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Lipid-protein surface films generated from membrane vesicles: selfassembly, composition, and film structure

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Abstract. Lipid-protein films at the air-water interface were generated from a variety of native vesicles and from vesicles derived from lipid extracts. A technique is described which is particularly suitable for the generation of films from small amounts of material at high yield and velocity. In all instances, 10 µl vesicle suspensions containing 25 µg protein yield at least 50 cm^2 film area at a constant surface pressure of 12 mN/m within minutes. Upon formation, surface films are separated from vesicles by use of shear forces. Complete separation is demonstrated by electron microscopy and surface pressure-area diagrams. The latter confirms previous conclusions that surface films generated from lipid vesicles are organized as a monolayer. Analysis of lipid-protein surface layers reveals that their lipid to protein ratios match those of the vesicles used, within a factor of two, irrespective of whether films are generated at high or low surface pressure. Surface denaturation of membrane proteins is shown to be effectively prevented when the film is generated and held at high surface pressure (≥ 15 mN/m). Upon surface pressure jumps from high to low values, denaturation kinetics revealed activation areas of 1.5 (± 0.2) nm^2.

Key words: Lipid-protein monolayer – Vesicle spreading – Surface pressure–area relation – Protein denaturation – Membrane adhesion – Membrane fusion

Introduction

In contrast to the extensive characterisation of lipid monolayers at the air-water interface there is no comparable systematic knowledge about lipid-protein surface films. Well characterised macroscopic layers containing intact proteins would be of considerable value for many assays in membrane research and for developments in biotechnology. For this purpose, a technique is required that allows surface film formation without protein denaturation or loss of function. This poses major problems when using conventional spreading from organic solvents or insertion of detergent solubilized membrane proteins into lipid monolayers. With the demonstration that surface films form spontaneously at the air-water interface of vesicle suspensions (Verger and Pattus 1976; Pattus et al. 1978a, b; Schindler 1979) an appropriate strategy became available to form lipid-protein surface films from any kind of vesicles, including native membrane vesicles. This strategy has, so far, been exploited for membrane reconstitution where two such vesicle derived monolayers are combined to planar membranes, either within a small circular hole in a thin teflon sheet (Schindler 1980) or at the tip of a glass micropipette (Schürholz and Schindler 1983). Reconstitution of several different integral membrane proteins (ion channel proteins) revealed that the viability of these proteins is conserved during selfassembly of surface films from lipid-protein or native membrane vesicles (for a review see Schindler 1989). However, there remain major questions to be answered on the way to a thorough understanding of the mechanism of vesicle spreading and resulting lipid-protein content and organisation. Such knowledge appears as a prerequisite for the evaluation and further exploitation of the possible uses of lipid-protein surface films.

In this respect, only a single lipid system (di-oleoyl-phosphatidyl-choline) has been analysed so far (Schindler 1979). The lipid films which formed at the surface of vesicle suspensions showed surface pressure-area isotherms identical to those of solvent spread monolayers, which has recently been confirmed for the same lipid by ellipsometry (Salesse et al. 1987). Different stages during monolayer self assembly from vesicles up to a final lipid exchange equilibrium (at surface pressure π_e) between the vesicle membrane and the monolayer have been demonstrated and described by a kinetic model (Schindler 1979). The predicted vesicle size dependence of π_e was later confirmed experimentally (Schindler 1980) and is supported by spectroscopic studies on lipid vesicles (Sun et al. 1986). Here we describe the first steps of extending the analysis

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to the self-assembly of multicomponent surface films formed from native membrane vesicles, from proteoliposomes, and from multicomponent lipid vesicles. We start with a phenomenological description of vesicle derived surface films, addressing the following questions of practical interest: how does film formation compare for different kinds of vesicles in terms of appropriately defined velocity and yield of the film formation; how do surface pressure-area isotherms appear for lipid-protein surface films (requiring separation of surface films from vesicles); under what conditions is protein conformation best maintained (requiring an assay for protein denaturation); how does the lipid-protein ratio in a surface film relate to the ratio in the sample used? In a second study (Schindler and Schürholz, submitted for publication) this more phenomenological description will be complimented by a mechanistic study of vesicle spreading by analysing observed film formation velocities in terms of energy barriers, i.e. electrostatic-, surface pressure or hydration-barriers.

Materials and methods

Soya bean lipid (SBL) was obtained from Sigma (Type III-S) and was partially purified (Kagawa and Racker 1971). Radioactive chemicals: ^{14}C-glycerol, ^{3}H-casamino acid, 1,2-di[1-^{14}C]oleoyl phosphatidylcholine (^{14}C-DOPC) were purchased from Amersham and octyl β-D-glycopyranoside from Calbiochem. Standard buffer: 10 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonate (HEPES) at pH 7.4 and 100 m*M* NaCl.

