Swelling of Polyelectrolyte Multilayer-Supported Lipid Layers. 1. Layer Stability and Lateral Diffusion

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The influence of solvents (water, ethanol/water mixtures) on the stability and mobility of polyelectrolyte multilayer (PEM)-supported lipid layers is investigated by the technique of fluorescence recovery after photobleaching (FRAP). Lateral lipid diffusion coefficients provide a measure of the interfacial interaction of lipid layer and polyelectrolyte multilayer cushion. An enhanced diffusion is found in solvent-swollen lipid layers in comparison to layers in air, with diffusion coefficients on the order of 10^{-10} cm^2/s. The effect of solvent is discussed in terms of solvent molecules screening the interaction of lipid and polyelectrolyte. The results of lateral diffusion in water-swollen PEM-supported lipid layers are interpreted in a model of headgroup-interaction dominated diffusion, whereas the chains are disordered and do not influence the mobility. Diffusion coefficients are governed by the nature of the lipid headgroup, and its respective electrostatic interactions: The ionic phosphatidic acid (DOPA) couples more strongly to the charged environment than the zwitterionic phosphatidylcholine (DOPC, DMPC). The above model explains the stronger binding of the PA headgroup in layers in water, whereas for layers in air the PC headgroup has a larger interaction. The distance dependence of the Coulomb versus dipolar interaction accounts for these results. Diffusion measurements are further performed in layers swollen in ethanol/water mixtures of varying composition. At an ethanol content below 50% the layers are stable and homogeneous. The extent of screening of the electrostatic interaction by solvent varies with its dielectric properties. At an ethanol content above 50% the state of PEM-supported lipid layers is inhomogeneous, which can be explained by either a partial dissolution of the lipid layer or a phase transition in the lipid layer. The findings on charged polymeric systems complement investigations of uncharged polymer-supported model membranes, and demonstrate the significance of local electrostatic interactions for issues of stability and dynamics.

Introduction

The coupling of lipid layers with biopolymers is ubiquitous and essential for the properties of biomembranes.[1−5] Supported lipid layers, i.e., layers attached to polymer layers, are a very interesting model system for biomembranes. The interest in the involved interaction in lipid/polyelectrolyte coupled systems results from the desire to make use of polyelectrolytes to stabilize liposomes, or to build lipid model membranes on ultrathin polymer support. Constructing stable supported lipid layer systems is of major importance for the development of model membranes, biosensors, and the simulation of cell surfaces.[6−11]

In addition, the contact plane is of interest from a fundamental viewpoint, since different types of interactions are involved, which can be varied via local and nonlocal effects, for example, the screening of the electrostatic interaction by solvent molecules. Hydration forces and even hydrophobic interactions can further influence the stability at the lipid/polymer interface. Investigating questions of interactions at the internal interfaces between supported lipid layers and polymers is thus of both practical and scientific interest.

Supported membrane model systems have been developed not only as planar but even as spherical structures, where a combination of lipid and polyelectrolyte layers can be built up as a composite capsule with walls made from polyelectrolyte multilayers.[12,13] Charged biomolecules, such as proteins and nucleic acids, combined into the walls of capsules can yield biomimetic systems for further research, forming an artificial cell.

In studies of lipid−polymer interactions, mainly uncharged hydrophilic polymers are employed as polymeric support.[14,15] Spin cast films of strongly hydrated biopolymers such as chitosan or agarose are current examples.[16,17]

Recent attention has also focused on charged polymeric supports: Here, polyelectrolyte multilayers prepared by self-assembly are particularly promising structures. Their advantage lies in their properties as well-defined monomolecular films, and in the versatility of combining different components on planar as well as on colloidal templates.[18,19] With very good control over the layer properties on a molecular level, PEMs are also particularly suitable for studies of fundamental aspects of interlayer interactions.

Concerning charged polymeric support, only few papers have so far dealt with interactions with supported membranes.[20,21]

We have recently systematically investigated the interactions and their implications for lipid dynamics in a lipid layer coupled to polyelectrolyte multilayers in air.[22] The charged segments of PEMs interact with the headgroup of adjacent lipid molecules, resulting in an enhanced layer stability and a reduced mobility of the lipid molecules. A very slow diffusion coefficient on the

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order of 10^{-12} cm^2/s is found and the mobility of the lipid layer is completely dominated by the electrostatic interaction between the lipids and the polyelectrolyte multilayer cushion, and by the number of lipid charges available for electrostatic interaction. This is suggested by the diffusion coefficients and further confirmed by the absence of phase transitions, and by the corresponding activation energies for diffusion.

It is expected that water (or other solvent) will have a large effect on the dynamics of PEM-supported lipid layers. Some lateral diffusion coefficients of phospholipid monolayers coupled to polyelectrolyte films have been determined in several solvents.[21] These preliminary data indicated that solvent has a large influence on the mobility of the lipid layers due to the different extent of reduction of the electrostatic interaction between lipid and polyelectrolyte. The influence of humidity on the lateral self-diffusion of supported lipid monolayers has been investigated systematically for a polysaccharide support.[16] There, a diffusion model was derived based on activated diffusion with an activation energy, which depends on the hydration state of the lipid headgroup. Upon assuming an exponential decay of the humidity-dependent disjoining pressure in the monolayer/substrate interface with respect to the equilibrium separation distance, the activation energy can be calculated.

