/DSPE-PEG2000 mixtures at lipopolymer molar concentrations between 30 and 100 mol %. There is no indication of a discontinuity if the curve shapes are compared in the range of the rheological transition. While the dashed line shows the center of the rheological transition, the solid lines indicate its width.

on the mean-field limit of the percolation theory.[21,22] Flory's and Stockmayer's approaches lead to different predictions regarding the nature of the gelation transition. While Stockmayer's approach predicts a discontinuity of the derivative of the isothermal osmotic compressibility, i.e., a third-order transition,[23] Flory's model predicts no thermodynamic transition at the gel point.[21] So far, the topic is still under discussion; for example, Tanaka and co-workers took over Stockmayer's model,[24] but others support Flory's approach.[25,26]

Because Langmuir film experiments allow the determination of the compressibility and its derivative in a very straightforward way, we are able to address the question about the nature of the gel transition for our quasi-two-dimensional physical network. This is shown in Figure 8A−C, where we compare the film pressure (Figure 8A), the compressibility C(Figure 8B), with

$$C = -\frac{1}{A}\left(\frac{\mathrm{d}A}{\mathrm{d}\pi}\right) \tag{5}$$

and the derivative of the compressibility (dC/dA) (Figure 8C), for different phospholipid−lipopolymer mixtures as a function of the lipopolymer area density, $\rho = 1/A_{\mathrm{lipo}}$. If we simply analyze the curve behavior in the region of the gel transition, thereby considering both the center of the gel transition (dashed line) and its transition width (solid lines), we obtain a direct answer about the nature of the physical gelation. Although we observe some overlap between the rheological and the high-film-pressure transition at the higher lipopolymer molar concentration of 80 mol %, there is no experimental evidence that the gel transition should be linked to a discontinuity of the compressibility and its derivative. While a discontinuity of the compressibility indicates a second-order transition, that of the derivative of the compressibility would indicate a third-order transition. Our statement becomes even more obvious if we consider the mixtures at 40, 50, and 60 mol % lipopolymer, which all show a rheological transition. In these cases, we observe no overlap between the high-film-pressure transition and the rheological transition. Again, we observe no indication of a second- or third-order phase transition at this point. Neither the compressibility nor its derivative shows a discontinuity. Our data, therefore, clearly support Flory's predictions.

Microphase Separation. Binary phospholipid mixtures of DSPE and DMPC are known to lead to a macroscopic phase separation because of their significantly different alkyl chain lengths.[27] Fluorescence microscopy measurements on DMPC/DSPE-PEG2000 mixtures containing 2 mol % of the fluorescently labeled phospholipid NBD-DMPE did, however, not show any macroscopic phase separation (data not shown). Microphase separations of domain sizes below the diffraction limit of an optical microscope are, on the other hand, still possible and seem to be quite likely, as the following simple arguments show.[4,5] As DSPE is covalently attached to a polymer chain, like for lipopolymers, the ability to phase separate is strongly dependent on the molecular area occupied by the far bulkier polymer chain. At large areas, the alkyl chains are not able to aggregate due to the steric hindrance caused by the polymer chains. As the polymer chains are forced into more stretched configurations, leading to a smaller area per lipopolymer, the point can be reached where the alkyl chains of adjacent lipopolymers start to "feel" and "attract" each other. In this case, they obtain a "limited" ability to phase separate. We use the term "limited" because the significantly larger cross-sectional area of the polymer (for DSPE-PEG2000 about 125−170 Å^2) and alkyl chain moieties (about 62 Å^2) very likely forces the alkyl chains of the lipopolymer into tilted configurations. We expect a microphase separation because only very small lipopolymer domains can be formed (e.g., aggregates of two or three lipopolymers embedded in a matrix of phospholipid molecules). Finally, a further compression of the monolayer will lead to a smaller lipopolymer domain size until the specific area per molecule, A_{rheo}, is reached where the PEG chains form a physical gel.

