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Interaction of bacterial surface layer proteins with lipid membranes: Synergysm between surface charge density and chain packing

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ABSTRACT

S-layer proteins from *Lactobacillus kefir* and *Lactobacillus brevis* are able to adsorb on the surface of positively charged liposomes composed by Soybean lecithin, cholesterol and stearylamine. The different K values for S-layer proteins isolated from *L. kefir* and *L. brevis* (4.22×10^{-3} and 2.45×10^{2} μM^{-1} respectively) indicates that the affinity of the glycosylated protein isolated from *L. kefir* is higher than the non-glycosylated one.

The attachment of S-layer proteins counteracts the electrostatic charge repulsion between stearylamine molecules in the membrane surface, producing an increase in the rigidity in the acyl chains as measured by DPH anisotropy. Laurdan generalized polarization (GP) shows that glycosylated causes a GP increase, attributed to a lowering in water penetration into the head groups of membrane phospholipids, with charge density reduction, while the non-glycosylated does not affect it.

The octadecyl-rhodamine results indicate that S-layer coated liposomes do not show spontaneous dequenching in comparison with control liposomes without S-layer proteins, suggesting that S-layer protein avoid spontaneous liposomal fusion.

It is concluded that the increase in stability of liposomes coated with S-layers proteins is due to the higher rigidity induced by the S-layer attachment by electrostatic forces.

1. Introduction

One of the outer surface components of cell envelopes of prokaryotic organisms, archaea and bacteria, are crystalline arrays of proteinaceous subunits, known as surface layers (S-layers) which are usually composed by single protein or glycoprotein species [1,2].

S-layers are typical surface structure in several *Lactobacillus* species, such as *L. acidophilus*, *L. casei*, *Lactobacillus brevis*, *L. buchneri*, *L. fermentum*, *L. bulgaricus* [3], *L. crispatus* [4], *Lactobacillus kefir* [5], *L. johnsonii* [6], *L. helveticus* [7] and *L. gallinarum* [8]. They spontaneously assemble into large regular arrays which endows them with immune stimulating and intrinsic adjuvant properties [9,10]. In addition, the potential of S-layers as antigen carriers for vaccine preparations [10,11], made these proteins interesting candidates for development of new kinds of oral vaccines. Recently Golowczyc et al. [12] showed that preincubation of salmonella cells with S-layer proteins from *L. kefir* changes the surface of salmonella, thus antagonizing invasion of cultured human enterocytes.

Isolated S-layer subunits of some microorganisms also assemble "*in vitro*", either in suspension or at liquid surface interfaces, such as lipid films including liposomes and solid supports [13]. Congruent with this observation, S-layer proteins from *L. brevis* and *L. kefir* have been shown to attach to positively charged liposomes [14].

A puzzling situation is that S-layer proteins adsorb to positively charged liposomes at pH 7, at which the protein present considerable exposed positive ionized groups. Therefore, other interactions counteracting the electrostatics should be involved and for this reason, it is important to take into account the composition of external groups of the proteins of different precedence. For example, S-layer protein from *L. kefir* is glycosylated [15] while S-layer protein from *L. brevis* is not [16]. Exposed carboxyl groups on the inner face of the S-layer lattice from *Bacillus* spp. interact by non covalent forces in addition to electrostatic interaction with the zwitterionic lipid head groups [17,18].

In order to characterize the interaction of glycosylated and non-glycosylated S-layers with lipid surfaces we have studied the changes near the polar head group and the glycerol backbone and

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in the non polar phase of the lipid chains in correlation with the charge density changes induced by the protein adsorption.

For these purposes, measures of ζ potential, fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) and generalized polarization (GP) using Laurdan were carried out in phosphatidylcholine liposomes.

2. Materials and methods

2.1. Chemicals

Soybean lecithin (SL), stearylamine (SA), cholesterol (Cho) and stearic acid were purchased from Sigma (St. Louis, MO, USA). The fluorescent probes: octadecyl-rhodamine B (R18), 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) were obtained from Molecular Probes (Eugene, OR, USA).