Here, we systematically investigate the lipid−polyelectrolyte interactions in dependence of the solvent environment: Lateral diffusion experiments are performed after swelling samples of different composition in water and in ethanol/water mixtures. The data are discussed in a local electrostatic model, where the properties of the local solvent environment control the interaction strength.

Materials and Methods

Polyelectrolytes. The polyelectrolytes used throughout this work were poly(ethylenimine) (PEI, $M_w \sim 55\,000$) and poly(allylamine hydrochloride) (PAH, $M_w \sim 70\,000$) as polycations and poly(styrene sulfonate sodium salt) (PSS, $M_w \sim 70\,000$) as polyanion. All polyelectrolytes were purchased from Aldrich Chemical Co. PEI and PAH were used without further purification, whereas PSS was dialyzed against ultrapure water (cutoff of $M_w > 14\,000$), and subsequently freeze dried. The water used in all preparation procedures was purified with a three-stage purification system (Seradest) and had a resistivity higher than 18.2 MΩ cm.

Lipids. The phospholipids used for monolayer formation were the zwitterionic 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 PC, DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and the negatively charged phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphate (Monosodium salt) (18:1 PA, DOPA). The fluorescent probe 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine (16:0−06:0 NBD-PC) was added to all lipid solutions at a molar ratio of l %. All the lipids and the dye probe were purchased from Avanti Polar Lipids Inc. (USA) except for DOPC, which was purchased from Sigma (Germany).

Preparation of PEM-Supported Lipid Layers. Glass or silicon substrates were cleaned by heating to 70 °C in a mixture of H_2O:H_2O_2 (p.a., 30% aqueous solution):NH_4OH (p.a., 28% aqueous solution) with a volume ratio of 5:1:1 to ensure the removal of organic impurities (RCA cleaning method). Since the substrates were negatively charged after cleaning, as a first layer, PEI was precoated to form a positively charged surface on the substrates by immersing the substrates into a 10^{-2} M aqueous solution of PEI for 30 min (all polymer concentrations given here refer to the monomer concentration). After PEI adsorption, the samples were washed in Millipore water three times (1 min for each washing) to remove excess polymer. After drying in air, further polyelectrolyte layers were deposited onto the PEI-coated substrates by alternatingly immersing the substrates into aqueous solutions of PSS or PAH, respectively, for 20 min, beginning with PSS. The adsorption of the PEMs was achieved in an automated procedure with 10^{-2} M aqueous solutions of the polyions containing 0.1 M NaCl. After each polyelectrolyte adsorption step, the substrates were rinsed again in pure water three times. After adsorption of each PSS/PAH layer pair, the samples were dried for 7 min at room temperature. Repeating this process five times for each layer pair, the desired PEMs with either PAH (total of 11 layers) or PSS (total of 12 layers) as the terminating layer were obtained.

Lipid monolayers were deposited by the Langmuir−Blodgett technique, using a Langmuir trough (NIMA). The glass substrates precoated by PEMs were first immersed into the aqueous subphase. The temperature of the water was maintained at 20 ± 1 °C by a water circulating system. The lipid monolayers were formed by spreading chloroform solutions of the lipid (10^{-3} M) containing a l% molar ratio of fluorescent probe NBD-PC onto the water surface. After 10 min, during which the solvent was allowed to evaporate, the monolayer was compressed at a rate of 30 cm^2/min to a lateral pressure of 30 mN/m. At this pressure, the monolayer was densely packed. While the monolayer was transferred to the substrate at a rate of 1 mm/min, the surface pressure was maintained by an electronic feedback system.

Fluorescence Recovery after Photobleaching (FRAP). The lateral diffusion of lipid layers coupled to the PEMs was measured by employing a custom-designed pattern TIR-FRAP, which had been introduced previously.[22] Briefly, an argon ion laser (Innova 90-6, Coherent, Inc. Santa Clara, USA) generated the excitation beam at 488 nm, which is a suitable excitation wavelength for the NBD-PC dye. A beam-splitter provided two laser beams with equal intensity and the same polarization. This provided an interference pattern of maximum contrast, which was formed at the measurement spot, where the focus of the beams is made to overlap by a lens. To achieve a sufficient signal-to-noise ratio, the experiment was performed under total internal reflection conditions by coupling both beams into the glass substrate at an angle of incidence greater than the critical angle of total reflection employing a prism. Thus the two beams were totally reflected at the glass surfaces and the evanescent field was selectively exciting molecules in the surface layer. Bleaching and observing the sample under total internal reflection (TIR), background signals not originating from the interface could be eliminated. This resulted in an improved signal-to-noise ratio of the setup.

A microscope objective (50×/0.50, LD, ZEISS) was used to collect the fluorescence emission from the sample. Through the objective and a mirror, the pattern image was focused onto the chip of a back-illuminated CCD camera (DV434-BV, ANDOR, cooled to −30 °C). The projection was such that the interference stripes were parallel to the long axis of the chip. Vertical binning was employed to improve the signal-to-noise, in particular to reduce the readout noise.

When the FRAP measurement was started, a series of images were taken with time after photobleaching, controlled by a computer. The image acquisitions and analysis have been described in detail elsewhere.[22] Data evaluation was achieved by spatial Fourier transform of the simplified one-dimensional diffusion equation. The diffusion coefficient was calculated from

the exponential fit of the Fourier component decays. Optimizing the setup for slow diffusion measurements by the features described above, it was possible to measure diffusion coefficients as low as 10^{-14} cm^2/s for monolayers with a dye content of only 1 mol %.

