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ARTICLE

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Probing the stability of S-layer-supported planar lipid membranes

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Abstract Isolated protein subunits of the crystalline bacterial cell surface layer (S-layer) of *Bacillus coagulans* E38-66 have been recrystallized on one side of planar black lipid membranes (BLMs) and their influence on the electrical properties, rupture kinetics and mechanical stability of the BLM was investigated. The effect on the boundary potential, the capacitance or the conductance of the membrane was negligible whereas the mechanical properties were considerably changed. The mechanical stability was characterized by applying voltage pulses or ramps to induce irreversible rupture. The amplitude of the voltage pulse leading to rupture allows conclusions on the ability of membranes to resist external forces. Surprisingly, these amplitudes were significantly lower for composite S-layer/lipid membranes compared to undecorated BLMs. In contrast, the delay time between the voltage pulse and the appearance of the initial defect was found to be drastically longer for the S-layer-supported lipid bilayer. Furthermore, the kinetics of the rupture process was recorded. Undecorated membranes show a fast linear increase of the pore conductance in time, indicating an inertia-limited defect growth. The attachment of an S-layer causes a slow exponential increase in the conductance during rupture, indicating a viscosity-determined widening of the pore. In addition, the mechanical properties on a longer time scale were investigated by applying a hydrostatic pressure across the BLMs. This causes the BLM to bulge, as monitored by an increase in capacitance. Compared to undecorated BLMs, a significantly higher pressure gradient has to be applied on the S-layer face of the composite BLMs to observe any change in capacitance.

Key words Crystalline bacterial surface layer · S-layer · Lipid membrane · Irreversible breakdown

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Introduction

Crystalline bacterial cell surface layers (S-layers) represent the outermost cell envelope component in organisms of almost every taxonomic group of walled bacteria and archaea [for a compilation see Sleytr et al. (1996, 1999) and references therein]. They are in most cases composed of a single type of protein or glycoprotein. Depending on the organism, their molecular weights range from 40 kDa to 200 kDa and they can exhibit either oblique, square or hexagonal lattice symmetry (Sleytr and Messner 1989; Beveridge 1994; Sleytr and Beveridge 1999). The S-layers in prokaryotic organisms have shown a broad spectrum of functions, including molecular sieves (Sára et al. 1992; Sleytr et al. 1996). The S-layer from *Bacillus coagulans* E38-66, used in the present study, represents a highly specialized supramolecular structure. The characteristic features can be summarized as follows: (1) composition of identical, non-glycosylated protein subunits with a molecular weight of 100 kDa; (2) oblique lattice symmetry with lattice parameters of $a = 9.4$ nm, $b = 7.4$ nm and base angle $\gamma = 80$; (3) a thickness of about 5 nm; (4) anisotropic topographical surface properties; (5) very precise molecular sieving properties due to 3.5 nm diameter pores; (6) a charge neutral, more hydrophobic characteristic of the outer surface and a net negatively charged inner surface (Sára et al. 1992; Pum et al. 1993).

Isolated S-layer (glyco)protein subunits are endowed with the ability to assemble into monomolecular arrays at many different interfaces (Pum and Sleytr 1996, 1999). Previous studies revealed that S-layer subunits from *B. coagulans* E38-66 recrystallize into large-scale coherent arrays on various planar lipid films generated either by the Langmuir technique (Pum et al. 1993; Schuster et al. 1998a; Wetzer et al. 1998) or on folded phospholipid bilayers (Schuster et al. 1998b).

Electrophysiological methods applied to planar lipid bilayers are appropriate for studying structure-function relationships of membrane-associated proteins (Hanke and Schlue 1993). Free-standing planar lipid membranes provide a good model matrix for ionophores and membrane proteins without the many complicating factors of biological cell envelope structures. However, a support which retains their functionality is useful (Zviman and Tien 1991; Sackmann 1996; Schuster et al. 1997) owing to the poor mechanical stability of these black lipid membranes (BLMs). One approach might be the application of recrystallized S-layers as supporting structures (Pum et al. 1993; Pum and Sleytr 1996).