Preparation of lipid vesicles

a) "Reverse Phase Evaporation (REV)". (Skoza and Papahadjopoulus 1978). This technique was used, slightly modified: 20 mg lipid was dissolved in 3 ml diethylether in a 50 ml round bottom flask. After addition of 1 ml of standard buffer the two phases were emulsified by bath sonication for 5 min. The ether was removed by rotary evaporation at 200 Torr until the gel broke, followed by application of 15 Torr for 2 min. After dilution with buffer to 2 (or 5) mg/ml the vesicle suspension was held at 50 Torr for 30 min.

b) Detergent-dialysis: lipid and β-octyl-glucoside (1 and 2% w/v, respectively) were dissolved in standard buffer and bath sonicated for 3 min. The detergent was dialysed for one day in a commercial dialyser (Lipoprep, Diachema AG, Rüschlikon, Switzerland; Zumbuehl and Weder 1981) followed by conventional dialysis against a 200 fold volume of buffer for one day with three changes.

Preparation of radio-labelled membranes

For the quantitation of protein to lipid ratios in vesicles and in vesicle spread monolayers, membranes were prepared with ^{3}H-labelled protein and ^{14}C-labelled lipid: a) *Escherichia coli B*^{e} cultures were grown at 37 °C in M9 medium (Studier 1969) without casamino acids by inoculation up to an absorbance of 0.08 at 550 nm. Then, 25 μCi ^{14}C-glycerol and 50 μCi ^{3}H-casamino acid were added to the cultures. The cells were harvested at an absorbance of 0.44 at 550 nm (5×10^8 cells/ml). From these cells, membranes were prepared (Osborn et al. 1972). Membrane fragments were separated on a linear sucrose gradient (30–50% w/w). b) For the preparation of proteoliposomes containing ^{3}H-labelled porin (matrix-protein) trimers and ^{14}C-labelled lipid, cells were grown as described above but without ^{14}C-glycerol. After breaking the cells with a french press, matrix-protein trimers (MX_3) were isolated (Schindler and Rosenbusch 1981) and lipopolysaccharides removed by gel filtration on a sephadex G 150 column. Homogeneity of MX_3 was judged by SDS-polyacrylamide gel electrophoresis and subsequent autoradiography. Incorporation of MX_3 into lipid vesicles was done by detergent dialysis (see above) in the presence of ^{14}C-di-oleoyl phosphatidyl-choline.

Monolayer formation at constant surface pressure

A small aliquot of vesicle suspension (10 μl of 2.5 mg protein/ml for all native membrane preparations used and of 2.0 mg lipid/ml for all lipid vesicle samples used) was distributed at the surface of a glass slide (2×3 cm^2 area). Wetting was facilitated by sandblasting or grinding the surface with diamond paste. It was cleaned using Na-dichromate-sulforic acid and bidistilled water. The glass slide was inserted at one end of a teflon trough (see Fig. 1 a). Trough dimensions were 40 cm (length) and 12 cm (width) with 0.5 cm buffer height. Initially, the surface barrier was positioned close to the glass slide. Surface film formation from membrane material after insertion of the glass slide was monitored by the area increase (barrier shift) at a pre-chosen constant value of the surface pressure measured by the Wilhelmy plate method (Gaines 1966) using an electronic feedback circuit between barrier position and surface pressure. This yields two experimental observables for comparison of spreading from different membrane samples: an initial slopes $(dA/dt)_0$ and a final area A_f (see Fig. 1 b). They are used to define a velocity v and a yield η of film formation. Values of v will be given in units of cm^2/min. Yield η will be given as A_f divided by total protein weight (25 μg) in units of cm^2/μg protein when native membrane samples are compared. For comparison of lipid vesicles samples, η can be directly expressed in % of maximal spreading, i.e. $\eta = 100\ A_f/A_{max}$, where A_{max} is determined from solvent-spread monolayers (area obtained for 20 μg lipid spread from lipid/hexane solutions to monolayers at corresponding surface pressure values). A_f values were taken at times, when the area increase had dropped to below 0.2 cm^2/min (the difference in area compared to 10 times longer times of spreading never exceeded 5%).

The spreading technique described has been developed especially to generate surface films from small amount of vesicles and to compare velocity and yield values for different vesicle preparations.

