ELSEVIER

Biochimica et Biophysica Acta 1418 (1999) 106–116

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Stabilizing effect of an S-layer on liposomes towards thermal or mechanical stress

C. Mader, S. Küpcü, M. Sára, U.B. Sleytr *

Zentrum für Ultrastrukturforschung und Ludwig Boltzmann-Institut für Molekulare Nanotechnologie, Universität für Bodenkultur Wien, Gregor-Mendelstr. 33, A-1180 Vienna, Austria

Received 10 December 1998; received in revised form 5 February 1999; accepted 11 February 1999

Abstract

Isolated subunits of the crystalline cell surface layer (S-layer) protein of *Bacillus stearothermophilus* PV72/p2 were recrystallized on positively charged unilamellar liposomes. Liposomes were composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol and hexadecylamine (HDA) in a molar ratio of 10:5:4 and they were prepared by the dehydration–rehydration method followed by an extrusion procedure. The S-layer protein to DPPC ratio was 5.7 nmol/μmol which approximately corresponds to the theoretical value estimated by using the areas occupied by the S-layer lattice and the lipid membrane. Coating of the positively charged liposomes with S-layer protein resulted in inversion of the ζ-potential from +29.1 mV to −27.1 mV. Covalent crosslinking of the recrystallized S-layer protein was achieved with glutaraldehyde. Chemical analysis revealed that almost all amino groups ($>95\%$) from HDA in the liposomal membrane were involved in the reaction. To study the influence of an S-layer lattice on the stability of the liposomes, the hydrophilic marker carboxyfluoresceine (CF) was encapsulated and its release was determined for plain and S-layer-coated liposomes in the course of mechanical and thermal challenges. In comparison to plain liposomes, S-layer-coated liposomes released only half the amount of enclosed CF upon exposure to shear forces or ultrasonication as mechanical stress factors. Furthermore, temperature shifts from 25°C to 55°C and vice versa induced considerably less CF release from S-layer-coated than from plain liposomes. A similar stabilizing effect of the S-layer lattice was observed after glutaraldehyde treatment of plain and S-layer-coated liposomes.

Keywords: Crystalline bacterial surface layer; S-layer; Liposome

1. Introduction

In many bacteria and archaea, two-dimensional crystalline arrays of identical protein or glycoprotein subunits (surface layers; S-layers) represent the outermost cell envelope component [1–3]. S-layers exhibit either oblique (p1, p2), square (p4) or hexagonal (p3, p6) lattice symmetry. Depending on the lattice type, one morphological unit cell is composed of one or two, four, three or six identical subunits. The constituent subunits generally show a molecular mass ranging from 40 000 to 200 000. In most bacteria, the S-layer subunits interact with each other and with the supporting cell envelope layer by non-covalent forces including hydrogen bonds, hydrophobic or charge interactions [1,2,4]. Previous studies have shown that S-layers from many bacillaceae could be detached from the underlying cell envelope layer and

* Corresponding author. Fax: +43-1-478-9112; E-mail: sleytr@edv1.boku.ac.at

0005-2736/99/$ – see front matter
PII: S0005-2736(99)00030-9

disintegrated into their constituent subunits by treatment with high concentrations of hydrogen-bond-breaking agents such as 5 M guanidine hydrochloride. During removal of the disrupting agents (e.g., by dialysis) the isolated S-layer subunits frequently reassemble into the same regularly structured lattices as are observed on whole bacterial cells. Recrystallization of the S-layer subunits into closed lattices may also occur on suitable surfaces such as poly-L-lysine-coated supports, Langmuir (L) lipid films or at the air/water interface [4–7]. Recrystallization and labelling experiments have further shown that S-layer lattices from bacillaceae are highly anisotropic structures with regard to their inner and outer surfaces. Generally, the outer surface is more hydrophobic than the inner one. The net charge of the inner surface is usually negative due to an excess of acidic amino acid residues, whereas the outer surface is charge neutral due to an equimolar amount of acidic and basic groups. High-resolution electron microscopic studies revealed that the inner surface of the S-layer subunits binds to positively charged head groups of L lipid films, indicating that electrostatic interactions are primarily involved [5–8]. The stabilizing effect of S-layer protein lattices on lipid films was recently demonstrated for both lipid bilayers and tetraetherlipid monolayers [9,10]. In a previous study, the recrystallization of the S-layer protein from *Bacillus coagulans* E38-66 on liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol and HDA was described [11].

In the present study, the S-layer protein from *Bacillus stearothermophilus* PV72/p2 was used for recrystallization on liposomes. The S-layer lattice shows oblique symmetry and is composed of subunits with a molecular mass of 97 000. The lattice constants are $a = 9.7$ nm, $b = 7.4$ nm and $\gamma = 80°$. The gene (*sbsB*) encoding this S-layer protein has been cloned and sequenced [12]. According to sequence data, the isoelectric point of the mature S-layer protein is 5.0. To obtain information on a possible stabilizing effect of an S-layer lattice on liposomes, comparative studies with plain and S-layer-coated liposomes were performed with regard to shear forces, energy stress and temperature shifts.

2. Materials and methods

2.1. Preparation of empty and carboxyfluoresceine (CF)-containing liposomes by the dehydration–rehydration method

Positively charged liposomes were prepared according to a modification of the dehydration–rehydration method originally described by Kirby and Gregoriadis [13]. The lipid mixture was composed of 20 μmol dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA), 10 μmol cholesterol (Sigma, St. Louis, MO, USA) and 8 μmol hexadecylamine (Fluka, Buchs, Switzerland). The components were dissolved in 2 ml chloroform and rotary evaporated to a dry film in a 25-ml round-bottomed flask at 60°C under vacuum. The dry lipid film was hydrated by addition of 1.5 ml distilled water and loosened from the glass wall during rotation at 60°C for 1 h. The solvent was removed by lyophilization overnight and preformed liposomes were stored at −20°C.