Prior to performing a diffusion measurement in water-swollen layers, a defined volume of water was placed on the surface of the sample and a thin microscope cover slide (thickness = 0.13 mm) was gently placed on top of the water to form a thin water layer on the surface of the sample. The sample was then left to equilibrate and diffusion experiments were carried out under water at 20 ± 1 °C. FRAP measurements were performed by the same procedure as previous measurements of lipid layers in air,[22] except that the interval between consecutive exposures was set to 2 s to allow the observation of faster processes.

The homogeneity of the dye distribution in the PEM-supported lipid layers was characterized by using the FRAP equipment as a fluorescence microscope. The images were acquired by the CCD camera, yielding a resolution of ~1 μm.

Results

The successful buildup of PEMs is monitored by X-ray reflectivity and by UV−vis spectroscopy. Both techniques typically show the thickness of the multilayers linearly increasing with the number of deposition cycles. The surface roughness of the PEMs is investigated by AFM, using samples prepared on silica wafers. For instance, for the samples $(PSS/PAH)_5$/PSS the surface roughness is about 1 nm over an area of 5 × 5 μm^2 (data not shown). Fluorescence images show a uniform emission, demonstrating that the dye probes are homogeneously distributed in the lipid layer.

Lipid Layers in Water: Stability. When lipid monolayers attached to PEMs are brought into contact with water, hydrophobic lipid tails become exposed to the water phase. The lipid/polyelectrolyte layer system is thus changed concerning the following aspects: (1) The surface energy is increased due to the change from a hydrophobic tail/air interface to a hydrophobic tail/water interface. (2) The hydration of the lipid layers, both in the chain and in the headgroup region, might change. (3) The chain order can be affected. All these changes are possible driving forces for a destruction of the lipid monolayer. Therefore, the stability of the monolayer is a critical issue, which we address here.

To monitor the layer stability, real time fluorescence images are taken by using the FRAP equipment to characterize the lateral distribution of the dyes. A homogeneous dye distribution is observed before placing water on the sample. After spreading water on top of the sample, the fluorescence intensity is still homogeneous. Only at a few positions on the sample was a single bright spot observed. Since the concentration of these spots is very low, they probably arise from defects in the layer, inducing a local dye aggregation. All diffusion data are taken on areas which do not contain such a spot.

Time-dependent fluorescence images show that the layer is stable in time over at least an hour (the time scale of the diffusion experiment), and the homogeneity does not change.

In general, a newly formed hydrophobic chain/water interface might rearrange to minimize the surface free energy. For a lipid monolayer on a hydrophilic surface swollen in hydrophilic solvents, dissolution in the form of vesicles can occur. Alternatively, bilayer patches could form, so that either PEMs or lipid headgroups become exposed to the hydrophilic solvent environment. If such a dissolution or rearrangement of the monolayer would follow the swelling, this would result in heterogeneous structures, such as large aggregates or domains at the interface. Such structures, if occurring on a scale of micrometers, which is the typical size of lipid domains, would cause inhomogeneities in the fluorescence images. However, no such domains or patches could be observed when PEM-supported lipid layers were swollen in water. This implies that at least on the micrometer scale, the monolayer remains stable. In the fluorescence images taken after water swelling, the intensity does not change over time. It can be concluded that the interaction energy of lipid headgroup with polyelectrolyte segments is large enough to stabilize the monolayer even in aqueous solvent environment. Therefore, all diffusion data are taken on the swollen film in the equilibrium state. Though the fact that a monolayer can be stable in aqueous environment might be surprising in view of the above energy arguments, stable lipid monolayers were also observed for similar systems, for example, DMPC on chitosan at 90% humidity,[16] and on polyacrylamide in water.[23] The stability is a consequence of the very strong interaction between lipid headgroups and charged polymer segments, which was demonstrated previously.[22]

TABLE 1: Diffusion Coefficients of DMPC, DOPC, and DOPA on Five Double Layers of PSS/PAH Swollen in Water with PAH as the Outer Layer, and on $(PSS/PAH)_5$/PSS with PSS as the Outer Layer, Respectively

	outer layer	
D [10^{-10}cm^2/s]	PSS(−)	PAH(+)
DMPC(±)	0.22 ± 0.03	0.24 ± 0.04
DOPC(±)	0.24 ± 0.02	
DOPA(−)	0.10 ± 0.02	0.11 ± 0.01

Lipid Layers in Water: Diffusion. Diffusion measurements are performed in different molecular combinations of the layered lipid/polyelectrolyte systems, where lipids are bound to either positively or negatively terminated polyelectrolyte multilayers. Several measurements are performed on each water-swollen layer, and the lateral diffusion coefficients are averages of at least 20 measurements for each type of layer system. The data evaluation and error considerations are similar to those for layers in air.[22]

The diffusion coefficients for each combination of lipid/polyelectrolyte investigated are summarized in Table 1. The diffusion coefficients are in the range of 10^{-11}−10^{-10} cm^2 s^{-1}, and are thus 3 orders of magnitude smaller than typical values in free, uncoupled lipid layers. Thus the coupling to the PEMs significantly decreases the mobility. In comparison to PEM-supported lipid layers in air, however, the diffusion is enhanced by 1−2 orders of magnitude.[22]