Previous studies showed that ionophores retain their functionality when they are incorporated in S-layer-supported lipid membranes. For example, valinomycin, a small ion-carrier, could pass through the pores of the S-layer lattice and was incorporated into the adjacent lipid membrane (Schuster et al. 1998a). The authors of another study demonstrated that staphylococcal α-hemolysin forms lytic pores when added to the lipid face of an S-layer-supported lipid bilayer (Schuster et al. 1998b). Owing to the intrinsic sieving properties of the S-layer lattice, no assembly was detected upon adding α-hemolysin monomers to the S-layer-faced side of the composite membrane. The two papers mentioned above clearly showed that S-layer-supported lipid membranes with incorporated valinomycin or α-hemolysin disrupted spontaneously after much longer periods of time compared to undecorated lipid membranes. The present paper reports on a more detailed insight into rupture behaviour and stability of S-layer-supported lipid membranes without the presence of ionophores or pore-forming proteins.

A recent study using a charge-pulse set-up reported that adsorption of a high-molecular polyelectrolyte to negatively charged lipid membranes resulted in a decrease of the breakdown voltage, whereas the same polyelectrolyte with a lower molecular weight shows no significant reduction (Diederich et al. 1998). Moreover, adsorption of high-molecular polymers resulted in a visible decrease of the rupture velocity. Furthermore, it was shown that for viscous films the charge-pulse technique allows determination of the two-dimensional viscosity of the membrane (Wilhelm et al. 1993).

In the present work the isolated S-layer protein of *B. coagulans* E38-66 has been recrystallized on artificial lipid membranes. Such composite S-layer/lipid membranes (Fig. 1) mimic the cell envelope of those archaea which possess S-layers as the exclusive cell wall component external to the plasma membrane (König 1988; Sleytr et al. 1996; Sleytr and Beveridge 1999). The aim of this work is to characterize the influence of the large-scale, coherent S-layer, recrystallized at the lipid/water interface, on the electrical properties, the mechanical stability and the rupture kinetics of the BLMs. Folded and painted BLMs were used in the present study and showed corresponding results. Knowledge of the intrinsic parameters which determine and characterize the stability and physical properties of S-layer-supported lipid membranes are important for further potential applications of these composite membranes.

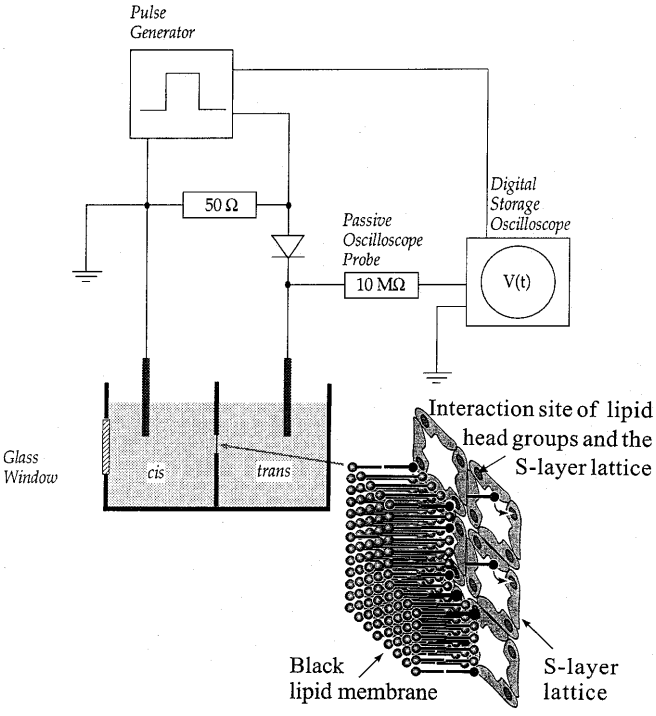

Fig. 1 Scheme of the charge-pulse instrumentation used to induce and record irreversible breakdown voltages of black lipid membranes. *Insert*: schematic illustration of the composite S-layer/lipid bilayer structure

Materials and methods

Painted BLMs were made from a 1% (wt/wt) solution of a mixture (molar ratio 10:4) of 1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine (DPhPC; Avanti Polar Lipids, Alabaster, Ala., USA) and hexadecylamine (HDA; Fluka, Buchs, Switzerland) in *n*-decane (Fluka) (Mueller et al. 1963). A custom-made polytetrafluoroethylene (Teflon) cuvette was separated into two compartments (*cis* and *trans*) by a septum with an orifice area of ~1 mm^2. The orifice was pre-painted with the same lipid mixture but dissolved in chloroform and dried for about 20 min. The compartments were each filled with 5 ml of standard buffer (2 mM $CaCl_2$, 10 mM KCl, adjusted with citric acid to pH 4.0). The front side of the *cis*

compartment contained a glass window, allowing optical observation of the membrane and its annulus. The thinning of the membranes was observed through a microscope (60-fold magnification).