2.2. Bacterial strains, growth conditions and isolation of the S-layer proteins

L. kefir JCM 5818 and *L. brevis* JCM 1059 were grown to mid-log phase in 250 ml of MRS broth (Biokar Diagnostics, Beauvais, France) at 37 °C, harvested by centrifugation (5.000 × *g*, 15 min, 4 °C), and washed twice in physiologic solution. The S-layer proteins were extracted with lithium chloride solution (5 M LiCl) at 20 °C for 1 h. LiCl-extracted S-layer proteins were dialysed against distilled water at 20 °C for 2 h, under agitation. Centrifugation at 16.000 × *g* for 20 min at 4 °C, by a modification of Jahn-Schmid et al. protocol [10], was carried out to eliminate large S-layer protein aggregates. The solution obtained did not show turbidity and was employed to titrate the liposomes. The S-layer protein content of the clear supernatant was evaluated by SDS-PAGE 12.5% and its concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, California, USA).

SDS-PAGE analysis of LiCl-extracted S-layer proteins from *L. brevis* and *L. kefir* respectively showed a single protein bands with an apparent molecular mass of 49.5 and 69 kDa, as was published in a previous work [14].

2.3. Preparation of liposomes with DPH, Laurdan or octadecyl-rhodamine

Positively charged liposomes were prepared mixing 625 nmol of soybean lecithin, 312 nmol of cholesterol and 62.5 nmol of stearylamine in 2 ml chloroform. For DPH control studies liposomes without SA, and liposomes with SL, cholesterol (in the same concentrations than previously) and equimolar ratios of negative and positive charges with 62.5 nmole of SA and 62.5 nmol of stearic acid were also prepared.

Lipids were dissolved and mixed with hydrophobic probes to achieve a final probe concentration of 8 mol% of R18, and 0.33 mol% of Laurdan and DPH. In order to optimize the R18 ratio in the liposomes for dequenching assays, different octadecyl-rhodamine-liposome ratios (from 1 to 10 mol%) were made.

The chloroform solutions were evaporated under nitrogen flow to eliminate solvent traces. The dry lipid film was rehydrated by addition of 1 ml H_2O under agitation above the stearylamine transition temperature (45 °C) for 1 h. After addition of the fluorescent probes, all samples were wrapped in aluminum foil to avoid fluorescence extinction.

2.4. S-layer coated liposomes

Liposomes prepared with and without probes as described above were coated by incubation of 1000 nmol of total lipids with 0.65 or 4.08 μmol of S-layer proteins from *L. kefir* or *L. brevis,* respectively, during 150 min under agitation at 22 °C. These protein/lipid ratios were chosen based on previous works in order to obtain the biggest changes with each protein [14]. With the aim to evaluate the effect of the amount of S-layer protein on the liposomes, incubations with different amounts of each S-layer protein were also carried out.

2.5. Electron microscopy (EM) by freeze-fracture

For freeze-fracture preparations, liposomes were extruded through membranes of 100 nm pore diameter and coated with S-layer proteins as described above. The samples were disposed on gold grids and frozen by rapid immersion in liquid propane cooled by liquid nitrogen. Freeze-fracture, etching and replication were performed in a BalTer BAF 060 (Balzers, Lichtenstein). Specimens were examined in Tecnai Spirit Twin (FEI Company) transmission electron microscope (TEM) at 120 kV.

2.6. Surface charge density (σ_0)

Surface charge density (σ_0 in [electron charges]/Å^2) were calculated for control and S-layer-coated liposomes from the ζ values previously published [14] at the different protein/lipid ratio by the equation

$$\sigma_0 = \varepsilon\kappa\Psi_0 \quad (1)$$

where ε is the dielectric permittivity of the electrolyte, κ (Å^{-1}) is the Debye-Hückel constant of the electrolyte, and ψ_0 (mV) is the surface potential of the liposome which can be considered equal to the ζ potential without serious error [19].

2.7. Steady-state anisotropy measurement with DPH

The fluorescent lipophylic molecule DPH partitions in the hydrophobic region in lipid bilayer [20] and it has been often used as a probe to detect the gel or fluid state of the hydrocarbon core of lipid bilayer of liposomes, biological membranes and whole cells, by monitoring the anisotropy $\langle r\rangle$ of its fluorescence [21,22].

DPH anisotropy measurements were done on S-layer-coated liposomes in a Perkin–Elmer Luminescence spectrometer Model LS 55 (Perkin-Elmer Corp./Applied Biosystems, California, USA), equipped with excitation and emission polarizers and a circulating water bath. The temperature was increased from 15 to 55 °C and was controlled inside the cuvette with a thermocouple within ±0.2 °C. Steady-state anisotropy $\langle r\rangle$ was calculated by using the following equation:

$$\langle r\rangle = \frac{I_{vv} - I_{vh}}{I_{vv} + 2GI_{vh}} \quad (2)$$

where I_{vv} and I_{vh} represent the fluorescence intensity obtained with the vertical and horizontal orientations of the excitation and emission polarizers. $G = I_{hv}/I_{hh}$ is a correction factor accounting for the polarization bias in the detection system.