From the above discussion, we know that the high-film-pressure transition and the rheological transitions are correlated phenomena. The behavior of A_{rheo} in Figure 7 seems to be understandable from the point of view of a simple free volume effect within the polymer moiety, with A_{rheo} being independent of the amount of phospholipid incorporated. What, however, causes the behavior of A_{fb} at π_{high} if we compare different phospholipid−lipopolymer mixtures? To explain this behavior, we will discuss the following situations for the phospholipid−lipopolymer mixtures: (1) ideal mixing behavior, (2) microphase separation where the area occupied by the alkyl chains of each lipopolymer remains unchanged, (3) microphase separation where the area per phospholipid within the mixture is the same as for a pure phospholipid monolayer at the same film pressure. These situations are summarized in Table 1 where several high-film-pressure-related parameters are listed.

Table 1. Experimental and Calculated Parameters of Phospholipid−Lipopolymer Mixed Monolayers at the High-Film-Pressure Transition Describing the Situations of Ideal Mixing and Microphase Separation

mol % DSPE-PEG2000	A_{fb}[a] [Å^2]	A_{lipid}[b] [Å^2]	n_{pl}[c]	$A_{fb} - A_{fb}$*[d] [Å^2]	$(A_{fb} - A_{fb}^*)/n_{pl}$[e] [Å^2]	$n_{pl}A_{DMPC}$[f] [Å^2]	$A_{fb} - A_{DMPC}$[g] [Å^2]
30	265	79.5	2.3	95	41.3	138	127
40	235	94	1.5	65	43.3	90	145
50	210	105	1	40	40	60	150
60	195	117	0.67	25	37.3	40.2	154.8
80	182	145.5	0.25	12	48	15	167
100	170	170					

[a] The area available for the two alkyl chains of each lipopolymer at the high-film-pressure transition. [b] The area available for the two alkyl chains of each phospholipid at the high-film-pressure transition. [c] The phospholipid−lipopolymer molar ratio. [d] The area available for alkyl chains of n_{pl} phosphlipids in scenario 2, where A_{fb}* is the area available for the two alkyl chains in the case of the pure lipopolymer monolayer. [e] The area available per phospholipid in scenario 2. [f] The area occupied by n_{pl} phosphlipids in scenario 3. [g] The area available for the two alkyl chains of each lipopolymer in scenario 3.

(1) In the case of ideal mixing, we make the assumption that the alkyl chains of lipopolymers and phospholipids occupy the same molecular area, A_{lipid}. As the A_{lipid} values for different lipopolymer molar concentrations in Table 1 show, there is no indication for a specific threshold value of A_{lipid} that could explain the behavior at the high-film-pressure transition. We do not consider this as a very likely scenario.

(2) By assuming that the alkyl chains of the lipopolymers occupy at the high-film-pressure transition the same area per molecule for both the pure lipopolymer and the phospholipid−lipopolymer mixture, we can simply determine the difference, $A_{fb} - A_{fb}$*, where A_{fb}* = 170 Å^2 is the value found for the pure DSPE-PEG2000. The area values of $A_{fb} - A_{fb}$*, which are also shown in Table 1, describe basically the remaining area available for phospholipid molecules. Because the number of phospholipid molecules occupying this area, n_{pl}, is known, we can now calculate the corresponding area per phospholipid molecule, $(A_{fb} - A_{fb}^*)/n_{pl}$. The values of $(A_{fb} - A_{fb}^*)/n_{pl}$ in Table 1 reveal an interesting result. The area per phospholipid molecule remains fairly constant at about 42 ± 6 Å^2 independent of the phospholipid−lipopolymer molar concentration. This might be the kind of threshold value we are looking for. On the other hand, this value is too small to be realistic because we expect at $\pi = 20$ mN/m a significantly larger area per phospholipid molecule than 42 Å^2.

(3) Another scenario is to assume that the area value per DMPC molecule at a corresponding film pressure should be the same within a phospholipid monolayer and a phospholipid−lipopolymer mixed monolayer. Figure 7 shows $\pi_{high} = 19.5 \pm 0.5$ mN/m being independent of the lipopolymer molar concentration. This film pressure corresponds to an area per molecule of about A_{lipid} = 62 Å^2, which is a far more realistic value for a film pressure of $\pi = 20$ mN/m. In this scenario, we should consider a change of the molecular area occupied by the alkyl chains of each lipopolymer molecule ($A_{fb} - (n_{pl}A_{DMPC})$) if we vary the lipopolymer molar concentration. Following our above arguments, we are clearly in favor of this scenario.

