Polysaccharide-Supported Planar Bilayer Lipid Model Membranes†

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Bilayer lipid membranes were deposited onto two different thin water swellable polymer cushions predominantly by Langmuir−Blodgett trough methods. Membranes consisting of zwitterionic lipids supported by agarose films were shown to be unstable, as observed by fluorescence microscopy, reflection interference contrast microscopy, and the impossibility of bilayer spreading (Rädler, J.; Strey, H.; Sackmann, E. *Langmuir* **1995**, *11*, 4539−4548) on the agarose surface. Chitosan, formerly observed to permanently coat liposome surfaces of zwitterionic lipid membranes (Henriksen, I.; Smistad, G.; Karlsen, J. *Int. J. Pharm.* **1994**, *101*, 227−236), was used to prepare thin films by spin-coating and was found to sufficiently adsorb and thereby stabilize planar bilayer lipid membranes. Bilayer spreading on a chitosan surface was observed, indicating the self-healing ability of chitosan-supported lipid membranes.

Introduction

Polymer interlayers have a great potential for effectively decoupling lipid membranes and the peripheral parts of integral membrane proteins from supports such as glass, gold, indium−tin oxide (ITO), or silicon devices.[3−5] Therefore, numerous efforts have been made to prepare suitable polymer cushions, physisorbed or chemically coupled to an underlying support and physisorbed or chemically coupled to the lipid membrane on top.

Lipid membranes that are either completely or partially chemically coupled to the polymer cushion are regarded to be advantageous to membrane stability. However, they increase the synthetic expense and considerably reduce membrane fluidity. A highly fluid membrane environment is generally assumed to be favorable, if not essential, for the activity of membrane proteins. Therefore, in the present work, membranes were prepared which were purely physisorbed to the polymer cushion.

Ringsdorf's lab was probably the first to work on physisorbed polymer-supported lipid membranes on a solid substrate.[6−9] In these works, polyethyleneimine (PEI) was used as an interlayer between a solid support and an amphiphile membrane (see also refs 10 and 11). Additionally, the polysaccharides dextran, cellulose[12−17] and polyacrylamide[18,19] were applied to prepare polymer-supported mono- and bilayer lipid membranes.

All of these systems share the fact that it is difficult or impossible to control or alter the membrane/substrate spacing by varying the thickness of the interlayer polymer cushion. The control of the membrane/substrate spacing is of importance for surface sensitive techniques, like methods using evanescent fields. Additionally, the electrical analysis of single membrane channels requires a large ionic reservoir (i.e., a polymer cushion of sufficient thickness).[20] The use of polymers, which are soluble under particular conditions and otherwise form a water-insoluble gel, is a way to avoid this problem. Both of the deposition methods, dip-coating and spin-coating, offer an extremely wide range of polymer thicknesses quite readily. Additionally, this approach is simple and time-saving, which is favorable for extensive physicochemical investigations of polymer-cushioned lipid membranes. Agarose and chitosan are polysaccharides that can be dissolved under special conditions and are insoluble under the conditions used for membrane preparation and analysis.

Probably due to the simple fabrication of the polymer cushion, agarose has been used extensively for supporting and thereby stabilizing black lipid membranes (BLMs),[21−23]

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which are obtained by the thinning of a lipid solution painted across a small aperture. BLM-like membranes were formed either on top of an agarose cushion exposed to electrolyte[24−29] or sandwiched between two agarose sheets.[20,30,31] Additionally, monolayer lipid membranes were transferred to agarose films by means of Langmuir−Blodgett transfer.[32] Interestingly, agarose-supported BLM-like membranes (i.e., lipid membranes coupled to a solvent-enriched lipid reservoir formed in an aperture) possess electrical properties which are comparable to those found for free-standing BLMs.[24,25,27,28,33] It was shown[29] that agarose-cushioned lipid bilayers allow the analysis of single ion channels, incorporated into the membrane. Other polymer-supported bilayer systems, however, showed electrical properties markedly different[34−37] from those of free-standing BLMs, which are regarded to represent an electrically "perfect" membrane. In the present work, we evaluated whether it is possible to prepare bilayer membranes on agarose cushions by Langmuir−Blodgett trough methods.

As an alternative to agarose, chitosan films were applied as membrane cushions. To the best of the authors' knowledge, this report is the first to describe the use of chitosan as a polymer cushion to support planar bilayer lipid membranes. However, chitosan has been utilized to coat liposomes with a monomolecular layer (so-called "chitosomes"), leading to an enhanced liposome stability.[2,38−40] We demonstrate that chitosan polymer cushions, which are as easy to prepare as agarose films, have major advantages over agarose for obtaining homogeneous and stable bilayer lipid membranes by Langmuir−Blodgett trough methods and by bilayer spreading from a lipid reservoir.