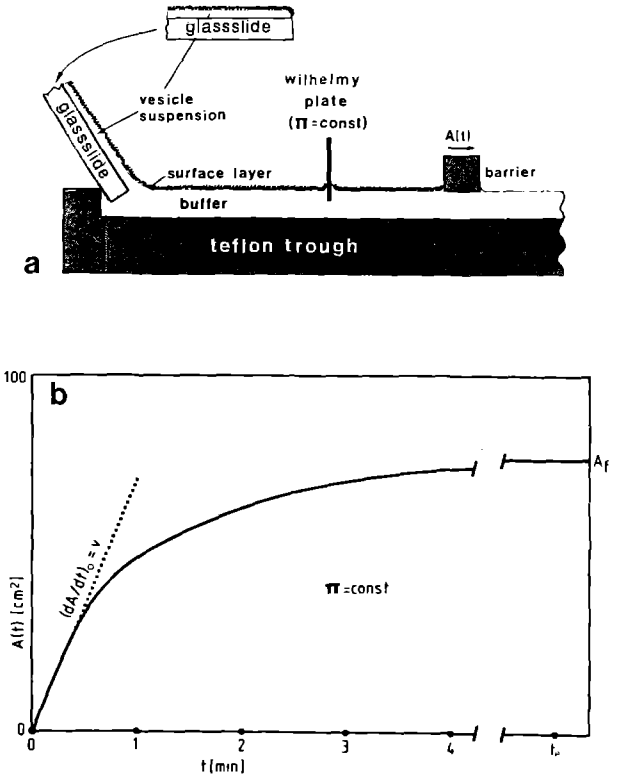

Fig. 1. **a** Schematic illustration of the technique used to form surface films from vesicles. **b** Time course of surface layer formation at constant surface pressure. Film formation is characterised by two parameters: the initial rate (velocity v) and the final area (A_f). In this example a SBL-REV sample was used at 2 mg/ml. The buffer contained 10 m*M* Hepes (pH 7.4) and 30 m*M* NaCl. Surface pressure was held constant at 2 mN/m

Table 1. Velocity (v) and yield (η) of surface layer formation from native membrane vesicles at a surface pressure of 12 mN/m. Concentrated membrane preparations (buffers are given in the references) were diluted about 10 times in standard buffer to 2.5 mg protein/ml. Aliquots of 10 µl were spread from the slide (see Fig. 1). The trough contained standard buffer. The given values represent means with maximum deviation for at least three independent experiments

Origin of native membrane vesicles	v (12) [cm²/min]	η (12) [cm²/µg prot.]	Reference
Electric organ of torpedo marmorata	30± 3	2.0±0.2	Sobel et al. (1977)
Sarcoplasmic reticulum (SR)	39± 8	2.3±1.2	Meissner et al. (1973)
Sub-mitochondrial particles (SMP)	110± 8	3.2±0.2	Deters et al. (1976)
Mitochondria from yeast	230±35	3.3±0.3	Deters et al. (1976)
Outer membrane *E. coli* – BZB	51±11	1.0±0.2	Osborn et al. (1972)
Erythrocyte ghosts	32± 4	1.0±0.1	Dawson et al. (1960)
Electric eel electroplaque	190±30	4.0±0.5	Grünhagen (1981)

Analysis of surface film composition

Analysis of film composition was carried out on surface films which had been separated from vesicles (see Results and discussion). At constant surface pressure conditions, defined monolayer areas (typically 100 cm²) were collected by aspiration through a short glass capillary into a glass vial. Quantitation of protein to lipid ratios was performed by double-counting radioactivity of ^{3}H-labelled protein and ^{14}C-labelled lipid both of the collected surface film and of the vesicles used to form the film. This yielded values for the ratio $\varrho(\pi)=(\text{prot/lip})_{\text{monolayer}}/(\text{prot/lip})_{\text{vesicles}}$, used to quantitate possible changes of the protein to lipid ratio during monolayer formation. Only for basophile cell membranes was amino acid analysis employed to quantitate protein content in collected surface films (Mazurek et al. 1984). The protein content was determined for different surface pressure values (π) during film formation and were normalized to densities at 20 mN/m using surface pressure-area relations. This yielded values for the ratio $\varrho(\pi_1)/\varrho(\pi_2)$ expressing possible changes of the protein to lipid ratio with changes of surface pressure values during monolayer formation.

Results and discussion

Velocity and yield of film formation

Spontaneous surface film formation from native membrane preparations appears to be a general phenomenon. Table 1 shows some examples for which velocity v and yield η has been determined. Even against a relatively high surface pressure (12 mN/m) spreading of native membrane vesicles yields sufficient areas for further analyses (30 to 230 cm²/min from 25 µg protein). Similar spreading velocities were found for vesicles made from crude lipid extracts (cf. Table 2). Table 2 shows, in addition, that spreading against a film pressure of only 2 mN/m is considerably faster and more efficient than against 12 mN/m. For spreading of soybean lipid vesicles the complete pressure dependence of v and η is given in Fig. 2. The velocity continuously drops with increasing film pressure; it reaches zero at the equilibrium pressure π_e (Schindler 1979) where the film pressure equals the spreading pressure. The plateau of η indicates maximal yield. It drops to lower values when, with increasing film pressure, the vesicle spreading becomes a slower process than the loss of vesicles by diffusion from the surface into bulk water.