For preparing carboxyfluoresceine containing liposomes (CFLs), preformed lyophilized liposomes were rehydrated with 250 μl 5 (6)-carboxyfluoresceine (CF; Molecular Probes, Eugene, OR, USA) solution (50 mg/ml distilled water; pH value adjusted to 7.5 with 1 N NaOH) to form CFLs. After treatment in a bath sonicator (Sonorex RK100, Omega Instruments, Vienna, Austria) for 1 min, CFLs were passed 10 times through symmetric polycarbonate membranes with decreasing pore sizes (400, 200 and 100 nm) using a LiposoFast mini extruder (Avestin, Ottawa, Canada). Non-entrapped CF was removed by applying the suspension to a Sephadex G-25 PD-10 column (Pharmacia, Uppsala, Sweden) which was equilibrated in 160 mM KCl solution and presaturated with empty liposomes. Turbid fractions were pooled and analysed for their liposome concentration by enzymatic determination of cholesterol [14]. Reference liposomes, termed empty liposomes (ELs) in the following, were prepared as described above except that 160 mM KCl solution was used instead of the CF solution.

2.2. Bacterial strain, growth conditions and isolation of the S-layer protein

B. stearothermophilus PV72/p2 was grown in continuous culture at 57°C under conditions previously described [15,16]. Cell wall fragments were prepared according to the procedure of Sleytr and Glauert [17] and the S-layer protein was extracted with guanidine hydrochloride solution (GHCl; 5 M in 50 mM Tris–HCl buffer, pH 7.2) at 20°C for 2 h. Subsequently, the suspension was centrifuged at 40 000×*g* for 20 min at 4°C and the supernatant containing the GHCl-extracted S-layer protein was dialysed against distilled water at 22°C for 2 h. To separate S-layer self-assembly products from soluble (monomeric and/or oligomeric) S-layer protein, the suspension was centrifuged at 40 000×*g* for 20 min at 4°C. The S-layer protein content of the clear supernatant was determined by the bicinchoninic acid (BCA, Pierce, Rockford, IL, USA) protein detection assay [18].

2.3. Recrystallization of the S-layer protein on liposomes

For recrystallization of the S-layer protein on CFLs, 6.25 ml of a solution containing 16 nmol/ml monomeric and/or oligomeric S-layer protein was mixed with 2.5 ml liposome suspension containing 4 μmol/ml DPPC and 500 μl of 500 mM phthalate buffer, pH 6.0. This mixture was supplemented with glycerol to achieve a final concentration of 300 mM after dilution to 10 ml. Recrystallization of the S-layer protein was carried out for 2 h at 22°C in a Test Tube Rotator, Type 3025 (GFL, Burgwedel) with a rotation speed of 10 min^{-1}. Excess of non-assembled S-layer protein was removed by centrifugation at 20 000×*g* at 4°C for 20 min.

2.4. Electron microscopy and determination of liposome size

Size distribution of liposomes was determined from micrographs of freeze-fractured preparations following the method of Hallett et al. [19]. For this purpose, aliquots of liposome suspensions were centrifuged at 16 000×*g* at 4°C for 10 min, resuspended in glycerol (25%) as cryoprotectant to achieve a final lipid concentration of 40 mM and equilibrated for 12 h at 4°C prior to freezing in Freon R22. Samples were fractured at −100°C and replicated in a Balzers BAF-400 freeze-etching unit (Balzers, Liechtenstein) using techniques described previously [20]. The size distribution of the liposomes was determined by image measurements of cross-fractured liposomes considering non-equatorial fractures according to Hallett et al. [19].

Ultrathin-sectioning and negative-staining of liposomes with uranyl acetate was performed as previously described [21]. Specimens were examined in a Philips CM 100 transmission electron microscope (TEM) at 80 kV using a 30-μm objective aperture.

2.5. Liposome size analysis and ζ-potential measurement by photon correlation spectroscopy

Plain or S-layer-coated ELs were resuspended in 25 mM phthalate buffer pH 6.0, containing 300 mM glycerol. The mean particle size and electrophoretic mobility of these samples was determined with a Malvern Zetasizer 4 system (Malvern, Malvern, UK) using a 5-mW helium/neon laser at 25°C. The liposome size distribution was characterized using the polydispersity index. This index ranges from 0.0 for entirely monodisperse up to 1.0 for completely polydisperse.

2.6. Glutaraldehyde treatment of S-layer-coated ELs

After recrystallization of the S-layer protein on ELs, the S-layer lattice was crosslinked with glutaraldehyde under different conditions (0.5, 1.5 and 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) at 22°C for 20 min). The reaction was stopped by the addition of Tris (final concentration of 0.5 M in the reaction mixture). After centrifugation of glutaraldehyde-treated ELs at 16 000×*g* at 4°C for 10 min, the pellet was washed with 100 mM phosphate buffer, pH 7.2. Crosslinking of the S-layer lattice was examined by extracting S-layer-coated ELs with sodium dodecyl sulfate (SDS) (5% SDS in distilled water; 5 min, 100°C) and by applying the SDS-extract to SDS–polyacrylamide gel electrophoresis. The extent of the reaction of glutaraldehyde with free amino groups of HDA was estimated by photometric determination of the ethanol extract-

able amount of HDA before and after the crosslinking reaction. For this purpose aliquots of native and glutaraldehyde-treated S-layer-coated EL samples were centrifuged (40 000 × *g*, for 15 min at 4°C) and the pellets were extracted three times with 1 ml of absolute ethanol. Pooled supernatants were dried in a speed vac concentrator SVC-100H (Savant, NY, USA) overnight. Dried samples were dissolved in 1 ml absolute ethanol and the amount of unmodified HDA was determined by the trinitrobenzenesulfonic acid (TNBS) test [22] which was modified to contain 5% Nonidet P-40 (Sigma, St. Louis, MO, USA) in the borate buffer.