In previous investigations by other authors, diffusion coefficients of supported lipid layers have mainly been measured in systems with small interactions: for monolayers of DMPC and DOPC on solid substrates coated with HTS, or in polymer-supported bilayers measured in water, diffusion coefficients on the order of 10^{-8} cm^2 s^{-1} were detected.[16,24] Bilayers of POPC and DPPC on silica particles showed similar values.[25,26] For lipid monolayers and bilayers deposited onto polyacrylamide gel of various compositions,[23] diffusion constants in layers in water varied in the range of 10^{-10}−10^{-7} cm^2/s. In a polymer-supported bilayer coupled by covalent tethering at both the polymer−substrate and the polymer−bilayer interface diffusion constants are in the range 10^{-8}−10^{-7} cm^2/s depending on the tethering density (lipopolymer/phospholipid molar ratio).[27] In contrast to these papers, the diffusion coefficients found here for the water-swollen coupled layer systems are much smaller, and rather agree with the order of magnitude in lipid monolayers in the gel phase on water surfaces (10^{-10} cm^2/s). The main

difference between our PEM-supported lipid layers and the systems mentioned above is a strong electrostatic interaction induced in the lipid/polyelectrolyte interfaces.

From a comparison of the different systems investigated in contact with water (see Table 1), the influence of the chain order and headgroup, respectively, can be extracted. For DMPC and DOPC on PSS as terminating polyelectrolyte layer, the diffusion coefficients are the same within error. Thus, the chain disorder induced by the unsaturated bond in the DOPC chain does not affect the diffusion. It can be concluded that the lipid chain region is rather disordered, such that additional disorder from unsaturated chain does not lead to an enhanced free area available for diffusion. On the other hand, for DOPC and DOPA, both containing the same unsaturated chains, but different headgroups, the diffusion coefficients differ significantly. The nature of the chain therefore has no influence on the diffusion. The headgroup binding appears to control the diffusion. The same trends were previously observed for lipid diffusion at ambient conditions in air.[22] Thus even when screened by water molecules, the lipid−polyelectrolyte interaction is large enough to govern the lipid mobility.

The sign of the charge of the last PE layer does not influence the diffusion. This is particularly interesting in the case of the negatively charged DOPA, where a similarly strong interaction occurs with a positively or a negatively charged terminating polyion layer, respectively. The same observation had been made for coupled layers in air.[22] There, we had interpreted these findings as a large flexibility of the segments of the outermost and the second outermost polyelectrolyte layer, allowing an interpenetration of the headgroup region into the terminating polyion layer. This provides the possibility of the headgroup to interact with both positive or negative segments.[22] In water-swollen layers, the flexibility of the polyelectrolyte chains, and thus the availability of charges with a sign opposite to the sign of the last layer, can be expected to be even enhanced due to an enhanced chain flexibility in the water-swollen state.

In comparison to PEM-supported layers in air ($D \sim 10^{-12}$ cm^2/s, ref 22), diffusion coefficients in water-swollen layers are increased by a factor of 40 for phosphatidylcholine lipids (PC) and by about 15 for phosphatidic acid (PA). The mobility in the present system is enhanced, probably induced by an increase of the number of hydration water molecules between the lipid headgroups and the corresponding polyelectrolyte charged segments. Another possibility may be a change of conformation of the lipid and polyelectrolyte multilayers by water.

Interestingly, while for layers in air the room temperature diffusion as well as the activation energies demonstrate a low mobility of zwitterionic DMPC or DOPC as compared to ionic DOPA,[22] in water-swollen layers the mobility of the zwitterionic PC lipids is enhanced as compared with that of the PA lipids. This issue will be taken up in the discussion below.

Lipid Layers in Ethanol/Water Mixtures. To systematically study the effect of the dielectric properties of the local environment on the lateral diffusion in lipid layers, experiments are designed to measure the diffusion in PEM-coupled lipid layers in contact with binary mixtures of water and ethanol. The advantage of choosing such binary mixtures is that the dielectric constant in such a solvent can be varied continuously by changing the volume ratio of the two components.[28] Thus, the dielectric constant ϵ in ethanol/water mixtures can be varied over a range of 24 (pure ethanol) to 80 (pure water).

Lipid Layers in Ethanol/Water Mixtures at Low Ethanol Content: Stability. When ethanol/water mixtures are placed on the surface of a supported lipid monolayer, dissolution of the lipid or bilayer patches may occur due to minimization of the surface energy of the newly formed hydrophobic chain/solvent interface. This process is more critical in ethanol/water mixtures as compared to water because of the higher solubility of lipid in ethanol. A dissolution of a large amount of lipid in ethanol/water mixtures would result in the observation of domains or aggregates in real time fluorescence imaging. However, the fluorescence images taken from samples swollen in ethanol/water mixtures with an ethanol content of less than 50% (v/v) show a homogeneous fluorescence similar to the layers in water. Thus it can be concluded that the majority of the lipid material remains at the PEM surface. Another evidence of the stability of the lipid layers is the fact that the diffusion coefficient does not change with time after solvent swelling. The stabilizing effect of PEMs on the lipid layer is thus also present under ethanol/water mixtures.