As discussed earlier, our experimental film balance and surface rheology data strongly indicate that the high-film-pressure transition is a necessary requirement for the occurrence of the rheological transition. Still, we have not provided a reasonable explanation for this interesting result. To address this problem, let us again analyze our storage modulus data in Figure 4B. Here, we use the fact that the density of network-forming junction points is directly related to the magnitude of the storage modulus, G_s', via $G_s' = kT\nu$, where kT is the thermal energy and ν is the density of junction points. This is done in Figure 4B, where we compare our experimental data of G_s' (dashed lines + markers) to theoretical values obtained from $G_s' = kT\nu$ (solid line). This enables us now to obtain a direct estimate of the number of junction points per lipopolymer molecule. Having done this in Figure 4B, we obtain a very surprising result. The comparison between experimental and theoretical data indicates that not more than about one junction point per lipopolymer molecule can be found in the post-gel regime. Because a polymer chain carrying only one "reactive" group is not able to form an infinite network, we again must consider the formation of microdomains to explain the existence of the observed quasi-two-dimensional physical network. In this scenario, the lipopolymer microdomains are held together by small clusters of condensed alkyl chains. One microdomain, which contains for steric reasons probably not more than 2−4 lipopolymer molecules, should then be seen as an entity carrying 2−4 "reactive" groups. This would now be sufficient to form a network. Monolayers of lipopolymers or phospholipid−lipopolymer mixtures can, obviously, form two-dimensional physical networks with unique structural properties. Our findings indicate that the network relies on two different kinds of junction points: cluster-forming alkyl chains and water-mediated PEG−PEG junctions between adjacent microdomains. From this perspective, we are now able to explain why a microphase separation (high-film-pressure transition) is a necessary requirement in order to observe the formation of the rheological transition at A_{rheo}.

PEG−DMPC Interaction. So far, our discussion has been focused on three aspects of our surface rheology experiments on DMPC/DSPE-PEG2000 mixtures: (1) the correlation between the rheological transition and the high-film-pressure transition, (2) the nature of the rheological transition, and (3) the relation between microphase separation and physical gelation. If we look, however, again into our rheology data in Figure 4A,B, another very interesting observation can be made. The curve behavior of both G_s'' (Figure 4A) and G_s' (Figure 4B) varies remarkably for different phospholipid−lipopolymer molar concentrations. If we compare, for example, corresponding values of the storage modulus, G_s', at a constant area per lipopolymer of $A_{lipo} = 150$ Å^2, which is well below the transition area, $A_{rheo} = 165$ Å^2, we observe a decrease of the magnitude of G_s' with increasing phospholipid molar concentration. Because the magnitude of G_s' is a measure of the strength of the physical PEG network ($G_s' \sim$ density of junction points), it becomes obvious that a larger amount of incorporated

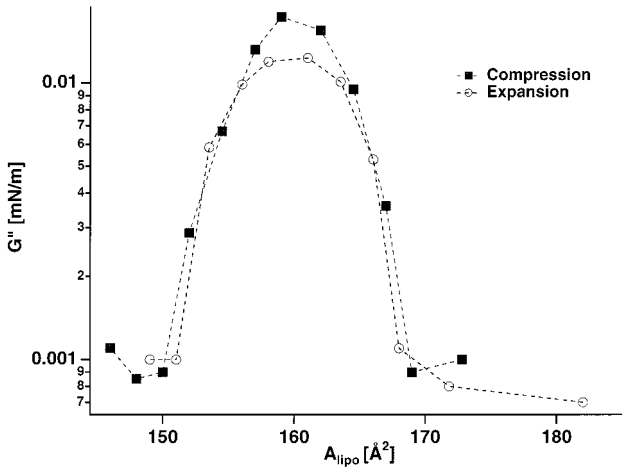

Figure 9. Comparison between compression and expansion curves of the loss modulus, G_s'', of a DMPC/DSPE-PEG2000 mixed monolayer at a lipopolymer molar concentration of 40 mol %. The data not only provide experimental evidence for the reversibility of the rheological transition but also show the strange hump behavior found for this lipopolymer molar concentration.