Methods and Materials

FRAP/Fluorescence Microscopy. For determining lateral self-diffusion coefficients, the method of fluorescence recovery after photobleaching (FRAP) of membrane labels was used as described.[14,41]

The central part of the FRAP apparatus was an inverted microscope (IX-70, Olympus, Hamburg, Germany), equipped with a mercury burner (HBO 100, Olympus, 100 W) for wide-field illumination and an argon ion laser (Innova 90/4, Coherent, Dieburg, Germany), which was operated at a wavelength of 488 nm (at a power of 1.2 W), for bleaching and fluorescence recovery measurements. Beam attenuation was performed by a combination of three pockels cells and four linear polarizers (Gsänger, Munich, Germany) in an alternating arrangement. Focus adjustment (and fluorescence microscopy) could be performed with the help of a light-enhancing camera (extended ISIS, Photonic Sciences, East Sussex, U.K.).

RICM. Reflection interference contrast microscopy (RICM) was performed as described in ref 42. The central part of the setup was an inverted microscope (IX 70, Olympus; the same microscope allowed for fluorescence microscopy and FRAP with the same sample). A high-pressure mercury burner served as the light source. A band-pass filter (546 nm, Zeiss, Göttingen, Germany) allowed for the selection of the intense line at 546.1 nm of the mercury lamp. A modified DIC cube (U-MDIC, Olympus) was equipped with two rotatable linear polarizers, needed for stray light suppression by means of the Antiflex technique. The microscope objective was a Zeiss Neofluar 63/1.25 (Zeiss, Göttingen, Germany). RICM images were recorded by means of a digital CCD camera (C 4742-95-12-NR, 1280 × 1024 pixels; Hamamatsu, Germany) which was controlled by the software HiPic 32 (Hamamatsu, Germany).

Giant liposomes for interference microscopy were prepared from a lipid stock solution in chloroform, which was dried on a Teflon disk and desiccated under vacuum overnight. Subsequently, 100 mM sucrose solution was added to the Teflon disk placed at the bottom of a glass beaker and left for at least 12 h at 40 °C. During that time, the lipid swelled and formed closed vesicles. A few milliliters of this vesicle dispersion was transferred into a measurement chamber filled with 100 mM inositol. The vesicles sedimented slowly to the bottom of the chamber due to the difference in density of sucrose inside the vesicle and inositol outside. In the case of experiments with giant multilamellar vesicles (liposomes), the measurement chamber consisted of a Teflon frame (10 × 30 × 40 mm) into which a hole was drilled (∅ 8 mm). The Teflon frame was closed at the bottom by a glass coverslip.

Langmuir−Blodgett Trough Methods. Lipid bilayer membranes were obtained by two successive depositions of lipid monolayers on a Langmuir−Blodgett (LB) trough. Monolayers were obtained by spreading a lipid solution (chloroform, 1 mg/mL) on a Langmuir−Blodgett trough equipped with a mechanical dipper and Wilhelmy balance (NIMA Technology Ltd., Coventry, U.K.). The subphase consisted of ion-exchanged Millipore filtered water (Millipore Milli-Q system, $R = 18.2$ MΩ cm). After the lipid solution was spread, the solvent was allowed to evaporate (for half an hour) and the film was compressed to the desired lateral pressure ($T = 25$ °C). After compression, the film was equilibrated for another half an hour and finally deposited at a speed of 4 mm/min. The second monolayer was transferred by vertically pressing the substrate through the compressed monolayer at the air/water interface (Schäfer transfer). For successful Schäfer transfers, the sample had to be dried thoroughly previously by means of a dry stream of N_2, as otherwise the barrier position on the LB trough indicated an insufficient lipid transfer.

Lipids. The lipids DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine), egg-PC (a natural lipid mixture), and cholesterol (3β-hydroxy-5-cholesten) were obtained from Avanti Polar Lipids, Alabaster, AL, and used without purification. The following fluorescence probes were applied for membrane labeling: *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE), and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), Molecular Probes, Leiden, The Netherlands. Fluo-

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rescent lipids were added to the lipid solution at a concentration of 1 mol % with respect to the host lipid. In all cases, fluorescent lipids were added to both bilayer leaflets.

Polymer Cushions. Chitosan and agarose were obtained from Fluka/Sigma/Aldrich, Seelze, Germany, and used without purification. Chitosan was dissolved in a 1% v/v acetic acid solution (99.8%, Riedel-de Haën, Seelze)[43] at a concentration of 1% w/w by stirring overnight. The solutions were filtered through syringe filters (Millex, Millipore Corp., Bedford, MA) with a pore size of 5 μm. Afterward, the solutions were centrifuged (Biofuge 22R, Heraeus, Germany) for 30 min at a speed of 11 400 rpm. Thin chitosan films were prepared by spin-coating the chitosan solution onto cleaned, hydrophilic silicon wafers (Wacker Siltronic Corp., Stuttgart, Germany; thickness, 600 μm; ⟨100⟩ orientation), which were cut into pieces of 25 × 40 mm. The wafers were thermally oxidized (IMM, Mainz; temperature, 800 °C) to yield an oxide layer thickness of 160 nm. In the case of RICM measurements, the polymer layer was deposited onto transparent glass substrates (Mettler Glas, Rettberg, Göttingen, Germany; length, 3.2 cm; width, 2.6 cm; thickness, 150−180 μm). Substrate cleaning was performed by sonication in a 2% v/v Hellmanex solution (Hellma, Germany), followed by thoroughly rinsing the substrates in Millipore water. Spin-coating was typically performed at a spinning speed of 3000 rpm, which yielded film thicknesses around 140 nm in the dry state, as determined by ellipsometry. To neutralize the films, the polymer-covered substrates were immersed for several hours in a borate buffer (pH = 9.22; Merck, Darmstadt, Germany) and afterward rinsed in Millipore water.