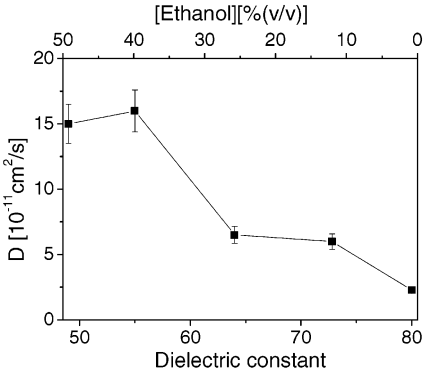

Figure 1. Diffusion coefficients of DMPC as a function of dielectric constant in water/ethanol mixtures. FRAP measurements are performed in different volume ratios of ethanol/water mixtures. The scale of the dielectric constant is a linear interpolation in the range of 0−50% (v/v). The solid line is a guide to the eye only.

TABLE 2: Diffusion Coefficients of DMPC, DOPC, and DOPA on Five Double Layers of PSS/PAH with PAH as the Outer Layer and on (PSS/PAH)$_5$/PSS with PSS as the Outer Layer, Respectively[a]

	D [10^{-10} cm^2/s]		
[ethanol] (%, v/v)	DMPC/PSS	DOPC/PSS	DOPA/PAH
0	0.22 ± 0.03	0.24 ± 0.02	0.11 ± 0.01
10	0.60 ± 0.10		
25	0.65 ± 0.13	0.70 ± 0.11	0.85 ± 0.12
40	1.60 ± 0.25		
50	1.48 ± 0.24	1.53 ± 0.22	1.36 ± 0.23

[a] The measurements were performed in samples swollen in a binary mixture of water and ethanol.

Lipid Layers in Ethanol/Water Mixtures at low Ethanol Content: Diffusion. FRAP experiments are performed for samples swollen in ethanol/water mixtures with an ethanol content of less than 50% (v/v), employing the same procedure as for measurements of water-swollen layers. The diffusion coefficients of DMPC measured in ethanol/water mixtures are shown as a function of the dielectric constant in Figure 1. Such measurements are also carried out for different combinations of lipid/polyelectrolyte. The diffusion coefficients of different systems are summarized in Table 2.

The diffusion coefficients of all combinations of lipid/PEMs in ethanol/water mixtures with an ethanol content of less than 50% (v/v) are on the order of 10^{-10} cm^2 s^{-1}, and are somewhat larger than those in water-swollen layers. For DMPC layers, the diffusion coefficients show an increase with decreasing dielectric constants. The main change occurs at ϵ between ~65

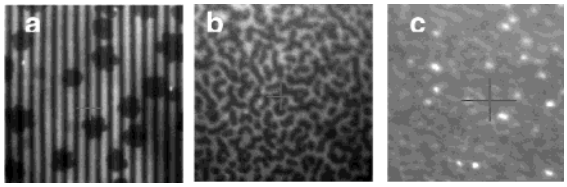

Figure 2. Fluorescence images of PEM-supported lipid layers taken by using the FRAP setup as a fluorescence microscope 1 h after swelling in solvent. The scale of the image is given by the period of the pattern in Figure 2a, which is 5 μm. (a) $(PSS/PAH)_5/PSS/DOPC$ in [ethanol] 75% (v/v), image acquired with excitation by an interference pattern, and $(PSS/PAH)_5/PSS/DMPC$ acquired with excitation by one laser beam, (b) in 100% [ethanol], and (c) in [ethanol] 75% (v/v).

([ethanol], 25%) and ~55 ([ethanol], 40%). The same trend of diffusion coefficients being increased at higher ethanol content is found for the other systems, see Table 2. Several influences can determine the interlayer interaction in the presence of solvents, for example the screening effect of the dielectric environment on the electrostatic interactions. Considering only the variation of ϵ with the composition of the aqueous phase would, however, result in an enhanced mobility at large ϵ. The partition of water and alcohol between the layers and the aqueous phase has to be taken into account, since it determines the local solvent properties within the layer. This will be discussed in detail further below.

Lipid Layers in Ethanol/Water Mixtures at High Ethanol Content: Stability. When ethanol/water mixtures with an ethanol content of above 50% (v/v) are brought in contact with the sample, interesting lateral structures are observed in the PEM-supported lipid layers. For example, after placing 20 μL of [ethanol] 75% (v/v) on DOPC coupled to PSS-terminated PEMs, dark domains start to appear in the fluorescence images as shown in Figure 2a. In similar experiments performed on PEM-supported DMPC layers, the fluorescence images show inhomogeneities in the fluorescence distribution appearing in the lipid layers as illustrated in Figure 2b,c. The domain patterns formed are characteristic for the particular lipid.

There are different explanations for the occurrence of such structures. In the fluorescence image the bright regions are indicating the presence of fluorescent probes, i.e., the lipid remains in a disordered phase, in which the dye probe is preferably dissolved. The dark domains are implying that the lipid layer in that region is either removed by solvent or forms a more ordered condensed phase, where the solubility of dye is lower.

In the former case a partial dissolution of the lipid layer has to be discussed: The ethanol molecules can penetrate to the interface of lipid/PEMs and then remove the lipid due to a reduced headgroup/polymer interaction. Since the chain aggregation is a collective process, this can lead to lateral heterogeneity.

In the latter case, the above phenomena might be induced by a phase transition of the lipid layer at high ethanol concentration. Thus there could be a coexistence of an ordered phase (black) and a disordered phase (bright) appearing in the lipid layers.