Separation of surface films from vesicles

For further analysis of surface films and for its practical uses (avoidance of protein unfolding, see below) it appeared necessary to separate surface films from vesicles at high surface pressure values.

This is rendered difficult, however, since vesicles tend to form a rather dense partition layer below surface films due to van der Waals forces (analyzed in detail for DOPC

Table 2. Surface layer formation from vesicles made from lipid extracts: velocity (v) and yield (η) at 2 and 12 mN/m surface pressure. Vesicles were prepared by the REV technique (see Materials and methods). 10 µl aliquots of vesicle suspensions (2 mg/ml) in standard buffer were spread from the slide as described. Number of experiments per sample was at least 3; errors represent maximum deviations about mean values

Origin of lipid extract	v (2) [cm²/min]	η (2) [%]	v (12) [cm²/min]	η (12) [%]	Reference
Electric organ of Torpedo marmorata	37± 8	>40	12± 3	21± 4	Popot et al. (1978)
Mitochondria from yeast	230±25	>60	48± 8	25± 4	Kates (1975)
Soybean (SBL)	330±25	>60	52±10	50±10	Kagawa and Racker (1971)
Human erythrocyte ghosts	235±30	>60	32± 6	54± 8	Kates (1975)
Electric eel electroplaque	>500	>60	210±25	63±10	Kates (1975)

Table 3. Lipid/protein composition of vesicle spread surface films

Membrane preparation	$\varrho(\pi) = \frac{\text{(prot./lip.) mon.}}{\text{(prot./lip.) ves.}}$	π[a] [mN/m]	Reference
Escherichia coli	0.9–1.0	5	[b]
Porin in PC vesicles	1.2–1.4	5	[b]
Microsacs from torpedo mermorata and lipid vesicles (1:1, 1:10, 1:100)	1.0–1.2	0–2	Schindler and Quast (1980)
Acetylcholine receptor in PC vesicles	0.7	15	Sobel et al. (1977)
Basophile cell (RBL) membranes			
a) 100 mM NaCl, 3 mM $CaCl_2$	$\varrho(0)/\varrho(15)=1$	0, 15	[b]
b) 500 mM NaCl	$\varrho(0)/\varrho(15)=2$	0, 15	
Erythrocyte membranes	1.0	2	Pattus et al. (1981)

[a] During surface layer formation from vesicles the surface pressure was held constant at the values given
[b] Preparation described in Materials and methods

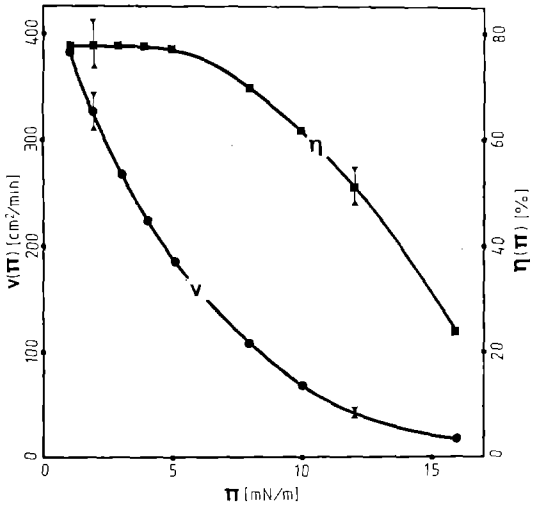

Fig. 2. v and η as a function of surface pressure. For calculation of v the area increases measured at constant π values were normalized to areas at 2 mN/m surface pressure. The % yield η (mg lipid in the monolayer per mg lipid in the vesicle suspension applied) was calculated from the area of monolayer formed. The mass-concentration was taken from the pressure-area curve of a hexane spread monolayer (see also Fig. 4). 10 µl of the vesicle suspension were distributed on the slide as described (Fig. 1). SBL-REV vesicles at 2 mg/ml were used in standard buffer. Dots and squares represent mean values of three experiments; representative error bars for high and low surface pressure indicate maximum deviations

vesicles, Schindler 1979). Application of the same assay to soybean lipid vesicles yielded a saturation value for the vesicle layer density of 0.25 µg/cm² above 0.1 mg/ml vesicle concentration.

Separation is reliably achieved, however, by use of shear forces as illustrated in Fig. 3. By appropriate handling of surface barriers B (Fig. 3a, for details see figure legend) a surface layer is formed between positions P_0 and P_1. Shear force is applied by a glass roller R which turns at frequency v (Fig. 3b). The resulting difference in surface pressure, $\Delta\pi$, is taken as a measure for the shear force and is adjustable by frequence v. The two barriers are then synchroneously moved from P_0, P_1 to P_0', P_1'. During this, vesicles are stripped off from the surface film at sufficiently high shear force. Insertion of a barrier at P_1 leaves a surface film between P_1 and P_1' for further analysis.