Folded bilayers were formed from a 1% DPhPC/HDA solution (molar ratio 10:4) in chloroform (Montal and Mueller 1972). The septum was made of a 25-μm-thick Teflon sheet (Goodfellow, Cambridge, UK) with orifices of 130 μm in diameter. The apertures were pretreated with a small drop of hexadecane dissolved in pentane 1:10 (v/v) (both Fluka, Buchs, Switzerland). The lipid mixture (5 μl) was spread on the aqueous surface of each half-cell. One of the two half-cells (*cis*-cell) was grounded; the other one (*trans*-cell) was connected by an Ag/AgCl electrode to a voltage clamp set-up (EPC 9, HEKA Elektronik, Lambrecht/Pfalz, Germany). Raising the level of the standard buffer within the two half-cells to above the aperture (final volume was 2.5 ml of standard buffer each) led to the formation of a folded DPhPC/HDA bilayer which was checked by measuring the membrane conductance and capacitance. All experiments were performed at room temperature (22 ± 2 °C).

Growth of *B. coagulans* E38-66 in continuous culture, cell wall preparation and extraction of S-layer protein with 5 M guanidine hydrochloride (GHCl) were performed as previously described (Sleytr et al. 1986). GHCl extracts were dialysed following this procedure and the self-assembly products were sedimented for 20 min at 40 000*g* at 10 °C. The clear supernatant containing the disassembled S-layer subunits or oligomeric precursors (1.8 mg of protein per ml) was used for all recrystallization experiments.

After forming the lipid bilayer the supernatant of the S-layer solution was carefully injected into one compartment of the cuvette to a final concentration of 0.3 mg protein per ml. The recrystallization process of the S-layer subunits on the membranes was allowed to last for about 3 h for all experiments. According to our experience the recrystallization process can be considered as completed within this time interval (Schuster et al. 1998b). In control experiments, S-layer protein was recrystallized on a lipid monolayer of the same composition as the bilayer lipid membranes. Subsequently the composite structure was transferred on carbon-coated electron microscope grids (Pum et al. 1993). On the negatively stained preparations, large coherent S-layer areas were observed by transmission electron microscopy (Philips CM12, Eindhoven, The Netherlands) as previously described (Pum et al. 1993). In further control experiments the S-layer lattice on lipid bilayer was also investigated on carbon-coated holey grids by transmission electron microscopy as described elsewhere (Schuster et al. 1998b). All experiments were performed at room temperature (22 ± 2 °C).

The current response from given voltage functions was measured to provide the electrical parameters of undecorated and S-layer-supported folded bilayers. The data handling was performed on a Power Macintosh 7600/120 personal computer by the Pulse + PulseFit 8.11 software (HEKA Elektronik). Statistical analysis was performed using the Microcal ORIGIN program. The settings of the two built-in Bessel filters of the EPC 9 amplifier for the current-monitor signal were 10 kHz and 1.5 kHz, respectively. A triangular voltage function (from +40 mV to −40 mV, 20 ms) was used to determine the capacitance of the folded membrane. The rupture voltage was determined by a voltage ramp with a slope of 10 mV/s (Fig. 2). This voltage ramp had a duration of 50 s and thus, the continuously raised voltage reached finally a value of 500 mV.

To determine the capacitance of the painted BLM, the membrane was charged within 10 μs to a voltage below the amplitude needed to induce defect formation. From the RC time of the exponential voltage decay via the parallel 10 MΩ resistance of the passive oscilloscope probe, the capacitance of the membrane can be determined (Fig. 1).

The change in surface and dipole potential of the BLM was monitored by a inner-field-compensation (IFC) apparatus similar to the one described by Sokolov and Kuz'min (1980). A sinusoidal voltage with an amplitude of 45 mV and an angular frequency of 1062 Hz provided by a lock-in amplifier (SR830 DSP, Stanford Research Systems, Stanford, Calif., USA) was applied to the BLM and caused small oscillating compressions of the membrane. The compressions modulated the capacitance of the BLM and lead to harmonics higher than the fundamental frequency in the capacitive current. The amplitude of the second harmonic component was detected, which is proportional to the difference in the boundary potential of both sides of the membrane. With a feedback circuit, asymmetric changes of the potential were followed (Bähr et al. 1998).