Agarose films were prepared by dipping clean substrates into a hot aqueous agarose solution of a concentration of 0.2% w/w. Upon quickly withdrawing, a thin polymer film remained on the substrate surface and formed a thin gel film upon cooling.[32]

Vesicle Fusion. Small unilamellar vesicles for vesicle fusion experiments were prepared by sonication. Lipid dissolved in chloroform was filled into test tubes and the solvent was evaporated while rotating the test tube so as to cover the inner walls by a thin lipid film. Subsequently, the lipid was thoroughly dried under vacuum overnight. Afterward, MilliQ water was added to the test tube and the lipid was allowed to rehydrate for at least 3 h at 40 °C. Sonication was performed by means of the tip sonicator Sonifier 250 (Branson, Danbury, CT), at a power of 30 W for 30 min, using a water bath for cooling. The clear dispersion was centrifuged (Labofuge 200, Haraeus) at 5300 rpm for 15 min, to remove sonicator debris. The vesicle dispersion was used directly after preparation. The dispersion was filled onto polymer-covered substrates and allowed to rest for several hours at a temperature of 30 °C (egg-PC) or 40 °C (DMPC). Afterward, the samples were washed by shaking them in pure MilliQ water.

Results

Bilayer lipid membranes were prepared on agarose and on chitosan polymer films by sequentially depositing two lipid monolayers onto polymer-covered glass substrates. The first transfer was performed by Langmuir−Blodgett transfer and leads, in all cases, to homogeneous supported monolayer membranes that were stable over the time course of the experiments (fluorescence images not shown).

The second monolayer was added by a Schäfer transfer.

We studied the influence of the membrane support type, the deposition temperature, and the membrane composition on membrane properties such as the following: homogeneity, stability, and self-healing (i.e., lateral bilayer membrane spreading), by fluorescence microscopy; the membrane/substrate interaction, by RICM; and the possibility of vesicle fusion and the fluidity of the supported bilayer, by FRAP.

Fluorescence Microscopy. *Agarose.* Figure 1 shows that lipid membranes consisting of DMPC on agarose cushions were laterally structured, indicating a partial fragmentation of the deposited membrane. The bilayer

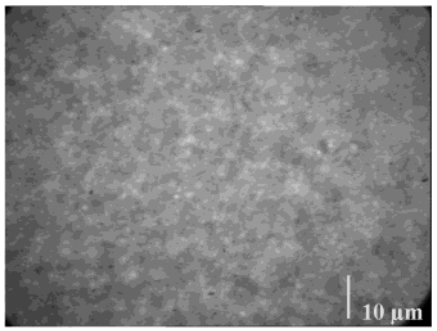

Figure 1. Fluorescence image of a DMPC bilayer membrane on an agarose cushion prepared by LB/LS transfer (lateral pressure, 35 mN/m; deposition temperature, 25 °C), using fluorescence dye DiI, directly after transfer.

deposition at a transfer temperature of 15 °C (below the main transition temperature of a DMPC bilayer, $T_t = 23.8$ °C) improved the initial bilayer quality, but a temperature increase to 25 °C again led to membrane fragmentation.

However, lipid bilayers of the same composition, deposited onto glass supports, always were found to show a perfectly homogeneous fluorescence at the resolution of the optical microscope and this state did not change with time (picture not shown).

One way to stabilize bilayer lipid membranes on solid substrates is the incorporation of cholesterol into the bilayer. The influence of cholesterol on the elastic properties of bilayers is well-known,[44] and it has been found that homogeneous bilayers on planar dextran cushions could be obtained only if the membrane contained a considerable amount of cholesterol.[19] This observation was explained[45] by a reduction of undulations and/or by an interaction of the polar OH-group of the cholesterol molecule with the underlying polysaccharide surface. Additionally, cholesterol compresses the membrane considerably, which enhances the electrical tightness of the bilayer.[46] However, in the case of agarose-cushioned lipid bilayers, the addition of cholesterol to DMPC membranes did not improve bilayer quality. Instead, directly after deposition at a temperature of 15 °C, large vesicles were observed on the surface and in the surrounding medium beneath the supporting surface (Figure 2, left part). However, the comparison of Figure 2 with Figure 1 reveals that fluorescent (i.e., bilayer-covered) areas are much more homogeneous when cholesterol is incorporated into the membrane. Dewetting of the homogeneous bilayer parts could be observed within minutes after membrane preparation (Figure 2, right part).