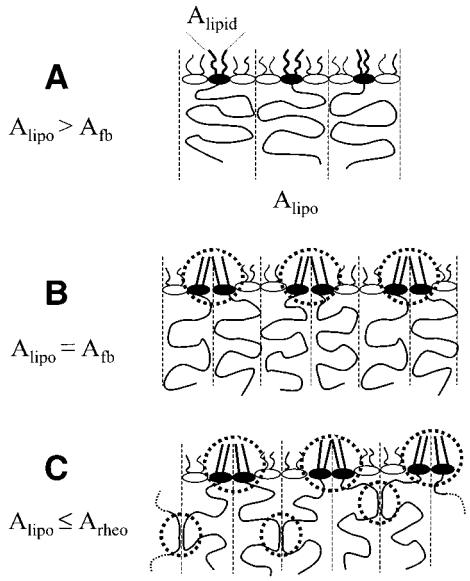

Figure 10. Schematic of a possible model to describe the physical network formation within a phospholipid−lipopolymer mixed monolayer. (A) At $A_{lipo} > A_{fb}$, there is no microphase separation. The alkyl chains of the lipopolymers are too far away to aggregate. (B) At $A_{lipo} = A_{fb}$, the center of the high-film-pressure transition, our data indicate a microphase separation between phospholipids and lipopolymers. The area mismatch between polymer and alkyl moiety allows no macroscopic phase separation. (C) At $A_{lipo} = A_{rheo}$, where A_{rheo} is independent of the amount of phospholipids incorporated, we observe the formation of a physical PEG network. The network is stabilized by two different kinds of junction points: (1) small clusters of alkyl chains of lipopolymers including 2−4 lipopolymers per cluster; (2) water molecules mediating the interaction between adjacent PEG chains via hydrogen bonding.

phospholipids leads to a weakening of the PEG network. Further evidence for this effect can be found in Figure 6, where the frequency behavior of $|G_s^*|$ is shown for different phospholipid molar concentrations at a constant area per lipopolymer, $A_{lipo} = 150$ Å^2. By comparing the curves representing 40, 50, 70, and 100 mol % DSPE-PEG2000, we observe at 40 mol % lipopolymer no indication for a physical network formation, as the comparison to the response of the needle without monolayer indicates. At 50 mol % lipopolymer, we find, however, clear signs for the formation of a physical network because both the magnitude of $|G_s^*|$ increases significantly and the slope of the curve becomes far smaller. At 70 and 100 mol % lipopolymer, we finally observe a further increase of the magnitude of $|G_s^*|$ but only slight changes of the curve slope. The observed behavior of $|G_s^*|$ between 50 and 100 mol % lipopolymer clearly indicates a weakening of the strength of the physical network if the amount of incorporated phospholipids is increased.

It is not very surprising to expect a weakening of the polymer network if the area per lipopolymer molecule becomes larger due to the expansion of the monolayer. It is, however, not quite obvious why we also observe such a weakening of the polymer network at areas $A_{lipo} < A_{rheo}$, if we compare different amounts of phospholipids at the same value of A_{lipo}. How do the DMPC molecules affect the PEG network? The behavior of G_s' in Figure 4B indicates a weakening of the physical network with increasing amount of phospholipids incorporated. Even the loss modulus, G_s'', in Figure 4A shows a similar behavior. One might argue that collective dynamic processes such as surface undulations of membranes or the collective dynamics of polymers could explain this behavior. Phenomena of collective dynamics are well-known.[28−31] We have, on the other hand, no further experimental indication for such an interpretation. Another, more likely, explanation can be given if we consider the "strange" behavior of the loss modulus G_s'' at 40 mol % lipopolymer, as shown in Figure 9. By going from larger to smaller area values (analogous to a compression of the monolayer), we observe first an increase of G_s'' until a maximum is reached. This is followed by a drastic decrease of G_s'', thereby even reaching the start value (before the transition) in the case of 40 mol % lipopolymer. As the corresponding expansion curve in Figure 9 nicely shows, this transition is reversible. We believe that the disruption of the network is likely related to the disappearance of the lipopolymer microdomains due to a change of thermodynamic conditions (film pressure). This not only explains the observed behavior in Figure 9 but also supports our earlier argument that the formation of the physical network depends on two kinds of associative interactions: (1) microcondensation to small clusters of lipopolymers; (2) water molecule mediation of the interaction between adjacent PEG clusters via hydrogen bonding.