The voltage $U(t)$ between both sides of the membrane was measured by a digital storage oscilloscope (LeCroy

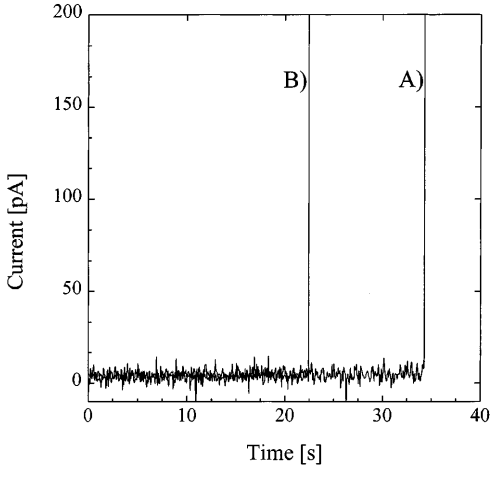

Fig. 2 Representative time course of the current during the application of a voltage ramp. The voltage was continuously increased with a slope of 10 mV/s. The voltage ramp was applied to an undecorated DPhPC/HDA membrane (*A*); and to a lipid bilayer membrane with an attached S-layer lattice (*B*)

9354A) using Ag/AgCl electrodes and a passive oscilloscope probe with a resistance of 10 MΩ. Using a fast pulse generator (DS345, 30 MHz Synthesized Function Generator, Stanford Research Systems), the membrane was charged with voltage pulses of 10 μs duration and increasing amplitude until rupture occurred. Typical experiments started with a comparatively small voltage of 200 mV and the amplitude was raised in steps of about 20 mV, applying several pulses per amplitude. The average of the voltages causing rupture of the BLM is called the breakdown voltage. The delay time is defined as the interval between the end of the applied 10 μs pulse and the irreversible formation of a pore, indicated by a change from an exponential to a superexponential voltage decay across the membrane (Diederich et al. 1998).

If the membrane resistance upon defect formation becomes smaller than the external 10 MΩ resistance, the discharge occurs mainly across the defect. For small defects the capacitance C of the membrane is approximately constant and the membrane conductance $G(t)$ can be expressed by

$$G(t) = -C\,\mathrm{d}/\mathrm{d}t \ln\, U(t) \quad (1)$$

If the radius $a(t)$ of the widening membrane defect is large compared to the thickness of the membrane, $G(t)$ can be approximated by the inverse access resistance:

$$G(t) = 2\kappa a(t) \quad (2)$$

where κ is the specific conductivity of the electrolyte in the cuvette. Equations (1) and (2) give a relation between the macroscopic observable transmembrane voltage and the microscopic pore radius. If the viscosity of the lipid film η can be neglected, $a(t)$ increases linearly in time and a constant velocity can be determined (Wilhelm et al. 1993). This quantity is called the rupture velocity. Adsorption of proteins or polymers can lead to a viscosity-determined rupture of the membrane. In this case, the two-dimensional membrane viscosity can be calculated by the following expression for the time-dependent pore radius (Wilhelm et al. 1993):

$$a(t) \sim \exp(\sigma t/4\eta) \quad (3)$$

where σ is the surface tension of the membrane/water interface per unit area.

Hydrostatic pressure differences were applied to the painted lipid bilayer by asymmetrically increasing or decreasing the volume of the aqueous solution in the Teflon cuvettes. Upon deformation the membranes change their area and possibly also their thickness, which can be monitored by a change in the capacitance. In addition, optical observations clearly showed bulging of the BLM towards the microscope when a pressure was applied from the *trans* compartment. The change in capacitance was detected by a change in the amplitude of the capacitive current across the membrane, owing to the sinusoidal voltage applied for the IFC experiments.

Results

Influence of the S-layer recrystallization on the electrical and optical properties of membranes

Previously it was shown that S-layer recrystallization depends on the charges of the lipid headgroups (Wetzer et al. 1998). To quantify this in a first set of measurements the influence of the S-layer lattice on the electrical properties of the membrane was investigated. S-layer subunits were added to one compartment of the cuvette and the change in the boundary potential of the lipid membrane during recrystallization was recorded with the IFC technique. Surprisingly, no significant changes in the potential could be observed. In addition, the capacitance of the membranes remained almost at their initial values during the crystallization of the S-layer protein on folded as well as on painted BLMs. The specific capacitance of the folded lipid bilayer was found to be 0.84 ± 0.05 μF/cm^2 before and 0.83 ± 0.05 μF/cm^2 after the recrystallization of the S-layer lattice, respectively. For undecorated painted lipid bilayers the measured capacitance was 0.81 ± 0.04 nF before and 0.77 ± 0.03 nF after the protein recrystallization. As the bilayer area of the painted membrane could only be roughly estimated by eye via the microscope, no specific capacitances are given. In addition, the painted BLM showed no changes in colour during the recrystallization of the S-layer.