Chitosan. Lipid bilayers supported by chitosan showed a much-improved initial bilayer quality directly after transfer (Figure 3). A slight structuring was observed after storage at 35 °C for several hours. The long-term stability of chitosan-cushioned membranes at increased temperatures could be increased even further by the addition of cholesterol. No decrease of membrane homogeneity was observed for DMPC membranes containing 30 mol % of cholesterol during storage overnight at 35 °C. However, as in the case of agarose-cushioned membranes, a considerable influence of deposition temperature (25 °C compared to 15 °C) on the initial bilayer quality was

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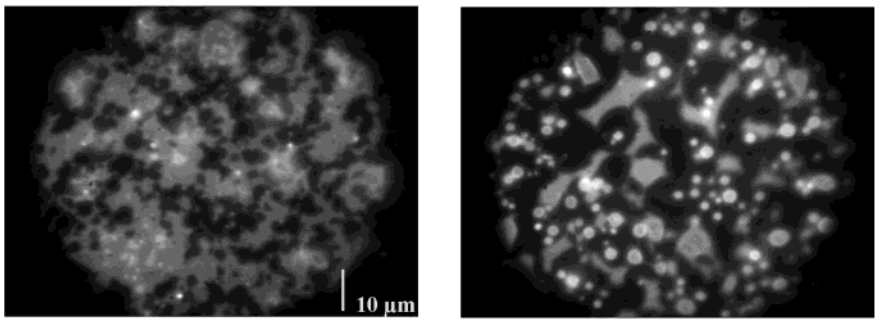

Figure 2. Fluorescence images of DMPC (30 mol % cholesterol) bilayer preparations on agarose cushions. Deposition temperature and observation temperature in both pictures: 15 °C. Left picture, directly after transfer; right picture, 10 min after transfer (different location on the same sample as compared to the left picture).

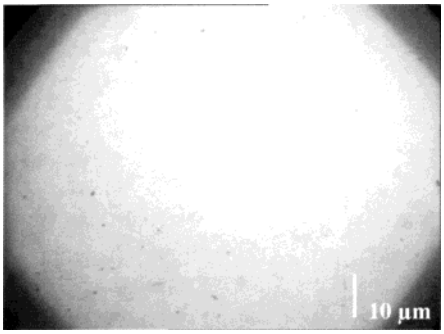

Figure 3. Fluorescence micrograph of a DMPC bilayer on a chitosan film, prepared by LB/LS transfer at a lateral pressure of 35 mN/m and deposition temperature of 15 °C, using fluorescence probe NBD-PE.

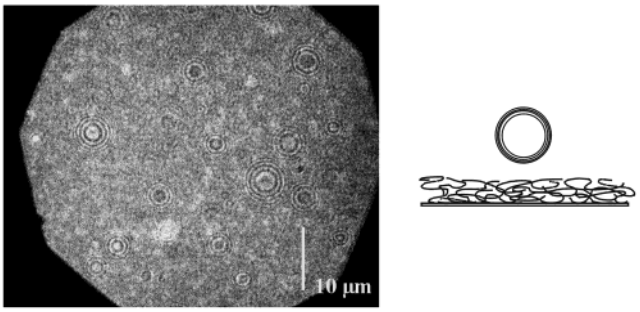

Figure 4. RICM image of DMPC vesicles hovering over an agarose surface (left); schematic drawing of a giant, multilamellar vesicle, hovering over the polymer surface (right).

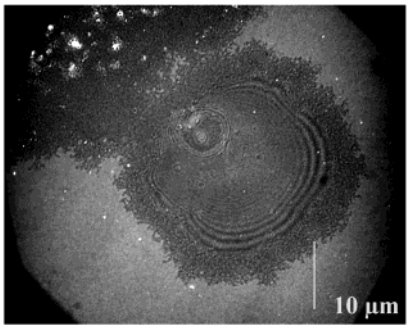

Figure 5. RICM image of giant multilamellar liposomes, partially spread on a chitosan surface at room temperature.

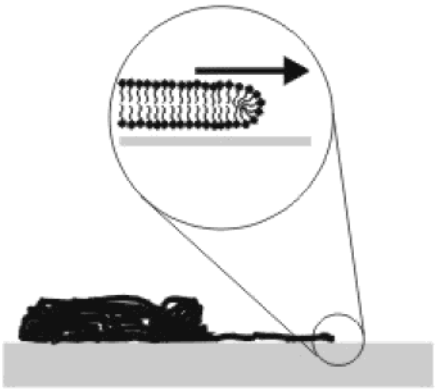

Figure 6. Bilayer spreading from a lipid reservoir, deposited onto a hydrophilic substrate from a solution. The substrate is immersed in water or a buffer solution afterward.

observed. A successful bilayer preparation was possible only at the lower temperature. This was the case even though the lipid layer was still in the fluid state at the lower temperature, due to the additional cholesterol content (at a cholesterol content higher than 30 mol %, no transition to a solid phase is found for DMPC/cholesterol mixtures[47]).

RICM Experiments. *Agarose*. To further analyze the interaction between phospholipid bilayers and agarose, giant multilamellar liposomes were added to the aqueous phase into which an agarose film was immersed. With the fluorescence microscopy results in mind, a strong adhesion of liposomes to the agarose surface could not be expected. Indeed, it was found by reflection interference contrast microscopy that liposomes were hovering over the agarose surface (Figure 4). Fast changes in height and position were observed, due to thermally activated movements of liposomes.[48] Height fluctuations were indicated by changes of the diameters of the interference fringes shown in Figure 4. Accordingly, the thermal energy was high enough to compensate for any attractive force between polymer and bilayer membrane, because an adhesion of giant liposomes to an agarose surface was never observed.