Effectiveness of vesicle removal by the glass roller was assayed in two independent ways: by measuring surface-area relations (Fig. 4) and by electron microscopy (Fig. 5). Figure 4a shows a typical observation made on lipid surface films without removal of vesicles (no glass roller, the same assay otherwise). A film, generated at 15 mN/m to an area of 75 cm² (starting point of the π-A recording in Fig. 4a) showed an irreversible increase of film area A when the film was relaxed to zero pressure for the first time. This irreversible increase in area is due to the

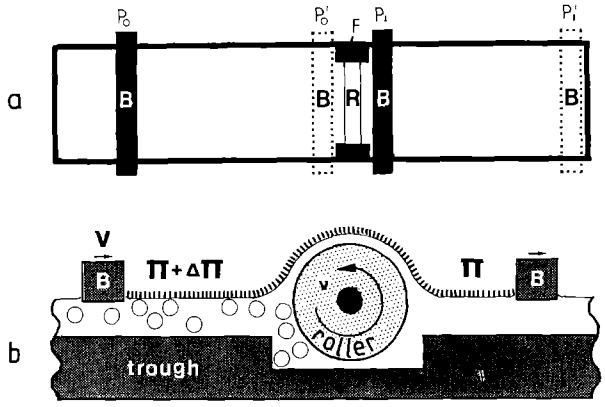

Fig. 3a, b. Separation of surface layer from vesicles by shear force. Vesicles are spread at the left end of the trough (Fig. 1) against one barrier, which moved automatically to hold surface pressure at a pre-chosen value (normally 15–20 mN/m) until position P_0' is reached (**a**). A second barrier is inserted at position P_0 and the glass slide is removed. For transfer over the roller, the surface pressure is transiently raised, a barrier is inserted at P_1 and the barrier at P_0' is removed. The surface layer expands over the roller to barrier at P_1 and the surface pressure is lowered to the pre-chosen value. The left barrier is then moved at a constant velocity (2 to 10 cm/min). The position of the right barrier is controlled by an electronic feedback maintaining the surface pressure to the right of the roller at the pre-chosen value. The shear force, generated at the roller and reflected by $\Delta\pi$ was controlled by two Wilhelmy balances (one on each side of the roller, not shown in **b**. π could be adjusted by the roller's degree of immersion and by the turning frequency v (about 0.1 Hz). For improved removal of vesicles adhered to the monolayer the aqueous compartment containing the roller. (**b**) was held at low ionic strength; volume flow was impeded by barriers at both sides of the roller reaching to just below the surface layer (not included in **b**). Rubber foam between trough and roller prevented flow of vesicles below the roller to the right side. B = barrier, R = roller, F = fitting of roller, π = surface pressure, v = frequency of turning roller, o = vesicles, dimensions: roller length 16 cm, diameter 3 cm; trough was 12 cm × 40 cm and 0.5 cm deep

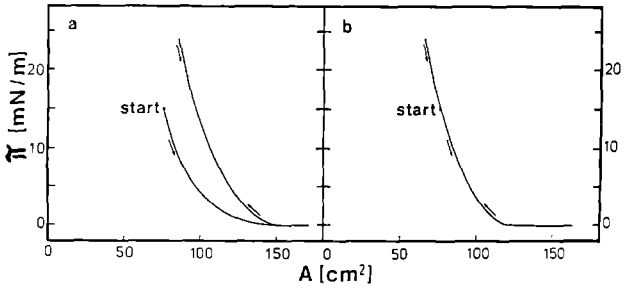

Fig. 4. π-A isotherms of lipid films without (**a**) and with (**b**) separation from vesicles. Surface films were formed from soybean lipid vesicles at 15 mN/m constant surface pressure. **a** When no shear force was applied an irreversible area increase was observed after relaxing the surface pressure to zero. Apparently these are vesicles adhered to the lipid film which spread and enlarge the film area during relaxation. This is taken as a reliable test for adhered vesicles when working with lipid material. **b** The same test after removal of vesicles with the glass roller ($\Delta\pi$ = 5 mN/m, bidistilled water below the roller). There is no detectable area increase. The point at 15 mN/m indicates the constant pressure after monolayer formation and is the starting point of the expansion. Cyclic expansion and contraction yielded the same curve as indicated by arrows. The π-A relation for SBL spread from hexane matched the curve shown to identity and is, therefore, not shown separately