Effect of recrystallized S-layer proteins on the stability of planar lipid membranes

In a different series of measurements, folded lipid membranes of DPhPC/HDA were made and voltage ramps were applied to induce irreversible rupture (see Materials and methods). Figure 2 shows representative experimental data for an undecorated (A) and an S-layer-supported lipid bilayer (B). The conductance of both membranes showed no significant change up to the moment when the spontaneous rupture of the membrane occured (Fig. 2). This voltage causing the spontaneous rupture of the membrane was found to be 367 ± 42 mV (number of experiments n = 5) for the undecorated BLM and 231 ± 70 mV (n = 5) for the S-layer-supported membrane. No correlation between the breakdown voltage and the capacitance of the membrane was observed (data not shown). This corresponds to previous results for the breakdown voltage of polylysine-decorated BLMs (Diederich et al. 1998).

In the following set of measurements a transmembrane voltage across painted BLMs was applied by short charge pulses. Figure 3 shows a representative time course of the transmembrane voltage during electric field-induced irreversible rupture of an undecorated (curve A) and an S-layer-supported lipid membrane (curve B). In good agreement with previous studies

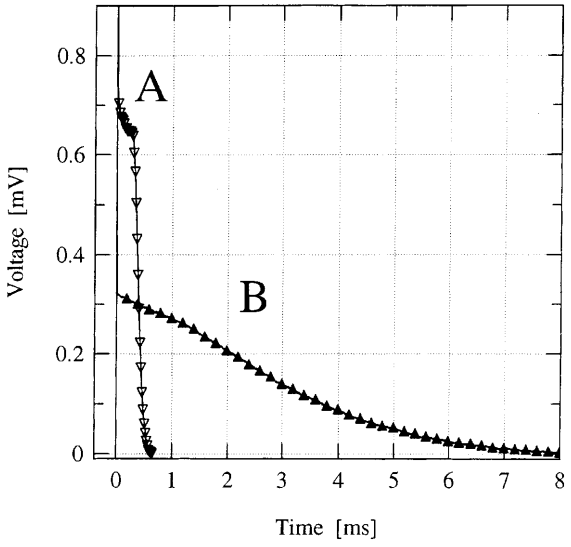

Fig. 3 Representative time course of the membrane voltage during electric field-induced irreversible rupture of an undecorated DPhPC/HDA membrane (*A*); and with an attached S-layer lattice (*B*)

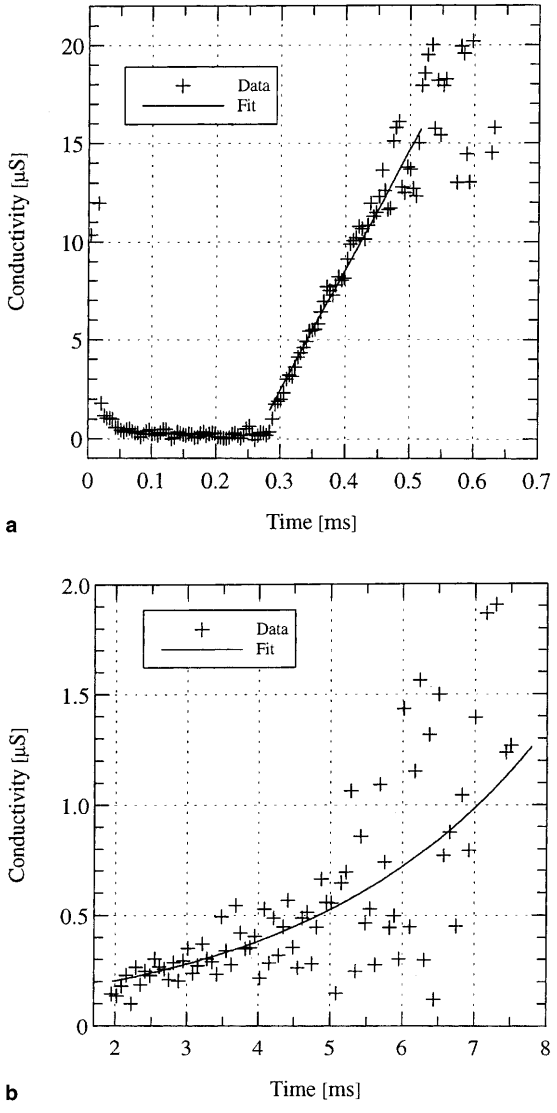

Fig. 4a, b Representative time course of the conductivity calculated from the two voltage versus time curves of Fig. 3. **a** corresponds to Fig. 3A, **b** to Fig. 3B. The *symbols* indicate the experimental data; the *solid lines* are the fitted curves

(Lindemann et al. 1997) the breakdown voltage for undecorated BLMs was found to be 625 ± 15 mV (n = 14). After recrystallization of an S-layer on one side of the BLM the breakdown voltage was considerably reduced to 310 ± 30 mV (n = 9).