Chitosan. According to the fluorescence microscopy observations described above, a higher interaction of chitosan with liposome surfaces is expected. Giant multilamellar liposomes consisting of DMPC were added to the aqueous phase above a chitosan film on a glass substrate. Indeed, an adsorption of liposomes was found. Liposomes, after the initial contact, flattened fast and finally ruptured. As multilamellar liposomes were used, the remaining lamellae could still be observed in the interferogram. Figure 5 shows an adsorption spot of a giant liposome on a chitosan surface. From Figure 5, it can be deduced that the polymer/liposome interaction is much stronger in the case of chitosan compared to agarose films. This stronger interaction explains the higher stability of planar chitosan-supported bilayer membranes.

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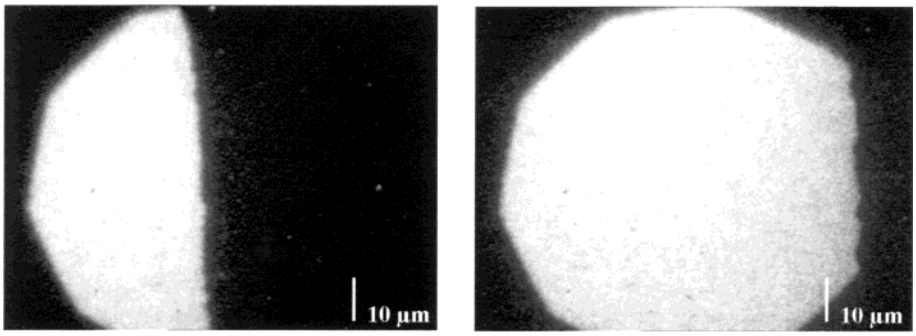

Figure 7. Fluorescence images of a spreading experiment on a chitosan surface. An egg-PC lipid reservoir was deposited onto the polymer film, and the substrate was immersed into MilliQ water. The right picture was taken 34 min after the left one.

Spreading Experiments. To further analyze the nature of the interaction between bilayer lipid membranes and polysaccharide films, spreading from a lipid reservoir on polymer films was analyzed. The effect of single bilayer spreading on planar surfaces was first observed by Rädler et al.[1] In that work, a lipid reservoir was deposited from a solution onto inorganic surfaces (like glass or MgF_2), thoroughly dried, and subsequently hydrated with water or buffer solution. The result was an advancement of single bilayers from the lipid reservoir, as shown schematically in Figure 6. Later it was found that such bilayer spreading is also possible on extremely thin (in the range of 10 nm), soft, and hydrated polymer cushions, such as dextran and cellulose.[15] Both polymers were assumed not to specifically interact with lipid membranes. Therefore, the energy gain by bilayer spreading was attributed to the van der Waals interaction between the membrane and the underlying (glass) substrate. Agarose and chitosan polymer cushions were considerably thicker than the above-mentioned polymer layers. A much-reduced van der Waals contribution is therefore expected.

While the advancement of bilayers on agarose cushions was not observed (zwitterionic lipids were used in all cases), Figure 7 shows that the spreading of a single bilayer from a lipid reservoir on chitosan is possible, which means that bilayers on chitosan possess self-healing properties. During the edge displacement of the bilayer, a roughening of the spreading front was observed (Figure 7).

To examine the kinetics of bilayer spreading, the time dependency of the edge displacement was analyzed. The length of the spreading monolayer was taken as the distance of the monolayer edge from the lipid reservoir (see Figure 6).

The kinetics of bilayer spreading can be described by means of the following equation:[1]

$$v(t) = \sqrt{\frac{W_A}{2\zeta}}\frac{1}{\sqrt{t}} = \sqrt{\frac{\beta}{t}} \qquad (1)$$

where W_A is the energy per unit area gained by spreading, ζ is a viscous drag coefficient, and β is a kinetic spreading coefficient.[1] Hence plotting of $\log(v)$ against $\log(t)$ should yield a straight line with a slope of -0.5.

From Figure 8, it can be deduced that a linear fit with a fixed slope of -0.5 successfully describes the spreading behavior on chitosan. From the interpolation to $\log(t) = 0$, the kinetic spreading coefficient was determined to be $\beta = 1.8 \times 10^{-13}$ $\mu m^2/s$.

Vesicle Fusion Experiments. Vesicle fusion onto hydrophobic or hydrophilic surfaces[49,50] is a common method for preparing solid-supported bilayer membranes. The formation of laterally fluid bilayers by vesicle fusion on a cellulose surface was observed.[51] However, using either agarose or chitosan, vesicle adsorption did not result in laterally connected membranes. When agarose films were subjected to a fluorescent-vesicle dispersion, shaking the substrate in pure water caused the fluorescence of the substrate to cease, implying that adhering vesicles were washed away. On chitosan films, adsorbed vesicles could not be washed away completely, but upon bleaching the fluorophores in a defined area (e.g., by means of intense laser light), no fluorescence recovery, which would indicate lateral connectivity, was observed.