2.8. Generalized polarization measurement with Laurdan (GP)

This method is based on the bilayer order-dependent fluorescence spectral shift of Laurdan which can be attributed to dipolar relaxation phenomena, originating from the sensitivity of the probe to the polarity of its environment [23]. Laurdan is located at the hydrophilic/hydrophobic interface of the membrane bilayer with the lauric acid tail anchored in the phospholipid acyl chain region [23,24].

Measurements and temperature control were done as described above for steady-state anisotropy. Emission intensity was then

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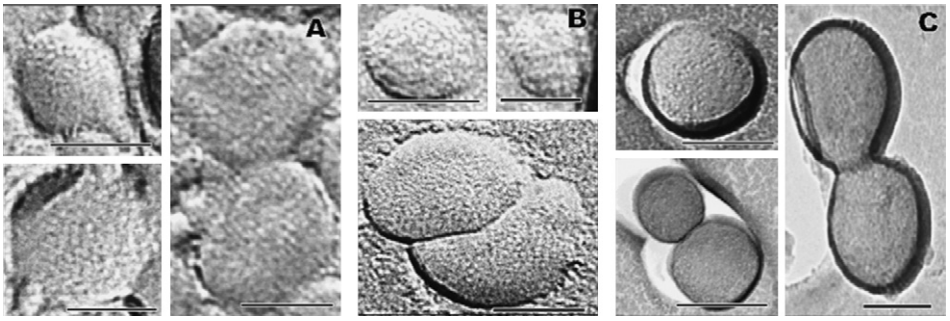

Fig. 1. Freeze-etching preparation of liposomes coated with S-layers proteins. Freeze-etching electron micrographs of liposomes coated with S-layer proteins from *L. kefir* (A), liposomes coated with S-layer proteins from *L. brevis* (B), and control liposomes without protein (C); Bars 80 nm.

acquired for several hundred seconds at 435 nm (I_{435}) and 500 nm (I_{500}) (excitation = 350 nm). Generalized polarization (GP) was calculated from the emission intensities using the following equation adapted from the work of Parasassi et al. [23]:

$$\mathrm{GP} = \frac{I_{435} - I_{500}}{I_{435} + I_{500}} \tag{3}$$

2.9. Octadecyl-rhodamine dequenching assay

The effect of the proteins on the liposome stability was monitored by the R18 dequenching assay [25,26]. In this assay the dequenching process of octadecyl-rhodamine after mixing one aliquot of R18 labeled liposome with one aliquot of liposomes without the fluorophore was followed by fluorescence. Control assays to determine the adequate probe/lipid ratio in which the fluorophore is quenched were made and, in good correlation with bibliography, fluorescence was completely quenched at 8 mol% of R18 in the lipid mixture. Different labelled: unlabelled aliquot ratios of 1:1, 1:2 and 1:4 also were tested at 37 °C in constant agitation.

The fluorescence changes were measured by fluorescence using a Luminescence Spectrometer LS 55 with an excitation and emission wavelengths of 556–570 nm, respectively. Results, expressed in percentage of dequenching, took 100% of dequenching as that measured after the disruption of liposomes by the addition of 0.1% Triton X-100 (final concentration) [27].

3. Results and discussion

Freeze-etching electron micrographs reveals that S-layer proteins from *L. kefir* and *L. brevis* are able to cover the whole external surface of lipid vesicles composed by SL, Cholesterol and SA, forming a uniformly rough array (Fig. 1A and B), in contrast to control liposomes which show a smooth surface (Fig. 1C).

The adsorption of the protein to the external surface of the liposomes can be followed by variation in the zeta potential ($\Delta\zeta$) as a function of the S-layer concentration [S].

The degree of coverage (θ) can be deduced from the following equilibrium:

$$k_1[S]^n(1-\theta) = k_2\theta$$

where k_1 is the specific rate of adsorption and k_2 the specific rate of desorption.

After rearrangement and taken the affinity constant as $K = k_2/k_1$ and $\theta = \Delta\zeta/\Delta\zeta_{max}$, Eq. (4) is obtained.