2.7. CF release from plain or S-layer-coated CFLs triggered by temperature shifts

The fluorescence of CF released from different liposome preparations in the course of temperature shifts was monitored directly using a Hitachi F-2000 fluorescence spectrophotometer with an excitation and emission monochromator at 470 nm and 514 nm, respectively. The fluorometer sample chamber was heated and cooled by an external thermostat and the sample temperature was followed by a thermocouple. Experiments were performed with plain and S-layer-coated liposomes before and after crosslinking with 0.5% glutaraldehyde as described before. Aliquots of all samples containing 15 nmol DPPC were adjusted to 800 μl with TBS (50 mM Tris and 140 mM sodium chloride, pH 8.0). The cuvettes with the samples were placed in the sample chamber and thermostated to 25°C. Subsequently, the selected temperature profile was applied and the fluorescence as well as the sample temperature were monitored over a period of 90 min. After an isothermal phase of 10 min at 25°C, liposome preparations were heated to 55°C twice within 40 min and subsequently cooled to the initial temperature within 40 min. Finally, CF remaining entrapped in the liposomes was released by addition of 50 μl of Triton X-100 solution (20% in distilled water) to achieve a final concentration of 1.25%.

2.8. CF release from plain or S-layer-coated CFLs triggered by shear forces

Aliquots of plain and S-layer-coated CFLs containing 18 nmol DPPC were diluted with TBS to a volume of 1 ml. The samples were stirred in the cuvette with a paddle mixer with a flat blade at constant stirring speed of 3000 min^{-1} (corresponding to Re 4500) for 20 min at 22°C. Fluorescence increase was determined in stirring time intervals of 1 min during the first 10 min and final measurements were carried out after 15 min and 20 min. CF remaining entrapped in the liposomes was released by adding 50 μl of Triton X-100 solution (20% in distilled water) to achieve a final concentration of 1%.

2.9. CF release from plain or S-layer-coated CFLs triggered by ultrasonication

Samples of plain or S-layer-coated CFLs in TBS containing 18 nmol DPPC/ml were sonicated for 20 min in a bath sonicator Sonorex RK 100 (Bandelin, Berlin, Germany) at 35 kHz and at a constant temperature of 26°C for 20 min. During the first 5 min of sonication, the fluorescence was measured in 30 s intervals, during the following 5 min, in 1-min intervals and during the next 10 min, in 5-min intervals. The remaining CF was released from the liposomes by adding 50 μl of Triton X-100 solution (20% in distilled water) to achieve a final concentration of 1%.

3. Results

3.1. Characterization of plain liposomes

Electron micrographs of negatively-stained and freeze-etched preparations were used to characterize the size, shape and lamellarity of liposomes. To determine the average vesicle diameter, all image radii from electron micrographs of freeze-fractured liposomes were measured, applying the factor $4/\pi$ to the mean image radius considering non-equatorial fractures according to Hallet et al. [19]. The average diameter of liposomes determined by this method was 150 nm with a variance for the size distribution of 390 nm^2 (number of measured liposome images $n = 407$). Negatively-stained and ultrathin-sectioned preparations (not shown) confirmed that the liposomes were spherical in shape and almost exclusively unilamellar. Size measurements by photon correla-

tion spectroscopy using the cumulants method for analysing auto-correlation functions revealed a *z*-average mean size of 180 nm by a polydispersity index of 0.12. The electrophoretic mobility of the plain liposomes was $+2.29\times10^{-8}$ m^2 V^{-1} s^{-1} from which a ζ-potential of +29.1 mV was calculated.

3.2. Recrystallization of the S-layer protein from B. stearothermophilus PV72/p2 on liposomes and characterization of S-layer-coated liposomes

After dialysis of the GHCl-extracted S-layer protein against distilled water at 22°C for 2 h and sedimentation of S-layer self-assembly products, the clear supernatant contained 20–25 nmol S-layer protein/ml. When liposomes were incubated with the clear supernatant containing the soluble S-layer protein, a clear correlation was observed between the extent of recrystallization of the S-layer protein on liposomes, the formation of self-assembly products and the molar ratio between the S-layer protein and DPPC as the major component of the liposomes. As shown by negative-staining, S-layer self-assembly products were formed when the S-layer protein was applied in great excess in comparison to DPPC (e.g., molar ratio S-layer protein/DPPC = 1:50). On the other hand, at a significantly lower molar ratio (e.g., S-layer protein/DPPC = 1:400), liposomes were not completely covered with an S-layer lattice after the same incubation period. A coherent S-layer on liposomes was obtained by incubation of 7.5 nmol S-layer protein with 1 μmol DPPC at 22°C for 2 h (molar ratio S-layer protein/DPPC = 1:133). The S-layer protein content of S-layer-coated liposomes was determined to be 5.7 nmol/μmol DPPC (S-layer protein/DPPC = 1:175) by the BCA protein detection assay. Considering that one morphological unit cell of the S-layer lattice occupies 68.5 nm^2, a total surface area of 0.24 m^2/μmol DPPC was calculated. Since the outer surface area of unilamellar liposomes containing 1 μmol DPPC was roughly estimated to be 0.23 m^2 by assuming molecular areas of 0.55 nm^2, 0.28 nm^2 and 0.22 for phosphatidylcholine, cholesterol and the HDA analogue stearylamine, respectively [23,24], these data were in good agreement with those from electron microscopic investigations that liposomes were completely covered with S-layer protein (Fig. 1). As derived from the roughness of the S-layer lattice in freeze-etching preparations, the S-layer subunits had bound with their smooth outer surface.