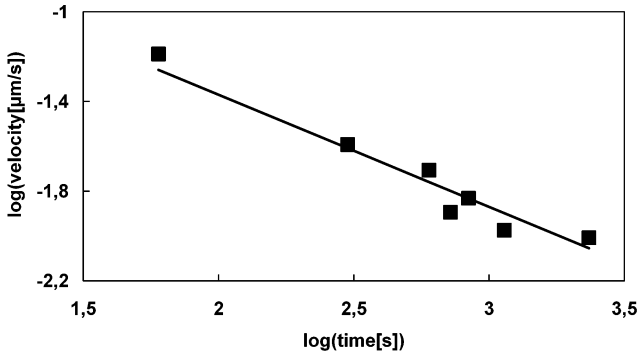

Figure 8. Double logarithmic plot of the spreading velocity against measurement time, for an egg-PC bilayer spreading on chitosan from a lipid reservoir; bold line, one-parameter fit with a fixed slope of -0.5.

Lateral Diffusion on Chitosan. In the case of agarose, no stable homogeneous membranes could be obtained by Langmuir−Blodgett trough methods. Chitosan-supported membranes, however, showed a sufficient stability for a characterization of membrane fluidity. By means of fluorescence microscopy, it was ensured that no membrane fragmentation occurred during a measurement series.

Figure 9 shows the temperature dependence of lateral diffusion coefficients in lipid bilayers on chitosan and on glass in an Arrhenius plot, as obtained by photobleaching measurements (FRAP). From the comparison, the following conclusions may be drawn. The magnitude of the lateral fluidity was not significantly increased by the presence of a hydrated polymer cushion, compared to a glass support. Furthermore, while the diffusion coefficients on chitosan- and glass-supported membranes were generally similar in magnitude, differences were found in the temperature region of the main phase transition of an

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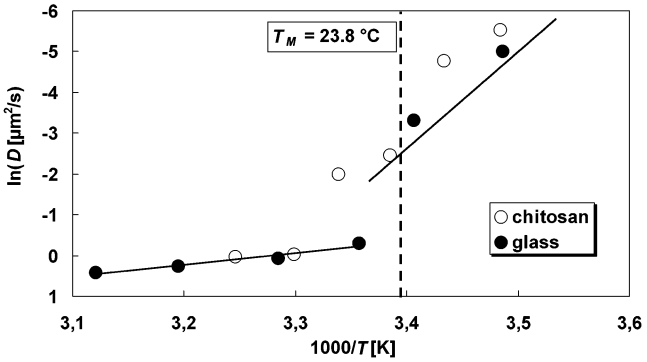

Figure 9. Comparison of the temperature-dependent lateral diffusion behavior of DMPC bilayer membranes labeled with NBD-PE, supported by glass and chitosan. In the case of glass, the phase transition as seen by lateral diffusion occurs around the phase transition temperature found in nonperturbed DMPC bilayers (line). However, in the case of chitosan, the phase transition occurs at a higher temperature. Each data point represents the average of three to five single measurements.

unperturbed DMPC membrane. The main phase transition of unperturbed DMPC bilayers occurs at 23.8 °C.[52] In the glass-supported bilayer, this first-order transition is indicated by a jump of the diffusion coefficients in an interval of 20.4−24 °C (Figure 9). This is in accordance with another report,[53] where this phase transition was observed around 20 °C (by FRAP experiments). A transition temperature close to the value for unperturbed bilayers was reported in ref 54 again for a glass-supported DMPC membrane.

However, in the case of chitosan, the jump of D occurred in the interval of 26.4−30.0 °C, which indicates that the presence of chitosan causes an increase of the phase transition temperature.

Discussion

In the following discussion, we first examine the observation of the failure to produce planar supported bilayer membranes on agarose or chitosan films by the method of vesicle fusion of small unilamellar vesicles. We proceed with a detailed examination of the physicochemical parameters which influence membrane/substrate interactions.

Vesicle Fusion Experiments. A common phenomenon found with chitosan and agarose polymer cushions was the fact that small unilamellar vesicles did not fuse on the polymer surfaces to yield a laterally connected, supported bilayer membrane. In the case of agarose, adherent vesicles could easily be washed away by means of flushing with water which did not contain vesicles. This observation is in agreement with the conclusion that lipid membranes are not sufficiently attracted by the substrate in the case of agarose films. In the case of chitosan films, vesicles could not be washed away completely, which again is in agreement with the conclusion that chitosan surfaces interact more strongly than agarose surfaces with lipid molecules bearing choline headgroups.

We found no fluorescence recovery after photobleaching involving a chitosan film that was treated according to the vesicle fusion protocol described in the methods and materials section. This indicates adsorbed vesicles that did not open to form a laterally connected bilayer.

The formation of planar supported lipid membranes by the method of vesicle fusion has been described to proceed by the following sequence: vesicles approach the surface, adsorb to the surface initially, and either rupture or fuse with each other before rupture takes place.