The degree of coverage (θ) due to both S-layer proteins adsorption to the external liposome's surface (Fig. 2) can be described by the following equation:

$$\theta = \frac{\Delta\zeta}{\Delta\zeta_{\max}} = \frac{[\text{S-layer}]^n}{K + [\text{S-layer}]^n} \tag{4}$$

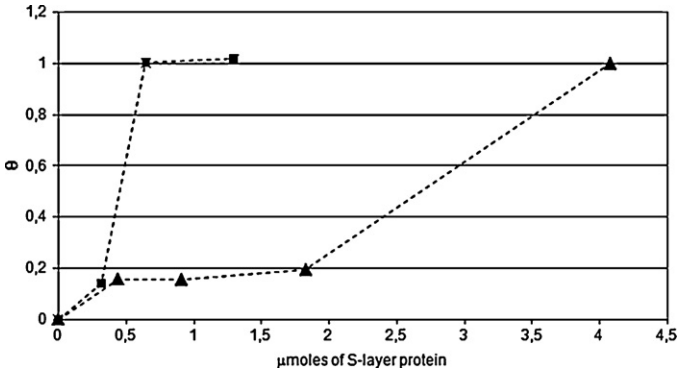

Fig. 2. Coated liposomes ratio (θ) as a function of S-layer protein added. Liposomes coated with S-layer proteins from *L. kefir* (■), liposomes coated with S-layer proteins from *L. brevis* (▲).

where $\Delta\zeta$ is the potential shift, K, the inverse of affinity constant and n, the heterogeneity parameter describing the width of energy distribution function. Although both proteins appear to reach a saturation value at a protein concentration of 0.5 μM, the plateau obtained with the glycosylated protein is higher than that corresponding to the non-glycosylated one from *L. brevis,* in the range 0–1.5 μmol of protein. This is consistent with different affinity sites available for each protein. The different K values for S-layer proteins isolated from *L. kefir* and *L. brevis* (4.22×10^{-3} and 2.45×10^{2} μM^{-1} respectively) indicates that the affinity of the glycosylated protein isolated from *L. kefir* is higher than the non-glycosylated one.

The differences in the affinity of both proteins are also reflected by the kinetics of adsorption (Fig. 3). After around 75 min. of incubation, *L. kefir* S-layer protein covers 60% of the surface while *L. brevis* proteins only 40%. The 100% of coverage is reached at times longer than 150 min for both proteins. As the standard deviation of the zeta potential measures are less than 5%, the differences are significative. In addition, the non linearity of the curves in the plots as a function of $t^{1/2}$ suggests that proteins attached to the liposomal surface induce a structural rearrangement on the membrane or protein structure.

We will focus on the changes at different levels of the membrane induced by the protein attachment. The isoelectric point of S-layer-proteins from lactobacilli are above 9.0 [16]. Therefore, the stabilization of the membrane-protein complex should imply charge redistributions.

The surface charge density (σ) of the liposomes in the aqueous media can be calculated from the values of ζ potential through the Gouy-Chapman equation (Eq. (1)) by:

$$\sigma = N^* e/AN \tag{5}$$

where e is the absolute value of the electric charge, A the area of the polar group, N^* the number of positively charged

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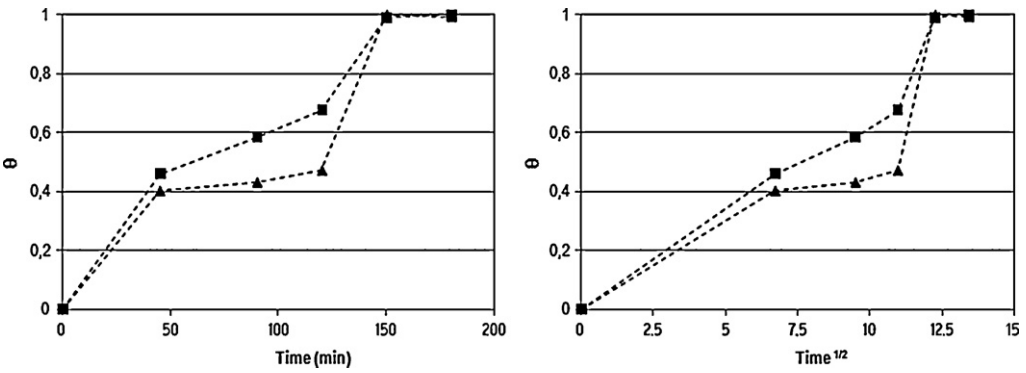

Fig. 3. Kinetic of S-layer coating liposomes. Coating of liposomes with S-layer proteins from *L. kefir* (■) or S-layer proteins from *L. brevis* (▲), as a function of time (A) or time 1/2 (B).

groups and N the total number of groups in the layer [19].