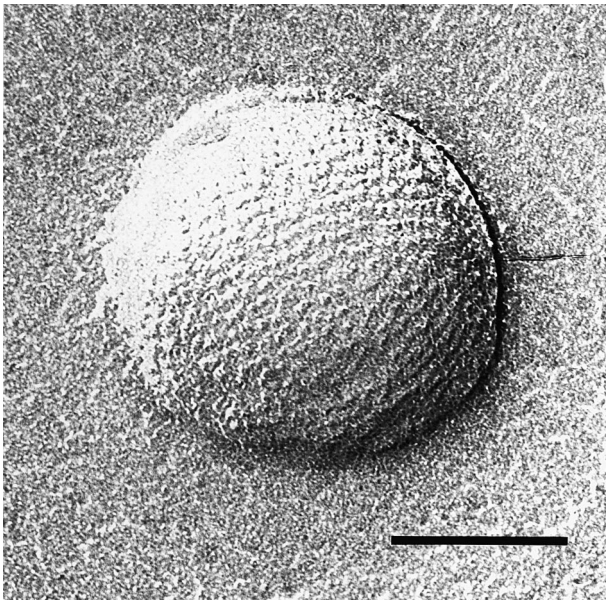

Fig. 1. Freeze-etched preparation of an S-layer-coated liposome which is completely covered with an oblique lattice. Bar = 100 nm.

As previously demonstrated, recrystallization of the S-layer protein was achieved only on liposomes composed of DPPC, cholesterol and HDA (10:5:1). No S-layer lattice was formed on liposomes deficient of either cholesterol or HDA [25].

Comparative particle size measurements of plain and S-layer-coated liposomes by photon correlation spectroscopy indicated a higher *z*-average mean size (320 nm) and an increased polydispersity index (0.22) for S-layer-coated liposomes. Since in freeze-etching preparations the average diameter of S-layer-coated liposomes was estimated to be at 150 nm ($n = 423$), these data differences can only be explained by the formation of small clusters of individual liposomes. The electrophoretic mobility of S-layer-coated liposomes was determined to be -2.13×10^{-8} m^2 V^{-1} s^{-1} resulting in a ζ-potential of −27.1 mV. This indicated that the presence of the S-layer caused an inversion of the liposome surface charge.

3.3. Crosslinking of the S-layer lattice on S-layer-coated liposomes with glutaraldehyde

On SDS-gels the S-layer subunits of *B. stearother-*

mophilus PV72/p2 migrated as a single protein band with an apparent molecular mass of 97 000. Crosslinking of the S-layer lattice on liposomes with glutaraldehyde was confirmed by the absence of a protein band corresponding to S-layer subunits on SDS gels. To investigate the amount of amino groups of HDA involved in the crosslinking reactions, S-layer-coated liposomes were treated with glutaraldehyde concentrations of 0.5%, 1.5% and 2.5%, respectively. Subsequently, the preparations were extracted with ethanol, and free amino groups were determined by the TNBS test. After crosslinking with 0.5% glutaraldehyde, the portion of extractable unmodified HDA was in the range of 5%. Increasing the glutaraldehyde concentration to 1.5% reduced the extractable unmodified HDA to 2% of the total amount. No HDA could be extracted from samples treated with 2.5% glutaraldehyde.

3.4. Comparison of the thermal and mechanical stability of plain and S-layer-coated liposomes determined by CF release

Encapsulation of CF as a hydrophilic marker molecule was performed by applying the dehydration–rehydration method [13]. After down-sizing and removal of unentrapped marker from CFLs by gel permeation chromatography, the concentration in the pooled fractions was assayed to be approx. 4 μmol DPPC/ml. The amount of enclosed marker was determined by fluorescence measurements before and after the total release of entrapped CF from liposomes by addition of Triton X-100 solutions. Because of self-quenching of concentrated CF solutions, the entrapped marker did not significantly contribute to the fluorescence of samples [26]. Following the addition of Triton X-100, CF release led to a strong increase in fluorescence intensity which corresponded to 220 μg CF/ml or to 55 μg CF/μmol DPPC. These measurements enabled the calculation of the encapsulation efficiency of the marker into unilamellar liposomes. Considering the applied amount of CF (12.5 mg) and the lipid yield which decreased to 60% during the liposome preparation procedure, the encapsulation efficiency was in the range of 9%. The thermal as well as mechanical stability of liposome samples was expressed by the CF release caused by temperature shifts, shear forces or ultrasonication. The amount of released CF (calculated in percent of the entrapped amount considering the temperature dependence of CF fluorescence) and the actual sample temperature were plotted against the time of exposure. As shown in Fig. 2, the measurements revealed a direct correlation between released CF and the applied temperature profile. During the first heating cycle native plain liposomes showed a marker release of 14% within the temperature range from 25°C to 55°C, whereas during the same temperature range the CF release of native S-layer-coated liposomes was only approx. 4%. During the second heating cycle, the behaviour of native plain or native S-layer-coated liposomes closely resembled that of the first cycle, with a total release of 24% and 6% CF, respectively. Under the same conditions, an increased CF release was observed for liposomes treated with glutaraldehyde (Fig. 3). However, after treatment with glutaraldehyde, S-layer-coated liposomes showed a lower marker release during the first and second heating cycle (21% and 31%) compared to plain liposomes (34% and 47%). For all preparations, a continuous

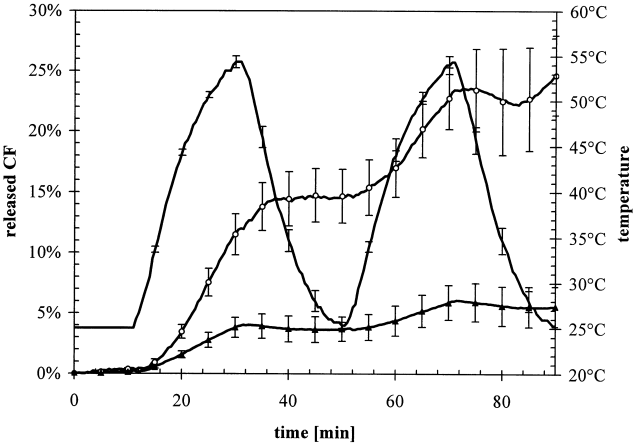

Fig. 2. CF released (given in percent of the entrapped amount) from S-layer-coated liposomes (▲) and plain liposomes (○) was measured as a function of the applied temperature profile consisting of an initial isothermal phase at 25°C (0–10 min), followed by two cycles of heating from 25°C to 55°C (10–30 min; 50–70 min) and cooling to 25°C (30–50 min; 70–90 min). Liposomes were suspended in TBS. Points represent the averages of three independent measurements. Error bars indicate standard deviations of the means. In comparison to S-layer-coated liposomes (4% and 2%, respectively), a significantly higher CF release was observed for plain liposomes during both heating cycles (14% and 10%, respectively).