The formation of a homogeneous supported bilayer that covers the whole substrate surface was suggested to be due to a lateral spreading and fusion of a bilayer;[1] on the other hand, it was proposed that a closed bilayer forms by the coalescence of single, substrate-supported bilayer patches which grow by the fusion of vesicles with the high-energy edges of these bilayer patches.[61] Since lateral spreading of lipid bilayer membranes from a lipid reservoir and the adsorption of vesicles on the chitosan surface were observed, we conclude that the adsorbed small unilamellar vesicles did not rupture.

Physicochemical Parameters Influencing the Stability of Supported Bilayer Membranes. In the present work, a considerable difference of the stability of lipid bilayer membranes on agarose and on chitosan polysaccharide cushions was observed, while monolayer lipid membranes were found to be homogeneous and stable in both cases.

To interpret these observations, it is necessary to separately examine the physicochemical parameters which influence membrane/substrate interactions.

Spreading Parameters of Mono- and Bilayer Membranes. Since a chemical fixation is absent, the stability of a substrate-supported, physisorbed membrane is determined by the spreading parameter, S, which is calculated from the interfacial energies, γ, of the surfaces indexed in eq 2.[19]

$$S = \gamma_{\text{polymer,water}} - (\gamma_{\text{polymer,lipid}} + \gamma_{\text{lipid,lipid}} + \gamma_{\text{lipid,water}}) \quad (2)$$

The spreading power, S, has to be positive in the case of a stable membrane. Since $\gamma_{\text{polymer,water}}$ for a highly hydrated polymer cushion is on the same order as $\gamma_{\text{water,water}}$, which is zero, the spreading power is expected to be small or even negative.[19] On the other hand, the stability of monolayers on a hydrated polymer support is much higher compared to that of bilayers, since in that case

$$S = \gamma_{\text{polymer,vapor}} - (\gamma_{\text{polymer,lipid}} + \gamma_{\text{lipid,vapor}}) \quad (3)$$

and $\gamma_{\text{polymer,vapor}}$ is of the same order as $\gamma_{\text{water,vapor}}$. Therefore the spreading power is positive. The deposition of a second monolayer onto a polymer-cushioned monolayer is a critical step, which, according to eq 2, may be followed by membrane decomposition.

By the complementary methods of fluorescence microscopy, reflection interference microscopy, and bilayer spreading, it was shown that membranes on chitosan were considerably more stable than lipid membranes supported by agarose films.

Electrostatic Interactions. The observed higher stability of lipid bilayer membranes on chitosan might be due to the positive zeta potential of the chitosan film. The pK_a value of the glucosamine segments is in the range 6.3−7,[55] which leads to a slight protonation in the case of the polymer immersed in MilliQ water (pH = 5.5). Bilayers with choline headgroups, on the other hand, have a slightly negative electrostatic potential across the membrane/water interface. This potential arises from an overcompensation of the lipid headgroup dipole potential by a water dipole potential, caused by the ordering of water molecules

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near the bilayer surface.[56] Furthermore, there might be an interaction of amino groups on the surface of chitosan films with the phosphate group of the phosphatidylcholine headgroup. An additional electrostatic attraction arises by the use of the negatively charged membrane probe NBD-PE. However, it has been shown that chitosan forms a stable coating layer even on zwitterionic lipid vesicles.[2] This behavior of chitosan is remarkably different from that of uncharged, water-soluble polymers. Poly(ethylene glycol) (PEG) for example does not bind to the surface of phosphatidylcholine membranes, and the exclusion from the membrane surface leads to a dehydration of liposomes, often accompanied by phase separations.[57] While PEG acts as a liposome fusogen, chitosan-coated liposomes (chitosomes) are stable and the polymer coating does not desorb during washing by centrifugation.[2]

Membrane Undulations: The Effect of Cholesterol and the Deposition Temperature. Even in the case of favorable electrostatic interactions, the membrane stability on a support may be considerably reduced by a disjoining pressure due to undulations, that is, thermally driven collective out-of-plane movements of the bilayer membrane,[44,58]

$$P_{\mathrm{und}} = \frac{3}{4}\frac{kT}{K_c d^3} \qquad (4)$$

where P_{und} is the disjoining pressure due to undulations, k is Boltzmann's constant, T is the temperature, K_c is the bending modulus of the bilayer, and d is the membrane/substrate distance. The bending modulus of a bilayer depends on the membrane phase state and is smaller in the case of a membrane in a fluid lamellar phase compared to a gel phase.[64] This could lead to a higher stability of gel-phase membranes supported by a solid substrate, compared to a fluid membrane. As undulations are thermally excited, temperature reduction leads to a stabilization of the supported membrane. This membrane stabilization is crucial, especially during the Langmuir–Schäfer (LS) transfer, as strong flow in the subphase can cause bilayer rupture.

Another way of suppressing bilayer undulations is the addition of cholesterol, which was observed to enhance membrane quality on a bilayer lipid membrane supported by dextran.[19] However, in the case of agarose-supported bilayers containing cholesterol, vesicle budding and fission were observed at the low deposition temperature of 15 °C (Figure 2). This can be explained by the influence of cholesterol on the phase transition temperature of the bilayer. At 15 °C, a DMPC bilayer is in the liquid condensed state, while no transition to the liquid condensed state is found for a cholesterol content above 30 mol %.[47] Another reason for an increased dewetting tendency upon addition of cholesterol is the comparably high roughness of the agarose cushion.[59] While a bilayer with a low bending modulus could embed itself into a rough surface structure, this embedding becomes more difficult for a bilayer with an increased bending modulus (as is the case for cholesterol-containing bilayers). The result would be a decreased contact area and hence a decreased sticking of the membrane to the support.