A likely scenario to explain the rheological and high-film-pressure transition behavior is illustrated in Figure 9A−C. For $A_{lipo} > A_{fb}$, we expect no microphase separation because the lipopolymer alkyl chains are too far away to aggregate (Figure 9A). At $A_{lipo} = A_{fb}$, the center of the high-film-pressure transition, we expect a microphase separation between phospholipids and lipopolymers. The area mismatch between polymer and alkyl chain moiety allows no macroscopic phase separation (Figure 9B). At $A_{lipo} = A_{rheo}$, where A_{rheo} is independent of the amount of phospholipids incorporated, we observe the formation of a physical PEG network. The network is stabilized by two different kinds of junction points: (1) small clusters of alkyl chains of lipopolymers including 2−4 lipopolymers per cluster and (2) water molecules mediating the interaction between adjacent PEG chains via hydrogen bonding (Figure 9C).

Concluding Remarks

Our results on DMPC/DSPE-PEG2000 mixtures have shown that the incorporation of phospholipid molecules into the lipopolymer monolayer leads to several new insights: (1) The correlation between high-film-pressure and rheological transitions can be explained. (2) We obtain new insights into the nature of the rheological transition. (3) On the basis of the existence of microphase separations, there is now a better structural understanding of the formation of these quasi-two-dimensional physical networks. (4) The remarkable interaction between the phospholipid and polymer layers provides a new perspective of the phospholipid−lipopolymer monolayer as a composite material at the air−water interface. Nevertheless, there are still some open questions. Our molecular understanding of the physical PEG network is, for example, still limited. Right now, we have no detailed knowledge about the exact molecular structure and the dynamical behavior (lifetime) of its network-forming junction zones. Another closely related topic, in which we are currently interested, is whether the observed physical gelation found for PEG lipopolymers is a PEG-specific or a more general phenomenon typical of other lipopolymers carrying different polymer chains.

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Nomenclature

A = average area per molecule at the surface, Å^2/molecule
A_{lipo} = average area per lipopolymer molecule, Å^2/molecule
A_{fb} = average area per molecule at which the film-balance transition occurs, Å^2/molecule
A_{lipid} = average area per alkyl chain pair after assuming ideal mixing between lipopolymers and phospholipids, Å^2/molecule
A_{pl} = average area per phospholipid molecule after assuming microphase separation between lipopolymers and phospholipids, Å^2/molecule
A_{rheo} = average area per molecule at which the rheological transition occurs, Å^2/molecule
A_{DMPC} = average area per DMPC molecule, Å^2/molecule
AR = amplitude ratio; the ratio of the resulting strain amplitude to the applied stress amplitude, m/mN
G_s^* = complex dynamic surface modulus, mN/m
G_s' = real part of G_s^*, the surface storage modulus, mN/m
G_s'' = imaginary part of G_s^*, the surface loss modulus, mN/m
n_{pl} = number of phospholipids per lipopolymer molecule
γ_s = surface strain applied to the monolayer (dimensionless)
δ = phase difference between the strain and the stress, rad or deg
σ_s = surface stress applied to the monolayer, mN/m
π = surface pressure, mN/m
π_{high} = surface pressure at which the high-pressure film-balance transition occurs, mN/m
π_{rheo} = surface pressure at which the rheological transition occurs, mN/m
ρ = area density, 1/Å^2
ω = frequency, rad/s

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