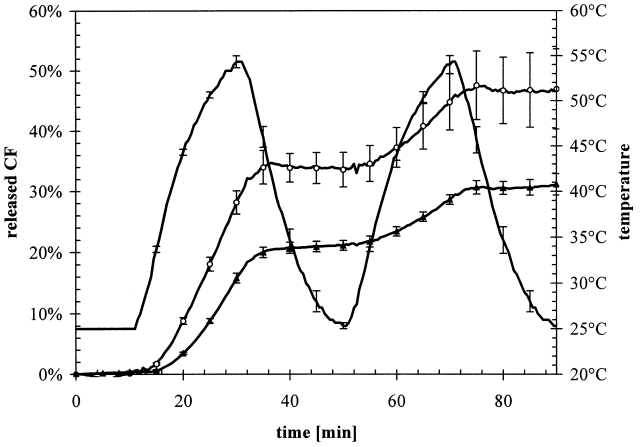

Fig. 3. CF released (given in percent of the entrapped amount) from glutaraldehyde-treated S-layer-coated (▲) and plain liposomes (○) was measured as a function of the applied temperature profile consisting of an initial isothermal phase at 25°C (0–10 min), followed by two cycles of heating from 25°C to 55°C (10–30 min; 50–70 min), and cooling to 25°C (30–50 min; 70–90 min). Liposomes were suspended in TBS. Points represent the averages of three independent measurements. Error bars indicate standard deviations of the means. In comparison to S-layer-coated glutaraldehyde-crosslinked liposomes (21% and 10%, respectively), a significantly higher CF release was observed for crosslinked, plain liposomes (34% and 13%, respectively).

increase in CF release was observed during the heating periods without significant maxima at discrete temperatures. This was in contrast to the behaviour of plain or S-layer-coated liposomes with a lower cholesterol content (7 mol% in comparison to 26 mol% in the standard preparation) in which the permeability displayed a pronounced anomaly in the transition region (data not shown). Furthermore, a lower standard deviation of the mean was found for data obtained with native or crosslinked S-layer-coated liposomes in comparison to data obtained for the corresponding preparations of plain liposomes.

In Fig. 4 and 5, CF released (in percent of the entrapped amount) from plain or S-layer-coated liposomes is plotted against duration of mechanical stress. CF released in the course of stirring resembled typical saturation curves reflecting non-steady-state conditions (Fig. 4). During the whole experiment, CF released from plain liposomes was about 2.9 times higher than that from S-layer-coated liposomes. The plot of CF released upon sonication of plain liposomes (versus time) also reflected a saturation curve, whereas for S-layer-coated liposomes a slightly sigmoidal curve was observed (Fig. 5). After 20 min sonication, S-layer-coated liposomes released approximately 50% less CF in comparison to plain liposomes.

4. Discussion

The possibility to recrystallize S-layer subunits isolated from various bacillaceae on liposomes led to novel types of liposomes [11]. In their basic structural principle S-layer-coated liposomes resemble the cell envelopes of Gram-negative archaea which possess S-layers as the exclusive cell wall component external to the plasma membrane [2]. Since most archaea grow in habitats with extreme environmental conditions with regard to pH, temperature and osmotic pressure, a general stabilizing effect of S-layer lattices on lipid membranes was expected [2]. In this study, the effect of an S-layer coat on liposomes on the release of entrapped CF under thermal and mechanical stress factors was investigated. Therefore, the S-layer protein isolated from *B. stearothermophilus* PV72/p2 was recrystallized on positively charged liposomes. Since spherical, unilamellar liposomes were used in the present study, it was assumed that the

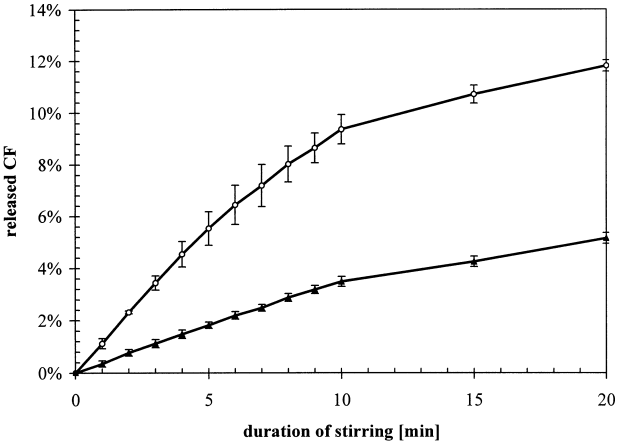

Fig. 4. CF released (given in percent of the entrapped amount) from S-layer-coated (▲) and plain liposomes (○) was measured during stirring of the suspension at 3000 min^{-1} and 22°C. Liposomes were suspended in TBS. Points represent the averages of two independent measurements. Error bars indicate standard deviations of the means.