The values in Table 1 denote that liposomes containing 6 mol% stearylamine have a positive net charge of 3.7×10^{-4} (charges/Å^2). Considering that for pure phosphatidycholine (SL) the surface charge density of 1.43×10^{-2} (charges/Å^2) corresponding to 1 charge per lipid, it is reduced to 7×10^{-3} (charges/Å^2) with the presence of cholesterol. Then, the values of charge density reported in Table 1, is ca. 1 charge each 20 lipids which is near to the ratio of 1/17 for charged lipids to total lipids in the preparations containing 6 mol% of SA. Interaction of S-layer proteins with the charged lipids results in a decrease of the surface charge density in the liposomes to 7×10^{-5} (charges/Å^2) as was shown in Table 1. This corresponds to one charged per 100 lipids. Therefore, more than 90% of the charges are neutralized by the addition of 0.65 μmol of S-layer protein from *L. kefir* or 4.08 μmol of S-layer protein from *L. brevis*.

This surface charge neutralization enhances the lateral interactions between the chains. As observed in Table 1, the decrease of surface charge density, promoted by both S-layer proteins, was accomplished by an increase in the anisotropy ⟨r⟩ values. However, the GP values increase for *L. kefir* S-layer protein remains unchanged with the attachment of *L. brevis* S-layer protein (at the temperature that assays were carried out, 25 °C). Thus, charge neutralization affects defined regions of the membrane structure.

The DPH anisotropy increases with the addition of both S-layer proteins to SA containing liposomes at low and high temperatures (Fig. 4). The highest values of anisotropy were obtained when liposomes were incubated with 0.65 μmol of S-layer proteins from *L. kefir*, or with 4.08 μmol of S-layer protein from *L. brevis* at both temperatures studied (25 and 45 °C) (Fig. 4).

A similar connection between the surface charge density decrease and the increase of membrane anisotropy is found in

Table 1
Surface charge density (σ_0), anisotropy (⟨r⟩) and generalized polarization (GP) values from liposomes with and without S-layer proteins from *L. brevis* or *L. kefir*. All measurements were carried out at 25 °C.

S-layer protein of *Lactobacillus kefir*				S-layer protein of *Lactobacillus brevis*			
μmol	σ_0 (e/Å^2)	⟨r⟩	GP	μmol	σ_0 (e/Å^2)	⟨r⟩	GP
0	0.00036948	0.188 ± 0.0084	0.468 ± 0.030	0	0.00036948	0.188 ± 0.0084	0.468 ± 0.030
0.32	0.00030743	0.193 ± 0.0065	0.500 ± 0.054	0.91	0.00029944	0.191 ± 0.0058	0.465 ± 0.1
0.65	−0.00007051	0.213 ± 0.0055	0.545 ± 0.037	1.83	0.00028205	0.206 ± 0.0058	0.469 ± 0.072
1.30	−0.00007756	0.198 ± 0.0045	0.542 ± 0.045	4.08	−0.00008085	0.210 ± 0.0050	0.478 ± 0.045

SD stands for standard deviation. Each point represents 20 measures of the same liposome of a sample divided in three independent tubes in the same conditions. Two different batches of liposomes were tested. For fluorescence, the final data correspond to the average of five spectra taken in each condition which was repeated with a different batch in an independent assay.

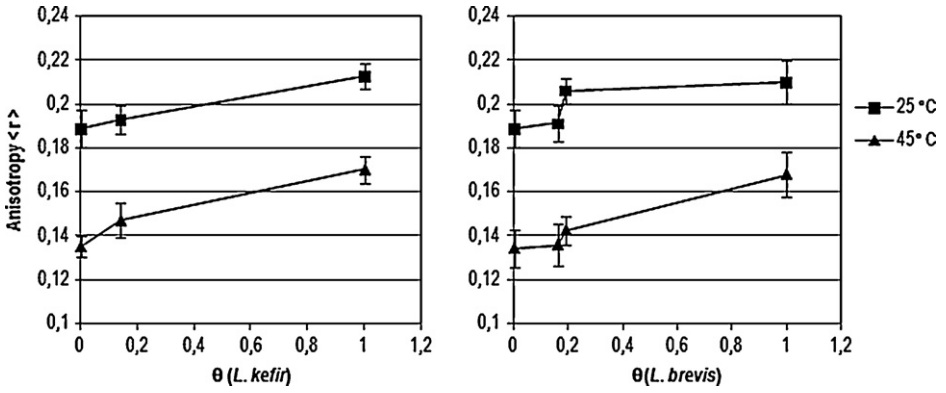

Fig. 4. DPH fluorescent emission anisotropy as a function of θ at 25 and 45 °C. Amounts added to 1000 nmol of total lipids. Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the mean values.