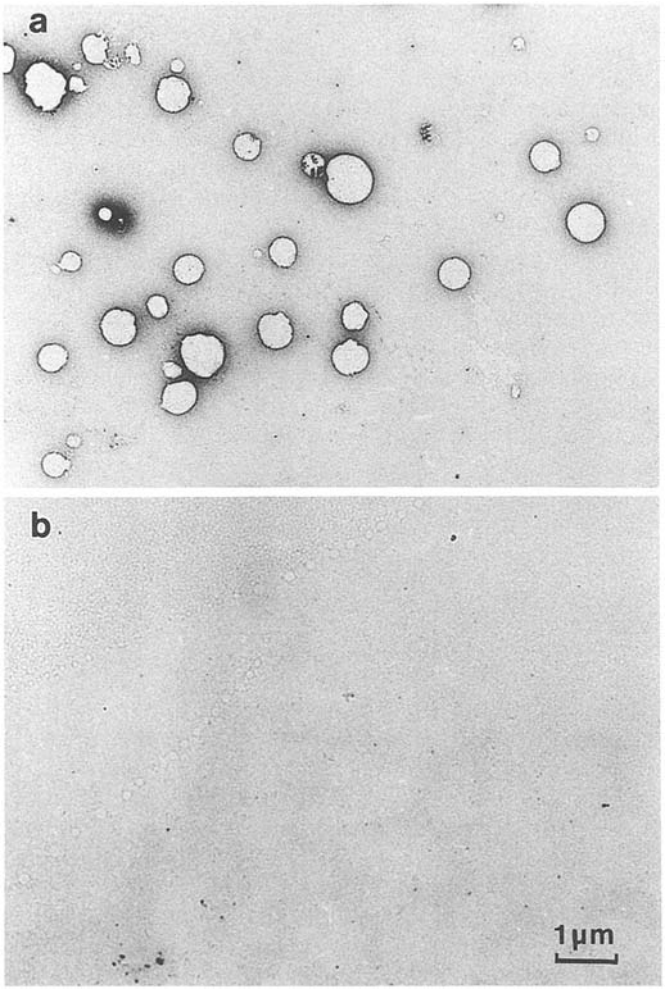

Fig. 5. Electron micrographs of a surface film without (**a**) and with (**b**) separation from vesicles. The surface films, generated from microsacs of Torpedo marmorata, were transferred to electron microscope grids (coated with a carbon film as a support) at 30 mN/m and negatively stained with uranyl acetate (1%). Surface pressure difference due to shear force (**b**) was 5 mN/m. The compartment of the roller contained bidistilled water. **a** No roller was applied at otherwise identical conditions

spreading of residual vesicles from just below the surface facilitated by the reduction of surface pressure to zero (cf. Fig. 2). This spreading reservoir is readily exhausted so that subsequent π-A relations are reversible. No irreversible area increase is observed after shear force application (Fig. 4b) indicating that vesicles had been removed effectively. Electromicrographs of negatively stained monolayers confirmed this conclusion. Without shear separation, vesicles were clearly resolved under all conditions, with typical densities of 10^5 to 10^7 vesicles/mm^2. At appropriate shear force conditions, specified below, not a single vesicle was usually seen per mm^2 in searches over whole grids. This is exemplified in Fig. 5 for surface films spread from postsynaptic vesicles (microsacs) prepared from the electric organ of Torpedo marmorata fish (Sobel et al. 1977). Microsacs, adhered to the surface film (Fig. 5a), were completely removed by shear forces (Fig. 5b).

These two assays, π-A relations and electron microscopy, were employed to find conditions for complete separation irrespective of the vesicle material used, i.e., native membrane vesicles or lipid vesicles. For this, it appeared sufficient to fulfil two conditions: *(1)* the $\Delta\pi$ value, as a measure for the shear force during separation, should be ≥ 5 mN/m and *(2)* the ionic strength of the aqueous phase at the glass roller should not exceed 10 mM of univalent cations (Na^+ or K^+) or (and) 10 μM of divalent cations (Ca^{++} or Mg^{++}). Adjustment of shear force by frequency ν is to a much higher accuracy and stability than required for the purpose of separation. By use of volume stops the aqueous environment of the glass roller can be held in effective separation from the adjacent aqueous phases without impeding the flow of the surface layer. This is of practical importance since spreading velocity v increases with increasing ionic strength (Schindler and Schürholz, submitted for publication) whereas separation is best at low ionic strength.

Lipid-protein composition of surface films

Surface films formed from vesicles and separated from residual vesicles were analyzed with respect to their protein to lipid composition. Table 3 summarizes the data so far obtained including previously published results. In all instances, the surface layer had been removed by aspiration through a glass capillary tube with as little buffer volume as possible (typically 100 cm^2/4 ml) directly into vials used for quantitation, either scintillation counting or amino acid determination (see Materials and methods for details). The protein to lipid ratios in the surface layers roughly corresponded to that of the vesicles used to form the surface layers ($\varrho \approx 1$). When the vesicle suspensions were mixtures of native membrane vesicles (microsacs) and lipid vesicles (soybean lipid) the resulting surface layers reflect the overall vesicle composition (see data for the acetylcholine receptor). Most remarkably, protein to lipid ratios did not significantly depend on the surface pressure during vesicle spreading (cf. data for the acetylcholine receptor, in PC vesicles and in microsac membranes, and the data for basophile cell membranes). Depending on the particular membrane and on salt conditions, protein to lipid ratios in the monolayer were found to be both higher and lower compared with those in vesicles, but these deviations were always within a factor of 2. A detailed analysis of surface layers spread from erythrocytes revealed that all integral components of the membrane were present in the surface layer and with the same stoichiometries as in the erythrocyte membrane (Pattus et al. 1981).