Kinetic Spreading Coefficient. Bilayer spreading of a single membrane from a hydrated lipid reservoir allows characterizing membrane/support interactions.[15] On agarose cushions, no advancement of a bilayer membrane was observed, while a spreading of a bilayer membrane was found on chitosan cushions (Figure 7). The possibility of bilayer spreading is considered to be important for practical applications of solid-supported bilayer lipid membranes, since it leads to self-healing properties of such systems.[1] The kinetic spreading coefficient of an egg-PC bilayer membrane on chitosan at room temperature was determined to be $\beta = 1.8 \times 10^{-13}$ μm^2/s. This value is about 2 orders of magnitude smaller compared to the spreading of a DMPC bilayer on glass and 1 order of magnitude smaller compared to DMPC bilayers spreading on cellulose or dextran films.[15] The smaller value could be explained by higher friction due to a dehydration of the membrane/substrate gap in the case of chitosan-supported membranes (see below). Additionally, the driving force for spreading might be smaller compared to that for glass supports or ultrathin polymer films, since an energy gain by the van der Waals interaction between membrane and glass substrate is much smaller due to the much-increased bilayer/substrate separation distance (the chitosan films used in the present work had a thickness of 140 nm in the dry state). During the spreading of bilayers on chitosan surfaces, a roughening of the bilayer edge was observed by fluorescence microscopy. Such a roughening was also found in the case of spreading on glass surfaces and was attributed to line pinning, induced by the substrate roughness.[1] This edge roughening is in contrast to monolayer spreading on the same substrates, where no significant line roughening was observed.[60]

The fact that no spreading of bilayers on agarose films was observed can, according to eq 1, be explained by a small energy gain by bilayer spreading W_A, which is in accordance with the results from LB-trough experiments and RICM. However, the impossibility of bilayer spreading on agarose cushions could also be due to a high drag coefficient ζ on agarose films.

Temperature Influence on Membrane Fluidity. FRAP experiments revealed an increased main phase transition temperature of a DMPC membrane supported by chitosan, as compared to data for free or glass-supported DMPC bilayer membranes (Figure 9). A similar effect can be observed in the case of DMPC bilayers supported by polyacrylamide films.[19,62] However, differential scanning calorimetry measurements of a mixture of multilamellar vesicles composed of DPPC and chitosan, immersed in a phosphate-buffered saline (PBS) buffer, provided no evidence for an increased main phase transition temperature due to DPPC/chitosan interactions.[63] DPPC, however, has a higher main phase transition temperature than DMPC (40 °C[63] compared to 23.8 °C). The phospholipid/chitosan interaction is likely to be temperature dependent and might also depend on ionic strength. Therefore, the findings described above[63] cannot be compared to our results.

An increase of the main phase transition temperature of the DMPC bilayer could be due to a slight dehydration of the membrane surface, relative to an unperturbed bilayer. This increase would then be a result of closer

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packing of lipid headgroups in the proximal leaflet, caused by headgroup dehydration. Such dehydration could be due to the attractive chitosan/lipid interaction mentioned above.

Conclusion

Lipid bilayer membranes consisting of zwitterionic lipids and deposited onto agarose cushions by Langmuir–Blodgett methods were shown to be unstable. The bilayer instability was indicated by (a) membrane decomposition upon storage as observed by fluorescence microscopy, (b) missing adhesion of giant multilamellar liposomes to the agarose surface as measured by RICM, (c) the impossibility of bilayer spreading from a lipid reservoir on the agarose surface, and (d) the fact that small unilamellar vesicles could be completely washed away from the agarose surface by gently shaking in pure water. In light of these observations, the stabilization of black lipid membranes by supporting them using an agarose cushion can only be regarded as a hydrodynamic one, in terms of the suppression of mechanical distortions.

Bilayer lipid membranes cushioned by thin chitosan films were shown to possess an increased stability compared to agarose-cushioned membranes. This higher stability was shown by (a) the observation of homogeneous bilayer membranes by fluorescence microscopy, (b) a strong attraction of zwitterionic, giant unilamellar liposomes leading to vesicle rupture, and (c) the fact that single bilayer sliding on chitosan surfaces from a lipid reservoir deposited onto the polymer cushion was observed.

Chitosan-supported lipid membranes provide easy to prepare model membrane systems. They allow for study of the bilayer itself and for the incorporation and examination of membrane proteins.[59] Fundamental biological processes may be mimicked with the help of these supported model membrane systems. The advantages of the chitosan cushions described in the present work are simple preparation and controllable film thickness, which provides, for example, access to polymer-cushioned membranes for biological laboratories, where facilities allowing for extensive chemical synthesis are missing. By the presence of a soft and hydrated polymer cushion, it is probable that integral membrane proteins may be incorporated into these model membrane systems in a functionally active form.

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