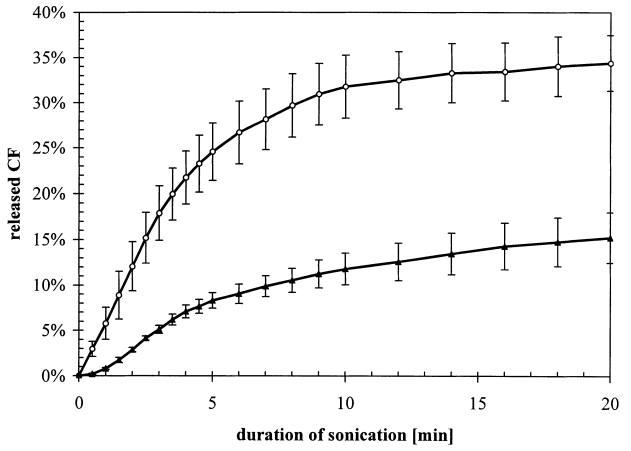

Fig. 5. CF released (given in percent of the entrapped amount) from S-layer-coated (▲) and plain liposomes (○) was measured as a function of sonication time at 26°C. Liposomes were suspended in TBS. Points represent averages of five independent measurements. Error bars indicate standard deviations of the means.

release of CF under different stress factors primarily depended on the presence or absence of the S-layer lattice and was not influenced by the effects of additional lipid membranes.

Measurements of particle size distributions by photon correlation spectroscopy led to larger diameters for S-layer-coated liposomes than those estimated from electron microscopic preparations. On the other hand, the two techniques showed only relatively small differences in the size distributions of plain liposomes. These differences may be explained by the different types of averages (intensity weighted and number average, respectively) and a higher uncertainty of the microscopic estimations using practicable numbers of liposome images in the random samples. The twofold difference in particle size of S-layer-coated liposomes as estimated by photon correlation spectroscopy or electron microscopy can further be explained by the fact that in micrographs of freeze-etched preparations, the mean diameter of individual vesicles is estimated whereas by photon correlation spectroscopy, the average size of particles in suspension is determined including clusters of liposomes. In fact, a certain proportion of aggregated S-layer-coated liposomes was always observed in negatively-stained preparations. Thus, the increased polydispersity index observed for S-layer-coated liposomes may be attributed to this aggregation.

The coating of positively charged liposomes with the S-layer lattice was accompanied by inversion of the vesicle charge. The ζ-potential changed from +29.1 mV before to −27.1 mV after recrystallization of the S-layer protein. This observation was qualitatively confirmed by phase partitioning experiments in polyethylene glycol/dextran two-phase systems with low as well as high electrostatic potential differences ($\Delta\psi$) between the two phases [27]. In the charge-insensitive, low $\Delta\psi$ system, the plain vesicles accumulated mainly at the interface, whereas S-layer-coated liposomes partitioned in the dextran-rich, lower phase. In the charge-sensitive, high $\Delta\psi$ system, plain liposomes were in the relatively negative dextran-rich phase whereas S-layer-coated liposomes were enriched at the interface (data not shown). These results confirmed the data from ζ-potential measurements, showing that surface charge inversion occurs after coating of the positively charged liposomes with an approximately 5 nm thick S-layer [28]. The net negative vesicle charge may arise by a shielding effect of the S-layer on the HDA charges as well as by the higher contribution to the ζ-potential of charges localized at some distance from the interface.

The crystalline protein lattice on liposomes can be exploited for a great variety of surface modifications. As it was shown for both S-layer ultrafiltration membranes and S-layer carrying cell wall fragments [29], the physicochemical surface properties of S-layer lattices can be changed by chemical modification reactions and functional molecules can be covalently attached to S-layer lattices in well-defined position and orientation [29]. In both cases intermolecular crosslinking of the S-layer lattice with glutaraldehyde is required for generating a stable protein matrix. With S-layer-coated liposomes it had to be assumed that also amino groups from membrane incorporated HDA are involved in the crosslinking reaction with glutaraldehyde [11]. Since crosslinking the S-layer lattice on S-layer-coated liposomes with glutaraldehyde under the selected conditions resulted in the modification of nearly all amino groups of HDA, it was concluded that HDA at the inner leaflet of the lipid membrane was also involved in the crosslinking reaction and that glutaraldehyde passed through the liposomal membrane. Nevertheless, the crosslinking reaction caused no release of CF and the vesicles remained intact. The observed high reactivity of ami-

no groups from incorporated HDA combined with the capability of glutaraldehyde to span various distances due to different degrees of polymerization suggests that heterologous crosslinking between S-layer protein and HDA had occurred. Since in the course of heterologous crosslinking, parts of the membrane lipids would be fixed to discrete positions in the S-layer lattice, this modification would significantly modulate the lateral diffusion of free lipid molecules and consequently the fluidity of the whole membrane as predicted for the semifluid membrane model [6].

For studying the influence of the S-layer lattice on the stability properties of liposomes under thermal and mechanical challenges, the release of the encapsulated hydrophilic marker CF was investigated. During temperature shifts from 25°C to 55°C and vice versa, plain liposomes released about four times the amount of CF in comparison to S-layer-coated liposomes, whereas after chemical fixation with glutaraldehyde, the release of plain liposomes was only about 1.5 times higher. Glutaraldehyde-treatment generally induced an increase in marker release. No discrete maxima in CF release during temperature increase were observed. In contrast, liposomes with a significantly lower cholesterol content (e.g., 7 mol%) revealed a pronounced anomaly in passive permeability which is characteristic for main phase transition. High amounts of cholesterol are well known to suppress this anomaly [30]. Hence, the observed continuous CF efflux during temperature shifts is attributed to the high cholesterol content (26 mol%) in standard liposome preparations.