The application of charge pulses allows also detection of the time course of the membrane rupture. During the process of rupture, the conductance increased linearly for undecorated BLMs (Fig. 4A) and allowed with Eqs. (1) and (2) the calculation of the rupture velocity to 19 ± 1 cm/s (n = 11). In contrast, the conductance increased exponentially in time during an irreversible breakdown after recrystallization of an S-layer on the lipid membrane (Fig. 4B). With Eqs. (1) and (3) the two-dimensional viscosity of the S-layer/lipid membrane was estimated to be $1.9 \pm 0.5 \times 10^{-6}$ N s/m (n = 10).

Another independent quantity which can be determined in electroporation experiments is the delay time. It is defined as the time after the end of the applied pulse and the point at which the voltage shows a deviation from the relaxation determined by the parallel resistance and the capacitance of the membrane. The delay time was scattered, as reported recently for other composite systems (Diederich et al. 1998). Sometimes, the delay time was difficult to estimate as there was no sharp bend in the rupture curve. In most experiments the delay time of undecorated BLMs was shorter than 400 μs. In the presence of the recrystallized S-layer protein, all measured delay times were higher than 800 μs.

Deformation of the BLM by hydrostatic pressure gradients

The application of a hydrostatic pressure gradient causes the lipid membrane to bulge to the side of lower pressure. The increase in membrane area is monitored by an increase in capacitance. This requires a flux of lipid from the rim into the bilayer membrane and expansion of the area. Undecorated BLMs expanded rapidly upon application of hydrostatic pressure. For a difference in the buffer level between both compartments of about 1.7 mm (corresponding to the addition of 650 μl buffer to one side) the capacitance reached about five times the initial value for symmetrical conditions within 5–10 min. Restoring the same pressure on both sides reduced the capacitance reversibly to the initial value within seconds. Asymmetrically decorated membranes showed different behaviour, depending on the side of pressure application. Increasing the buffer volume on the side without S-layer, it took about the same amount of added buffer to start a continuous deformation of the composite BLM as for undecorated membranes. However, in the begin-

ning, the capacitance signal increased one order in magnitude slower in time compared to the undecorated BLM, but later on the capacitance increased to four times its initial value. This indicates that the initially coherent S-layer disintegrated into patches. Interestingly, the increase was reversible also for the decorated lipid membrane. After balancing the pressure, the capacitance returned within seconds to the initial value. The increase in capacitance again was slower than for an undecorated BLM upon further application of the same pressure gradient. In contrast, applying the pressure from the S-layer face of the composite BLM, significantly higher amounts of added buffer (more than 800 µl corresponding to a buffer level difference of about 2.1 mm) were needed to see any change in the capacitance and the membrane disrupted before larger changes in area could occur.

Discussion

In the present study the S-layer protein of *B. coagulans* E38-66 was recrystallized on planar phospholipid bilayers. No significant differences in the recrystallization of the S-layer protein were found between painted and folded membranes. The absence of a significant increase in the conductance is in accordance with previous studies suggesting that recrystallization of the S-layer protein does not impair the integrity of the lipid membrane (Schuster et al. 1998a, b). In addition, X-ray measurements show only minute rearrangements in the hydrophobic part of the monolayer (Weygand et al. 1999), which indicates that at least no major S-layer protein domains penetrate into the hydrophobic part of the lipid film. This does not exclude a possible orientational effect on the lipid headgroups during the protein crystallization. The authors of a previous study dealing with lipid monolayers showed a significant increase in surface pressure upon S-layer protein recrystallization (Diederich et al. 1996). This observation is probably caused by intensive interactions between the S-layer protein and the lipid headgroups (Weygand et al. 1999).