Such lateral structures are not observed when DOPC or DMPC lipid layers are deposited on glass and immersed into water/ethanol mixtures. Here, the ethanol completely removes the layers by dissolution. This is another evidence for the PEMs stabilizing the lipid layers to a certain extent. This stabilization is sufficient to result in intact layers up to a critical hydrophobicity of the organic solvent.

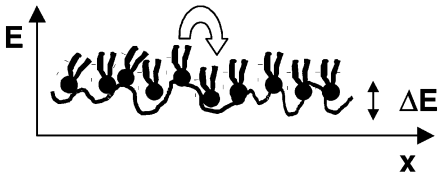

Figure 3. Schematic illustration of the potential energy surface in the model of local binding of the lipid to the polyelectrolyte.

Discussion

Stability of PEM-Coupled Lipid Monolayers. In water and in ethanol/water mixtures below the critical ethanol content ρ_E, the stabilizing effect of PEMs is sufficient to keep the lipid layer intact. This stability can be explained by the strong electrostatic interaction deduced from the diffusion coefficients discussed above. Opposed to this, for weakly interacting substrates, the equilibrium structure for a monolayer immersed into water typically is a coexistence of bilayer patches with uncoated surface. In this case the monolayer is rearranging to avoid the formation of a hydrocarbon chain/water interface, which would have a high surface energy. The typical surface energy of a hydrocarbon chain/water interface is 20 mN/m. Assuming a molecular area of 50 Å^2, this results in a binding energy of about 0.3 kT only. This energy is smaller than the activation energies, which are on the order of 10 kT, as determined for the lipid motion on PEMs in air.[22] Thus, in the strong coupling case investigated here, the interaction of the headgroups with the polyelectrolyte is apparently large enough to stabilize the monolayer under the solvents employed.

Mechanism of Diffusion in Supported Lipid Layers. In conclusion from the diffusion coefficients determined in air[22] and in various solvents, a general picture of the coupled lipid/polyelectrolyte layer system is evolving: Lipids in contact with a polyelectrolyte multilayer interact with the charged segments of the polyions. The polyelectrolyte chains in PEMs were shown to exhibit diffusion coefficients less than 10^{-16} cm^2/s,[29] whereas lipid molecules in monolayers or bilayers typically show diffusion coefficients of $10^{-8}-10^{-7}$ or 10^{-10} cm^2/s for a fluid phase or a gel phase, respectively.[30] Thus the polyelectrolyte can be assumed immobile as compared to the lipid, and the motion of the lipids is controlled by their interaction strength with the immobile polyelectrolyte. The lateral distance of the lipid molecules can be estimated by the charge separation on a polyelectrolyte chain, assuming that the charge density determines the number of lipids to be bound. Typical charge separations along the chain are between 5 and 10 Å, depending on the molecular structure of the polyelectrolyte. Therefore in the case of loose packing of the lipid chains and sufficient free area, the diffusion process can be described by a model of jumps of the headgroups between binding sites on the polyelectrolyte as shown in Figure 3.[21] In such a picture, the energy ΔE for a jump to the next local energy minimum is the limiting parameter for the diffusion process. Temperature-dependent diffusion measurements on layers in air resulted in an activation behavior and thus support the above picture. Activation energies for layers in air amounted to $\Delta E \approx 10$ kT.[22] The activation energy can be taken as a parameter to characterize the energy required to break up the interaction of a lipid molecule bound to a polyelectrolyte segment. This release of the local interaction is assumed as the key step for the diffusion process.

Hydration. When the sample is brought into contact with water, water molecules can diffuse across the lipid layer, as had been shown for bilayers.[31] Thus, a new hydration equilibrium of the headgroup/polyelectrolyte interfacial region is

established. There are some parts of the phospholipid headgroup region that may be particularly involved in the interaction with water molecules: the ester and phosphate oxygen of all phospholipids, and the charged tetramethylammonium groups for PC.[32,33] The headgroup hydration will influence the strength of the lipid−lipid interaction between neighboring headgroups, and also the interaction with polymer segments.

Water molecules are found even in the hydrophobic region, and can form an interchain hydration there.[34] It is supposed that water molecules existing in the hydrophobic region would cause some disorder in the chain. For the current system, the effect on the diffusion can be neglected, since the diffusion is dominated by the strong interfacial interaction in the PEM-supported lipid layers. Therefore only hydration influences on the headgroup−polyion interaction are discussed here.

The water present at the interface of lipid and polyelectrolyte screens the electrostatic interaction. The reduced interaction leads to a faster lateral diffusion of the lipid. The result of faster lateral diffusion in water-swollen layers therefore is consistent with a higher hydration of the headgroup region as compared to lipid layers in air. The reduction of the interfacial interaction by water can be due to (a) a change of dielectric properties of the environment or (b) a distance change between the lipid layer and the supporting polyelectrolyte layer. The effect of both is discussed in the following model in terms of the local electrostatic forces governing the binding energy.

Model of Local Electrostatic Interactions. As discussed above, local electrostatic interactions are determining the diffusion in PEM-coupled lipid layers. For the DOPA headgroup containing a single charge, Coulomb interaction with the nearest polyion segment applies. The electrostatic interaction is then described by the Coulomb law

$$E = \frac{1}{4\pi\epsilon\epsilon_0}\frac{Q_1Q_2}{r} \tag{1}$$

where Q_1 and Q_2 are the charges of the interacting segments, r is the distance of the charges, and ϵ is the dielectric constant of the medium.