Since S-layers are highly porous structures [2], their presence at the vesicle surface cannot be a serious obstacle for the passage of small molecules such as CF. On the other hand, according to the semifluid membrane model, the S-layer lattice may have the potential to alter the permeability properties and phase behaviour of the underlying lipid membrane. As derived from Fourier transform infrared spectroscopy and differential scanning microcalorimetry measurements [31,25], the crystalline S-layer lattice increases the intermolecular order of the lipids and induces a tighter lipid packing which results in a certain degree of inhibition of the fluidity of the lipid membrane. These phenomena were attributed to specific interactions of lipid head groups with defined protein domains, reducing the lateral mobility, thereby driving the lipid into a phase of higher intrinsic order [31]. Since the interactions between distinct S-layer protein domains and lipids are effective over the whole temperature range, the higher order of lipids will directly affect the transmembrane permeability which explains the observed decrease in marker release compared to plain liposomes. As boundary defects in the interfacial regions between different phases are considered to be responsible for high transmembrane permeability, the increased marker release in glutaraldehyde-treated liposomes in which more than 95% of the HDA had reacted was attributed to phase separation phenomena. This interpretation is supported by previous differential scanning microcalorimetry studies in which deconvolution of heat capacity functions indicated that overlapping transitions appear after crosslinking with glutaraldehyde [25].

Liposome preparations were also challenged mechanically by applying high speed stirring or ultrasonication. Since in the case of stirring the corresponding Re number indicated the beginning of the laminar to turbulent transition region, shear forces are thought to be the main stress factor. In a comparison of plain and S-layer-coated liposomes CF release was investigated as a function of stress duration. As derived from the differences in the initial slopes (about 0.4–0.5 times lower than those observed for plain liposomes) under both stress factors, CF release was much lower from S-layer-coated than from plain liposomes. Higher permeability of liposomes under shear stress is not a consequence of increased convective mass transfer but is related to lipid bilayer structure modifications [32]. Application of fluid force may increase the number of bilayer packing defects through which passive transport can occur [32]. Furthermore, especially during sonication, deformations will arise resulting in changes of vesicle shape, membrane tension and possibly rupture and closure with re-equilibration of internal and external solutes as well as simply liposome disintegration. S-layers represent an additional coherent layer with considerable rigidity and stiffness in comparison to the plain lipid bilayers. Consequently, they stabilize the shape of the vesicles and improve their resistance against external perturbations. The observed slight, sigmoidal shaped release dependence on duration of ultrasonication for S-layer-coated lip-

osomes indicated a time-cooperative behaviour. Apparently, a threshold of work had to be put into the system before marker release was significantly increased. Thus, the behaviour of S-layer lattices under sonication may reflect memory effects observed with a great variety of solids exposed to stress-inducing pretreatments. The higher reliability of measurements generally observed for S-layer-coated liposomes, as reflected in lower values for the standard deviation of the mean, may also indicate a better defined system which is less sensitive to experimental deviations.

In conclusion, the recrystallization of the S-layer protein on liposomes stabilizes the lipid membrane not only by supporting it mechanically through an additional layer but also by increasing the intrinsic order of lipids through interactions between S-layer protein domains and lipid head groups. S-layer-coated liposomes were shown to resist temperature changes as well as mechanical challenges much better than plain ones. Furthermore, they provide a highly ordered immobilization matrix for the oriented anchoring of biologically active macromolecules or ligands [33] and a high potential for chemical modifications to adapt surface properties for specific requirements [34]. This is one of the reasons why S-layer-coated liposomes are expected to be useful for a broad range of applications [35]. Improved stability will be a particularly important feature for liposome technologies, in which the vesicles have to resist process steps like stirring, pumping or re-suspension. Among medical applications, increased stability will be particularly important in formulations which have to be administered as aerosols, in which liposomes will be exposed to heat, shear or sonication stress through the applied nebulizers, and for injectable liposome preparations to optimally withstand the fluid shear forces occurring in the mammalian vasculature.

Acknowledgements

We thank Dr. Jürgen Siekmann, Baxter-Immuno AG, for photon correlation spectroscopy measurements, and Aida Medovic for her technical assistance. This work was supported by grants of the Austrian Science Foundation (project S72/02) and the Federal Ministry of Science and Transport.

References

[1] T.J. Beveridge, S. Koval, Advances in Bacterial Paracrystalline Surface Layers, Plenum Press, New York, 1993.
[2] U.B. Sleytr, P. Messner, D. Pum, M. Sára (Eds.), Crystalline Bacterial Cell Surface Protein, Landes Company, Academic Press, Austin, 1996.
[3] U.B. Sleytr, Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly and function, Int. Rev. Cytol. 53 (1978) 1–64.
[4] M. Sára, U.B. Sleytr, Relevance of charged groups for the integrity of the S-layer from *Bacillus coagulans* E38-66 and for molecular interactions, J. Bacteriol. 175 (1993) 2248–2254.
[5] D. Pum, M. Weinhandel, C. Hödel, U.B. Sleytr, Large scale recrystallization of the S-layer of *Bacillus coagulans* E38-66 at the air/water interface and on lipid films, J. Bacteriol. 195 (1993) 2762–2766.
[6] D. Pum, U.B. Sleytr, Large-scale reconstitution of crystalline bacterial surface layer proteins at air/water interface and on lipid films, Thin Solid Films 244 (1994) 882–886.
[7] D. Pum, M. Sára, U.B. Sleytr, Structure, surface charge, and self-assembly of the S-layer lattice from *Bacillus coagulans* E38-66, J. Bacteriol. 171 (1989) 5296–5303.
[8] B. Wetzer, A. Pfandler, E. Györvary, D. Pum, M. Lösche, U.B. Sleytr, S-layer reconstitution at phospholipid monolayers, Langmuir 14 (1998) 6899–6906.
[9] B. Schuster, D. Pum, O. Braha, H. Bayley, U.B. Sleytr, Self-assembled α-hemolysin pores in an S-layer-supported lipid bilayer, Biochim. Biophys. Acta 1370 (1998) 280–288.
[10] B. Schuster, D. Pum, U.B. Sleytr, Voltage clamp studies on S-layer supported tetraether lipid membranes, Biochim. Biophys. Acta 1369 (1998) 51–60.
[11] S. Küpcü, M. Sára, U.B. Sleytr, Liposomes coated with crystalline bacterial cell surface protein (S-layer) as immobilization structures for macromolecules, Biochim. Biophys. Acta 1235 (1995) 263–269.
[12] B. Kuen, A. Koch, E. Asenbauer, M. Sára, W. Lubitz, Molecular characterization of the second S-layer gene *sbsB* of *Bacillus stearothermophilus* PV72 expressed by oxidative stress, J. Bacteriol. 179 (1997) 1664–1670.
[13] C. Kirby, G. Gregoriadis, Dehydration–rehydration vesicles: a simple method for high yield drug entrapment in liposomes, Bio/technology 2 (1984) 979–984.
[14] Methoden der enzymatischen Lebensmittelanalytik, Lebensmittelanalytik Boehringer Mannheim, 1983, p. 13.
[15] M. Sára, B. Kuen, H.F. Mayer, F. Mandel, K.C. Schuster, U.B. Sleytr, Dynamics in oxygen-induced changes in S-layer protein synthesis and cell wall composition in continuous culture from *Bacillus stearothermophilus* PV72 and its S-