In the present study the boundary potential, which represents the sum of surface and dipole potential of the BLM, was measured by the IFC technique. These experiments revealed no significantly altered boundary potential when the S-layer was attached to the BLM. Thus, within the experimental resolution, no significant electrostatic interactions between the S-layer lattice and the lipid membrane were observed. However, it cannot be excluded that some protein domains on the S-layer lattice interact electrostatically with some lipid headgroups. An indication for such interactions is that the S-layer protein E38-66 recrystallizes in the presence of Ca^{2+} ions on a zwitterionic DPhPC bilayer much slower (12–16 h) compared to a DPhPC/HDA bilayer containing positively charged surfactants (about 3 h). Further on, the recrystallization is poor on lipids with negatively charged headgroups (Wetzer et al. 1998). Note that in a study with actin, which also binds preferentially to positively charged BLMs again, no significant change in the transmembrane potential could be detected by the IFC technique (unpublished results). One speculative explanation for the absence of a change in the potential might be that the charges on the protein are too far away from the membrane surface to be detected with the IFC technique. Another explanation could be a lipid-condensing effect which would increase the surface potential and possibly also involve a change in the orientation of the lipid dipoles which could change the dipole potential of the membrane. However, this would be in disagreement with studies on lipid monolayers finding only minute rearrangements in the hydrophobic part of the monolayers (Weygand et al. 1999) and with our preliminary studies on lipid monolayers at the air/water interface which show no significant change of the surface potential upon S-layer recrystallization (unpublished results).

The rupture of membranes can be induced by many techniques, e.g. via application of osmotic stress, mechanically applied pressure with micropipettes, with electric field pulses (Needham and Hochmuth 1989) or via a pressure gradient. Rupture is inhibited by an energy barrier necessary for the formation of the initial defect (Winterhalter 1996). The probability for defect formation depends via a Boltzmann factor exponentially on this energy barrier. The application of a transmembrane electric field causes so-called Maxwell forces which have to be balanced by a mechanical stress and depend quadratically on the transmembrane voltage (Winterhalter 1996). Recently the equivalence between an electric field-induced stress and a mechanically applied one was shown (Needham and Hochmuth 1989). Both can reduce the energy barrier necessary for defect formation and destabilize the membrane. The breakdown voltage under voltage clamp conditions for folded BLMs was reduced from 367 ± 42 mV for the undecorated membrane to 231 ± 70 mV for the S-layer-supported membrane. The charge-pulse experiments on painted BLMs showed even a larger reduction of the breakdown voltage by the attached S-layer, from 625 ± 15 mV to 310 ± 30 mV, respectively. The smaller breakdown voltage for the folded BLMs is most likely due to the voltage clamp condition, in contrast to the charge-pulse technique for the painted BLMs. However, the results of both systems show that the recrystallized S-layer facilitates electroporation of the BLMs.

The surprising destabilizing effect of the S-protein recrystallization on the membrane might be the result of additional lateral stresses at the edges of the S-layer domains. This explanation is supported by studies indicating that the recrystallization of an S-layer results in a slight increase of the segmental chain order of the lipid molecules (Diederich et al. 1996; Weygand et al. 1999). According to the "semifluid membrane" model (Pum and Sleytr 1994, 1996), defined domains of the associ-

ated S-layer lattice may interact with lipid headgroups of the adjacent lipid layer. The partial penetration of the protein into the headgroup region might cause a dehydration of the lipid headgroups. In turn, especially for a lipid monolayer at low surface pressure, this may force the lipid chains into a state of higher molecular order (Diederich et al. 1996). Thus, one can speculate that the decrease in breakdown voltage is due to a change in membrane tension as a consequence of the asymmetric S-layer protein recrystallization.

In electroporation experiments the time scale of the electrocompressive stress is rather short and thus the S-layer lattice is not able to follow the change in the membrane deformation. In contrast, the application of a hydrostatic pressure gradient across the membranes gives complementary information on the flexibility of the BLM on a longer time scale. The bulging of the BLM requires flux of lipid from the lipid reservoir in the torus of the membrane to equilibrate the additional stress. This flux is likely considerably hindered after recrystallization of the S-layer and might be a reason for the slower bulging of the composite membrane. However, after removing the pressure difference, the bulge vanishes within several seconds both for the undecorated and the S-layer-supported membrane.