Structural aspects of vesicle derived surface films

Lipid films. When the surface pressure-area relation in Fig. 4b was compared with that of a monolayer spread from hexane (soybean lipid, same buffer) both characteristics matched to identity. Vesicle spreading results, therefore, in the formation of a monolayer by common criteria. This has been shown earlier for DOPC (Schindler 1979; Salesse et al. 1987). The data exclude the possibility that the surface film at the conditions applied is a lipid bilayer. This has previously been proposed only for very particular conditions of generating surface layers (at particular temperature values) and has so far only been demonstrated in the absence of salt (Gershfeld 1986, 1989). Also it appears very unlikely that the surface layer, before shear force separation, contains stable partly fused vesicles (vesicle outer layer fused with monolayer) as has been suggested (Kolomytkin 1987); such structures are not expected to be removed by the relatively weak shear forces which yield complete separation, nor to follow the observed ionic strength dependence of separation. In agreement with earlier findings (Schindler 1979) it is concluded, therefore, that spreading of lipid vesicles results in a lipid monolayer. Monolayer assembly occurs even when spreading is carried out at a relatively high surface pressure (15 mN/m) and monolayers can be separated from vesicles by the use of shear forces.

Lipid-protein films. Properties of surface films which self assemble from native membrane vesicles differ dramatically from those of lipid films. On the one hand, vesicles can be completely removed from lipid-protein surface films as from lipid films (see above; cf. Fig. 5). On the other hand, π-A isotherms of lipid-protein films, generated at a constant surface pressure of 15 mN/m and separated from vesicles, exhibit irreversible increases in area during π-A cycles, in contrast to lipid films (see Fig. 6). π-A cycles above 12 mN/m were reversible, although characterized by a small hysteresis at the same recording speed where lipid films do not show any hysteresis. As soon as surface pressure was relaxed for the first time and to a considerably lower value (5 mN/m in the figure), the film expanded irreversibly. After the surface pressure had been set for at least one minute to zero, the final π-A relation corresponded to an area increase by about 80%. Surface films derived from the different types of native membrane vesicles as listed in Table 1 all showed irreversible area increase between 50 and 250% of initial areas. The only exception were films generated from *Escherichia coli* outer membrane vesicles which showed

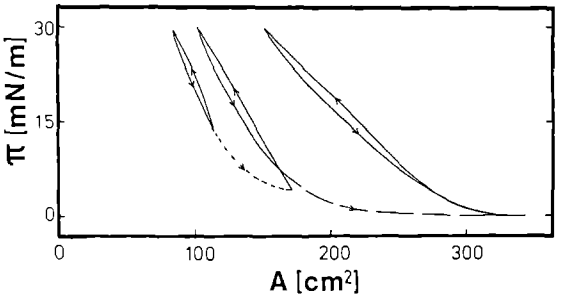

Fig. 6. π-A isotherm of a surface film generated from postsynaptic vesicles. A surface layer was formed at constant surface pressure (12 mN/m) from membrane vesicles of electric organ of Torpedo marmorata (microsacs). Vesicles had been removed as described in Fig. 3 and shown in Fig. 5b. Relaxation of surface pressure below 12 mN/m results in irreversible area increases which are attributed to protein unfolding. For details see text

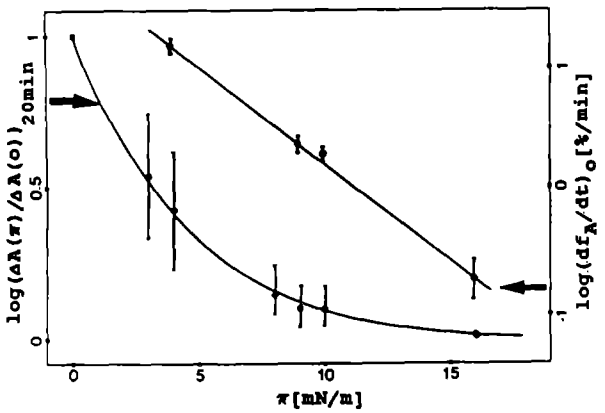

Fig. 7. Rate and extent of protein unfolding as a function of surface pressure. The time courses of irreversible area increases in lipid-protein surface films after surface pressure jumps were taken as a measure for protein unfolding. All jumps were from 20 mN/m to the pressure values indicated. The surface films were obtained from basophile cell membranes (Mazurek et al. 1984). They were generated at a constant surface pressure value of 20 mN/m and separated from residual vesicles. *Upper curve, right scale*: initial fractional area increase $[df_A/dt]_0$ upon pressure jumps. *Lower curve, left scale*: these data represent measured area increases 20 min after each jump. The maximal area increase for jumps to zero pressure, ΔA (o), was 2.4 times the initial area (filled square data point). The error bars include values of three experiments. Further details in the text