layer-deficient variant T5, J. Bacteriol. 178 (1996) 2108–2117.
[16] K.C. Schuster, T. Pink, H.F. Mayer, W.A. Hampel, M. Sára, Oxygen-triggered synchronized variant formation of the S-layer carrying *Bacillus stearothermophilus* PV72 during continuous cultivation, J. Biotechnol. 54 (1997) 15–28.
[17] U.B. Sleytr, A.M. Glauert, Self-assembly of the hexagonally and tetragonally arranged subunits of bacterial surface layers and their reattachment to cell walls, J. Ultrastruct. Res. 55 (1975) 360–377.
[18] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olsen, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[19] F.R. Hallett, B. Nickel, C. Samuels, P.H. Krygsman, Determination of vesicle size distributions by freeze-fracture electron microscopy, J. Electron Microsc. Techn. 17 (1991) 459–466.
[20] A. Robards, U.B. Sleytr, Low temperature methods in electron microscopy, in: A.M. Glauert (Ed.), Practical Methods in Electron Microscopy, vol. 10, Elsevier, Amsterdam, 1985, pp. 309–459.
[21] P. Messner, F. Hollaus, U.B. Sleytr, Paracrystalline cell wall surface layers of different *Bacillus stearothermophilus* strains, Int. J. Syst. Bacteriol. 34 (1984) 202–210.
[22] R. Fields, The rapid determination of amino groups with TNBS, in: C.H.W. Hirs, S.N. Timasheff (Eds.), Methods in Enzymology Enzyme Structure (part B), vol. 25, Academic Press, San Diego, 1972, pp. 464–468.
[23] F. Müller-Landau, D.A. Cadenhead, Molecular packing in steroid-lecithin monolayers, part II: Mixed films of cholesterol with dipalmitoylphosphatidylcholine and tetradecanoic acid, Chem. Phys. Lipids 25 (1979) 315–328.
[24] A.F. Mingotaud, C. Mingotaud, L.K. Patterson, Handbook of Monolayers vol. 1, Academic Press, San Diego, 1993, p. 390.
[25] S. Küpcü, K. Lohner, C. Mader, U.B. Sleytr, Microcalorimetric study on the phase behaviour of S-layer-coated liposomes, Mol. Membr. Biol. 15 (1998) 69–74.
[26] R.T. Chen, J.R. Knutson, Mechanism of fluorescence concentration quenching of carboxyfluoresceine in liposomes: Energy transfer to nonfluorescent dimers, Anal. Biochem. 172 (1988) 61–77.
[27] C. Tilcock, P. Cullis, T. Dempsey, B.N. Youens, D. Fisher, Aqueous two-phase polymer partitioning of lipid vesicles of defined size and composition, Biochim. Biophys. Acta 979 (1989) 208–214.
[28] D. Pum, U.B. Sleytr, Monomolecular reassembly of a crystalline bacterial cell surface layer (S-layer) on untreated and modified silicon surfaces, Supramol. Sci. 2 (1995) 193–197.
[29] M. Sára, S. Küpcü, C. Weiner, S. Weigert, U.B. Sleytr, Crystalline protein layers as isoporous molecular sieves and immobilization and affinity matrices, in: U.B. Sleytr, P. Messner, D. Pum, M. Sára (Eds.), Immobilized Macromolecules: Application Potentials, Springer, Berlin, 1993, pp. 71–86.
[30] D. Papahadjopoulos, K. Jacobsen, S. Nir, T. Isac, Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements concerning the effects of temperature and cholesterol, Biochim. Biophys. Acta 311 (1973) 330–348.
[31] A. Diederich, C. Sponer, D. Pum, U.B. Sleytr, M. Lösche, Reciprocal influence between the protein and lipid components of a lipid–protein membrane model, Colloids Surf. B: Biointerfaces 6 (1996) 335–346.
[32] S.R. Chakravarthy, T.D. Giorgio, Shear stress-facilitated calcium ion transport across lipid bilayers, Biochim. Biophys. Acta 1112 (1992) 197–204.
[33] S. Küpcü, U.B. Sleytr, M. Sára, Two-dimensional protein crystals (S-layers) as novel matrix for immobilization of IgG and applicability of S-layers as microparticles in immunoassays, J. Immunol. Methods 196 (1996) 73–84.
[34] S. Weigert, M. Sára, Surface modification of an ultrafiltration membrane with crystalline structure and studying interactions with selected protein molecules, J. Membr. Sci. 106 (1995) 147–159.
[35] U.B. Sleytr, M. Sára, Bacterial and archaeal S-layer proteins: structure–function relationships and their biotechnological applications, Trends Biotechnol. 15 (1997) 20–26.