When the experiments are performed under water, the lipid layers are highly hydrated due to the presence of water at the interface. As compared to layers in air, this leads to a change of the local dielectric constant. For example, a major change in the dielectric properties of polyelectrolyte multilayers in dependence of temperature, humidity, and deposition conditions was found:[35] The dielectric constant ϵ for the layer in the dry state is about 5−6, whereas ϵ is above 80 in hydrated layers prepared without salt, and even larger if the layers are assembled with salt. In our present case of layers swollen in water and highly hydrated, ϵ can thus be assumed to be up to 20 times higher as compared to PEMs in air. Assuming a similar ϵ in the lipid/PEM contact region, the increase of ϵ after swelling reduces the electrostatic interaction and facilitates the diffusion. A quantitative value of the Coulomb interaction energy is difficult to obtain, since absolute distances of the charges between the PA headgroup and the binding charged polymer segment are not known. A precise calculation would require estimates of the local dielectric constant and of the separating distance of the charges.

Differences between the zwitterionic and the ionic lipid diffusion can be explained by the different number of binding charges: Following the ideas of possible binding to the outermost as well as the second outermost polyion layer, as discussed above, the PC headgroup can interact with two

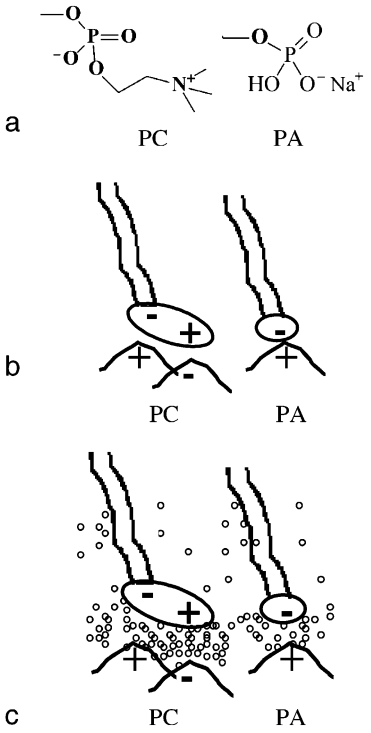

Figure 4. Chemical structures of phospholipid headgroups, zwitterionic phosphatidylcholine DOPC or DMPC ("PC", left), and ionic phosphatidic acid DOPA ("PA", right), part a. (b, c) Molecular picture of lipids and charged polymer segments in lipid layers in air (b) and in water (c). In water an increase of the interlayer distance is assumed.

different, oppositely charged polyelectrolyte segments, see Figure 4b. The interaction of PC with PEMs can thus be described by a dipole−dipole interaction:

$$E = \frac{u_1u_2}{4\pi\epsilon}\, f(\varphi)\,\frac{1}{r^3} \tag{2}$$

where u_1 and u_2 are the electric dipole moments, and r is their distance. $f(\varphi)$ is a function depending on the relative orientations of the dipole moments.

As discussed above, the dielectric constant is larger in water-swollen layers as compared to samples in air. The resulting reduction of the interfacial interaction in the coupled systems applies to the PA headgroup with Coulomb interaction in the same way as to the PC headgroup with dipole−dipole interaction. With only ϵ changing as the layers are brought from air to aqueous environment, the interaction energies should scale with ϵ as ϵ^{-1}, see eqs 1 and 2.

However, in comparison to layers in air, hydration enhances the diffusion coefficient by a factor of 40 for the PC and 15 for the PA headgroup, which is significantly differing.

Therefore, structural changes in the interlayer region need to be considered additionally: The hydration water may cause a change of distance between the lipid and polyelectrolyte. A further reduction of the interaction will occur due to the distance change after the sample is swollen in water. Assuming that the distance change after water swelling is the same for both PA and PC layers, comparing eq 1 for the PA headgroup with eq 2 for the PC headgroup, a larger decrease will result for the PC headgroup according to the distance dependence (r^{-3} versus r^{-1}) of the interaction, see Figure 4c. This might be one of the reasons why a faster diffusion for PC systems has been observed after sample swelling in water. However, a quantitative calculation cannot be performed because the distance of the polymer

dipole moment relative to the dipole moment of the lipid cannot be estimated from the experimental data up to now. A study of interlayer distances employing fluorescence resonant energy transfer (FRET), however, proves a distance increase due to water swelling, and is going to be published in a separate paper.[36]

Variation of the Solvent Dielectric Constant. On the basis of the comparison of water-swollen layers to layers in air, it was concluded that the local dielectric constants play a role in the mobility of the coupled lipid layer. In ethanol/water mixture-swollen layers, both water and ethanol molecules could diffuse into the lipid layer and stay in the headgroup region. The equilibrium amount of water and ethanol in the interfacial region depends on the ethanol content in the solvent and on the partition into the layer. The newly formed local solvation equilibrium then determines the local dielectric constant.

Quantitatively, an increase of the diffusion coefficient with increasing dielectric constant of the aqueous phase is expected according to the Coulomb or dipole−dipole interaction. This is not observed in the experiment and indicates that the solvent causes more subtle changes.