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liposomes with different surface charges without S-layer proteins (Fig. 5A). The anisotropy of SL/cholesterol liposomes is higher when SA is depleted. More interestingly, when SL/cholesterol liposomes were prepared with a 1:1 ratio of SA and stearic acid a result similar to that found for S-layer coated SA containing liposomes was obtained (Fig. 5B).

These results strongly suggest that the neutralization *"in situ"* of the positive charges of SA by the proteins affects the chain lateral packing reflected by the increase in anisotropy. The screening of the surface charges of SA by S-layer proteins decreases the charge repulsion of the head groups of SA favoring the lateral packing of the acyl chains.

Moreover, a detailed inspection of the data in Fig. 5 denotes that the increase in anisotropy, produced by S-layer proteins adsorption, is higher than that produced by the neutralization *"in situ"* of SA with stearic acid. It is possible that SA neutralization by S-layer residues fits much better in the surface array than the combination of amine and acid groups. The decrease in the acyl chains mobility by the surface charge density reduction takes place in the whole range of temperature independent of the protein glycosylation (Fig. 5). The difference between the effects of glycosylated and non-glycosylated proteins on positively charged liposomes visualized in the GP values (Table 1) suggests that protein interaction is taking place at the polar membrane region. The first one causes a GP increase with charge density reduction, while the non-glycosylated does not affect it. The effect of the glycosylation is relevant for membrane at low temperature (25 °C) being comparable at 45 °C (Fig. 6). As denoted in Fig. 7, GP values decreases smoothly with temperature, revealing no phase transition. However, in the whole range of temperatures, GP values of liposomes with *L. kefir* and *L brevis* S-layer proteins are higher than those found in control liposomes without protein.

GP values reflect local motion of polar molecules [28]. Lower values of GP can be attributed to water penetrating the head groups of membrane phospholipids [23]. Although water penetration would be aided by lipid motion in the membrane, it is especially sensitive to phospholipids packing [29]. The packing increase reduces the number of solvent molecules available to influence Laurdan. In Fig. 6, it is clear that the difference on GP between glycosylated and non-glycosylated proteins is significant at 25 °C. The addition of non-glycosylated protein did not affect the GP values at 25 °C. However, at 45 °C the GP values increase with the addition of both S-layer proteins (Fig. 6), an effect comparable to that found with DPH.

This denotes that S-layer condensation on SA containing liposomes is favored by the glycosyl moieties when temperature is decreased, which can be attributed to H-