The *cis*/*trans* asymmetry in the response to hydrostatic pressure is easily conceivable. The S-layer itself is asymmetric as the lipid-faced side is more corrugated (Pum et al. 1993), but most important the lipid membrane is covered only on one side with the S-layer lattice. Moreover, the S-layer protein presumably not only recrystallizes over the membrane but also on the Teflon septum in the surrounding of the hole, which is lipid-coated from the pre-painting. Thus, pressing the lipid membrane from the protein-faced side forces the crystalline S-layer through the small aperture suspending the membrane. At lower pressure gradients the lipid membrane might get shielded as the S-layer patches encounter one another like paving-stones and, thus, the underlying BLM cannot be expanded. Higher pressure gradients might bring about sharp bends and tear and thus the composite membrane disrupted before large changes in area occurred. Applying a pressure from the lipid face might result in bulging of the S-layer together with the BLM away from the Teflon septum. At the beginning, the attached S-layer may hinder the bulging of the BLM as the increase in capacitance is one magnitude lower than for undecorated BLMs. However, later on, the coherent S-layer may disintegrate into patches and subsequently drift away in the course of bulging of the BLM. It was not only possible to press the BLM against the S-layer, but also to reversibly distort the composite membrane to a large extent without breaking it.

Several experimental observations suggest that irreversible breakdown is a result of the widening of a single pore (Winterhalter 1996). The delay time was found to be shorter for the undecorated BLM ($<$400 μs) compared to the S-layer-supported lipid membrane ($\geq$800 μs). The delay times in the millisecond range for S-layer-supported BLMs imply a reduced ability of the lipid molecules to rearrange in a way which allows the pore to widen. This suggests an increased local stability. If we assume that the initial defect occurs preferably in uncovered membrane areas between S-layer patches and no longer on the whole membrane surface, the available area for defect formation is considerably reduced by the S-layer. As the probability for defect formation within a certain amount of time is proportional to the available area, this could explain the much longer delay times for the composite membranes.

Many parameters affect the kinetics of the irreversible breakdown. The two-dimensional membrane viscosity or an increase of the mass of the membrane are reflected in the opening velocity of the induced pore (Winterhalter 1996). The pore opening velocity of the painted undecorated BLM was found to be 19 $\pm$ 1 cm/s and is in good agreement with data known from literature (Lindemann et al. 1997; Diederich et al. 1998). Adsorption of polymers or polyelectrolytes like polylysine resulted in a significant decrease of the pore opening velocity, depending on the length or molar fraction of the polymer (Diederich et al. 1998). The attachment of an S-layer lattice results in a considerable decrease of the apparent pore opening velocity. Moreover, the rupture curves of the S-layer/lipid membranes indicate a viscosity-dominated pore widening in contrast to the inertia-dominated widening for the undecorated BLM. This allowed the determination of the two-dimensional viscosity of the composite S-layer/lipid membrane, which was found to be $1.9 \pm 0.5 \times 10^{-6}$ N s/m. Thus, S-layer-supported BLMs are significantly more viscous compared to BLMs with absorbed proteins like actin ($0.5–1.5 \times 10^{-6}$ N s/m) (Lindemann et al. 1997) or polyelectrolytes like high molecular weight poly-L-lysine ($0.1–0.5 \times 10^{-6}$ N s/m) (Diederich et al. 1998). In addition, a recent study with dextran-supported BLM with varying hydrophobic anchor-density showed that the number of direct contacts with the membrane also influences the viscosity (Diederich et al. 1999). For a dodecyl anchor-density of 3% and 6%, the viscosity was $0.01–0.05 \times 10^{-6}$ N s/m and $0.05–0.8 \times 10^{-6}$ N s/m, respectively, which is less viscous compared to the S-layer/lipid membrane. However, there is no evidence for hydrophobic anchors on the S-layer lattice but most probably protein domains of the S-layer which interact with lipid headgroups of the membrane (Weygand et al. 1999). The slow opening velocity and thus the calculated high viscosity might reflect a high number of contact sites (e.g. repetative domains of the associated S-layer lattice) per unit membrane, as also observed with polymers (Diederich et al. 1999). Another explanation might be a reduced mobility of the lipid molecules induced by the attached S-layer lattice. However, authors of a recent study reported that the lateral diffusion of fluorescence lipid probes were higher for S-layer-supported than for silane- or dextran-supported bilayers (Györvary et al. 1999), but caution must always be exercised in comparing different methods and types of lipid bilayers.

The ability to withstand large electric fields was reduced for the composite BLMs. On the other hand, the reduced pore opening velocity and the enhanced resistance against hydrostatic pressure gradients (from the S-layer face) indicate also a stabilizing effect. Thus, for distinct applications, S-layer-supported lipid membranes might open new strategies for improving the mechanical stability of lipid films on a local scale as required for solid-supported lipid membranes (Pum and Sleytr 1999).

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