no detectable irreversible area increases but a huge hysteresis loop with several reproducible sub-transitions (data not shown). These area increases certainly reflect irreversible unfoldung of membrane proteins (surface-denaturation). For a more quantitative description of denaturation we analyzed surface area increases upon surface pressure jumps from 20 mN/m (surface layers were generated at this pressure value) to lower π-values. For every jump a new surface layer was generated. Figure 7 shows data obtained from basophile cell membranes. The initial rates of area increase $(df_A/dt)_0$, given in percent of initial area per minute, steeply increased with decreasing π from only 0.2%/min at 16 mN/m to 15%/min at 4 mN/m. The data are approximated by an exponential dependence. Assuming the existence of an activated state for protein unfolding, the rate can be related to a "free area" or "activation area" $\Delta A^{\ddagger}$ for unfolding:

$$(df_A/dt)_0 = k_0 \exp\{-\pi \cdot \Delta A^{\ddagger}/kT\}$$

The straight line in Fig. 7 corresponds to an activation area of

$$\Delta A^{\ddagger} = (1.5 \pm 0.2)\ nm^2$$

The same value was indeed found for protein unfolding in films spread from postsynaptic vesicles (cf. Fig. 6), the major protein being the acetylcholine receptor. Similar activation areas have been reported for the unfolding of water soluble proteins at the air-water interface (Mac Ritchie 1978). The value of 1.5 nm^2 may be interpreted as the area of expansion of a structural unit of a membrane protein, such as tilting of a membrane spanning α-helix, which, beyond an area increase of $\Delta A^{\ddagger}$, leads to protein unfolding with "in plane" orientation of α-helices. Denaturation rate at zero surface pressure (k_0) was too high to be measured. The maximal area increase, ΔA_{max}, was 2.4 times the initial area. This value was only approached at surface pressure values close to zero. At 9 mN/m, for instance, the area increase with initial slope of about 2%/min levelled off already after 10 min to a constant value of 10% of ΔA_{max}; at 16 mN/m it levelled off to only 0.5% of ΔA_{max} within 20 min. This aspect has been included in Fig. 7 by the second set of data (left scale). The increase in area was measured 20 min after each surface pressure jump from 20 mN/m to π, which appeared sufficient in that $\Delta A(\pi)$ reached a constant value for all π values tested; i.e. $\Delta A_{max} = (\Delta A\ (o))_{20\ min} = 2.4$. These two findings together, the constant activation area and the π-dependent extent of denaturation, indicate that the fraction of proteins, which are able to unfold, decreases drastically with increasing surface pressure, which may be caused, for instance, by structural stabilization due to pressure induced protein aggregation. Such mechanistic insights of surface denaturation may be obtained from application of the assays described here to surface layers containing only single types of proteins with known secondary structures. The main emphasis here is to demonstrate that protein unfolding, as assayed by surface area increases, can be effectively avoided, when surface layers are generated against a constant surface pressure of $\gtrsim 15$ mN/m. This is in agreement with successful reconstitution of ion channel functions in plane membranes formed by combining two vesicle derived surface films (for a review of technique and results see Schindler 1989).

It appears fair to conclude, that the associate of transmembrane polypeptide chains of a membrane protein is held together when embedded in a dense lipid monolayer, instead of a bilayer. The polar part of the protein, exposed to air, is likely to still have its hydration layer of water bound which reduces tendencies to unfold. Also, it may well be that a boundary shell of lipids is retained by the protein part protruding out of the monolayer or more extending bilayer structures (surface pressure area diagrams of lipid-protein surface films do not discriminate irreversible areas increases at low surface pressure values due to protein unfolding from possible contributions from additional lipid from bilayer structures). Such structural details may be important for particular studies or uses of vesicle spread surface films, but they have no impact on the present level of argument.

The potentials of the described technique which allows one to form surface layers from native membrane vesicles with conserved protein structure may be exploited in many other connections besides in reconstitution studies: in efforts directed to physical chemical characterization of membranes, in biochemistry of surface reactions including pharmacological ligand binding studies, in spectroscopy and electromicroscopy as well as in membrane biotechnology. In these regards it may be of interest, that surface layers, derived from native membrane vesicles, can be easily transferred to supports as single layers, double- or multi-layers which will be presented elsewhere.

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