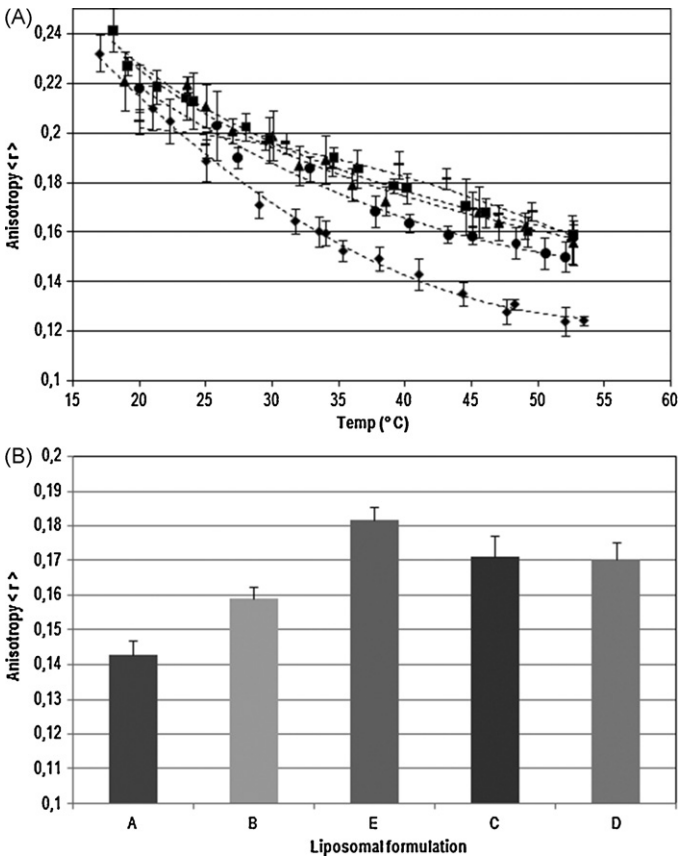

Fig. 5. (A) Fluorescent anisotropy of DPH as a function of liposomal composition. Control liposomes with a net positive charge density composed with soybean lecithin, cholesterol and stearylamine according to Table 1 (♦), liposomes with soybean lecithin and cholesterol without SA (▬); liposomes with equimolar ratios of positive and negative lipids composed by soybean lecithin, cholesterol, stearylamine and stearic acid (✹), liposomes coated with S-layer from *L. kefir* (■), liposomes coated with S-layer from *L. brevis* (▲). Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the mean values. (B) Fluorescent anisotropy of DPH as a function of liposomal composition at 37 °C. Control liposomes with a net positive charge density composed with soybean lecithin, cholesterol and stearylamine according to Table 1 (A), liposomes with soybean lecithin and cholesterol without SA (B); liposomes with equimolar ratios of positive and negative lipids composed by soybean lecithin, cholesterol, stearylamine and stearic acid (C), liposomes coated with S-layer from *L. kefir* (D), liposomes coated with S-layer from *L. brevis* (E). Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the mean values.

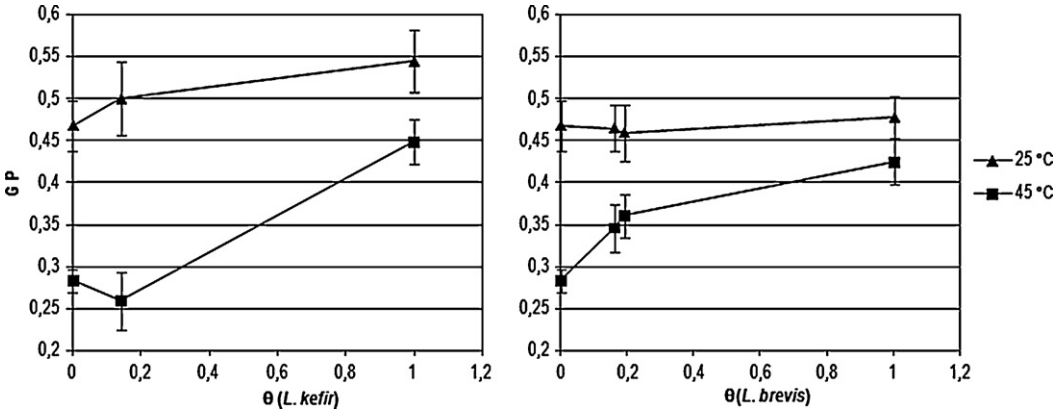

Fig. 6. Laurdan GP values as a function of the degree of coverage (θ) S-layer proteins at 25 and 45 °C. Amounts added to 1000 nmol of total lipids. Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the mean values.

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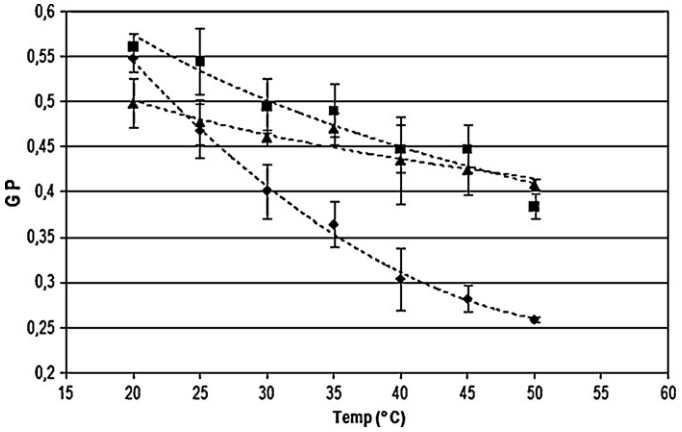

Fig. 7. Temperature dependence of Laurdan GP. Control liposomes (♦), liposomes coated with S-layer proteins from *L. kefir* (■), liposomes coated with S-layer proteins from *L. brevis* (▲). Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the mean values.

bond formation of these residues to gel domains on the membrane.

A four times higher amount of *L. brevis* S-layer protein is needed to obtain similar result than that found for S-layer proteins from *L. kefir*. This is an additional argument in favor that, besides the electrostatic forces no charged residues such as the glycosylated moieties can influence the charge arrangement due to S-layers proteins from *L. kefir*.