A strong swelling with increasing ethanol content is one possible explanation. In fact, several changes of the membrane structure were observed upon swelling in water−alcohol mixtures: Small alcohols can induce a loosening of the molecular packing.[37] Ethanol was shown to expand the headgroup region in bilayers.[38,39]

In addition, effects of preferential hydration of the headgroup region can be induced by the presence of the alcohol. It was observed by Westh et al. that the affinity of the membrane for the water is higher than that for small alcohols.[40] Therefore, the addition of the alcohol to the aqueous phase in combination with the partition of the water being modified can lead to strongly swollen layers.

Such a swelling may cause a larger distance increase of the interacting layers and lead to a larger reduction of the interfacial interaction. As a result, a fast diffusion is found in ethanol/water mixtures with higher ethanol content (see Table 2).

In addition, as a consequence of the swelling of the lipid/PEM interface, a dissolution of lipid molecules might occur. Even though no inhomogeneities of the dye distribution are observed after the sample was swelled in the ethanol/water mixtures containing less than 50% of ethanol, the dissolution of a few single lipid molecules cannot be completely ruled out. A homogeneous layer might remain even when a very small fraction of possibly up to a few percent of lipid dissolves in chemical equilibrium. Then, the layer would have a slightly lower packing density, so that the diffusion coefficient might increase due to this effect.

Both mechanisms, an ethanol-enhanced interlayer separation as well as the dissolution of a small fraction of lipids, can be attributed to a strong swelling of the lipid−polymer interfacial region. Since the lipid headgroup as well as the polyion backbones contain hydrophobic segments, it is possible that the ethanol uptake is rather large as compared to the water uptake.

Comparing different systems investigated in ethanol/water mixtures (see Table 2), DMPC and DOPC show the same diffusion coefficients within error, consistent with the findings for water-swollen layers or layers in air. Thus, again the chain has a negligible influence on the diffusion. However, no difference of D is detected for DOPA and DOPC in ethanol/water mixtures, unlike for layers in air and for water-swollen layers.

As discussed above, several factors, such as the local dielectric constant, the lipid−polyelectrolyte interlayer distance, and the possibility of partial dissolution, would cause a change in interfacial interaction and control the diffusion coefficients. These parameters may have a different influence on the PA or PC lipid layer. The combinations of all influences lead to an undetectable difference in the binding energy for PA and PC. Thus for the discussion of a mechanism of ethanol/water effects, all these possibilities should be taken into account. By the data extracted from FRAP experiments, we cannot distinguish the influence of all factors quantitatively. Fluorescence resonant energy transfer (FRET) experiments can determine distance changes and yield more quantitative information on this point.[36]

Conclusions

Diffusion experiments in water-swollen lipid layers are performed in dependence of the lipid headgroup charge (ionic or zwitterionic), the chain order, and the sign of charge of the terminating polyelectrolyte layer. The interfacial interactions lead to a constraint of the diffusion motion as compared to diffusion of free lipid monolayers in contact with water, and result in diffusion coefficients on the order of 10^{-10} cm^2/s. The results can be interpreted in a model of disordered chains, and headgroup-interaction dominated diffusion for PEM-supported lipid layers. Diffusion coefficients are governed by the nature of the lipid headgroup, where ionic lipids couple more strongly to the charged environment than zwitterionic ones. An enhanced diffusion is found in water or solvent-swollen layers in comparison to layers in air. The reduced interaction can be due to screening of the charges by water molecules, or furthermore due to an increase of the interlayer spacing. With such an increase, the distance dependence of the Columbic versus the dipolar interactions explains the finding of a larger mobility of DOPA as compared to DMPC or DOPC in air, whereas in water-swollen layers the DOPA mobility is reduced as compared to that of the PC lipids. Fluorescence resonance energy transfer (FRET) experiments, presented in a following paper,[36] provide evidence of the increase of interlayer distance due to hydration water.

The diffusion results on swollen layers show that PEM-supported lipid layers are stable under water and several other solvents. On the basis of the lateral diffusion measurements performed under different solvents, a reduction of the interfacial interaction induced by the solvents leads to a faster diffusion in the lipid coupled to PEMs. The diffusion results do not scale with the dependence of the Coulomb interaction on the dielectric constant, thus other factors have to control the binding strength, such as a distance variation induced by the swelling of the lipid/polymer contact region.

The results demonstrate that polyelectrolyte multilayer-supported lipid monolayers are a very successful approach for producing a stable model system of supported lipid layers. The effect of stabilization of polyelectrolyte multilayers on lipid layers is achieved by electrostatic interactions introduced into both the lipid−polyelectrolyte and polyelectrolyte−substrate interfaces. The stabilizing and protecting effect of PEMs can be concluded from the stability of the molecular assembly of lipid layers under water and under binary mixtures of water and ethanol (up to 50% v/v). The mobility of the lipid layers and the stability of the overall layer system have to compromise each other: The electrostatic interactions hinder the lateral diffusion of lipid layers, but are very beneficial for stabilizing the lipid layer. Though such strongly coupled systems might not be the ultimate choice to optimize biological function, they lead to a stabilization of the whole system, and form thus suitable model systems, which enable here very precise and

systematic investigations of the influence of lipid headgroup charge, lipid chain structure, and terminating polyelectrolyte layer of PEMs under different environments. Moreover, strongly coupled layers might be helpful in systems where a strong immobilization of proteins in model membranes is required.

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