A further evidence of increase in packing due to S-layer attachment to SA liposomes is given by the assays using the fluorescent probe octadecyl-rhodamine, R18. The fluorescence of this probe is completely quenched at 8 mol% (data not shown). The dilution of the probe can be observed as a fluorescence increase. This would imply a decrease in the lipid packing. In our study, the mixing of labelled liposomes with another liposomal batch of a similar composition without the probe produced a 60% fluorescence increase after 200 min incubation time (Fig. 8). This fluorescence increase can be interpreted as a consequence of the dilution of octadecyl-rhodamine probe when lipids of the labeled and non labeled population mix spontaneously.

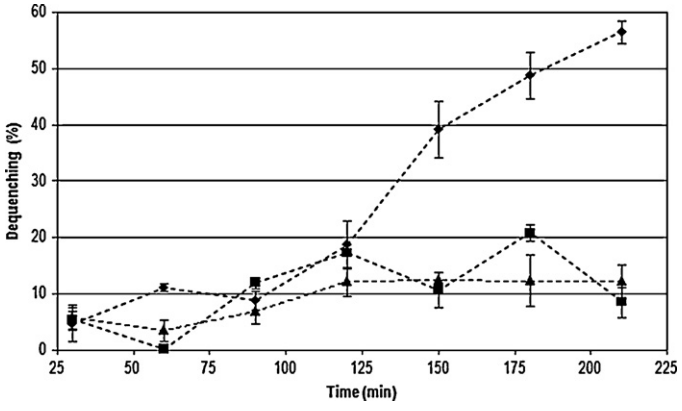

Fig. 8. Dequenching of octadecyl-rhodamine in positively charged liposomes. Percentage of dequenching of control and S-layer-coated liposomes labeled with 8 mol% of octadecyl-rhodamine by dilution with similar aliquot of liposomes without probe. Control liposomes (♦), liposomes coated with S-layer proteins from *L. kefir* (■), liposomes coated with S-layer proteins from *L. brevis* (▲). Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the mean values.

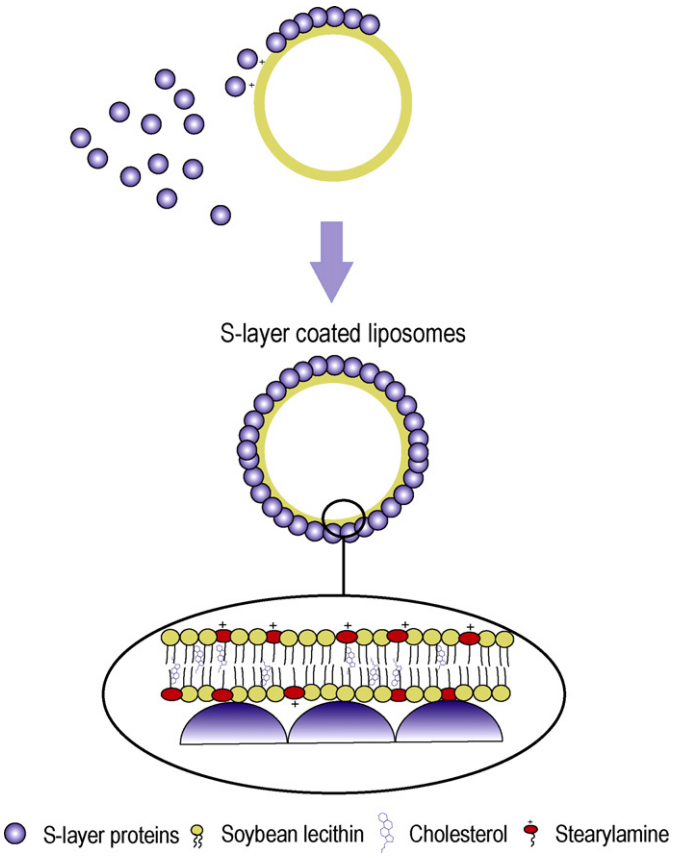

Fig. 9. Schematic representation of the S-layer protein adsorption to the external surface of liposomes containing phosphatidylcholine, cholesterol and stearylamine.

When liposomes were coated with *L. kefir* or *L. brevis* S-layer proteins, the fluorescence increase is significantly attenuated (Fig. 8). The dequenching observed is less than 20% and can be ascribed to inspecific diffusion of the probe from labelled membranes to none labelled ones [30] (Fig. 9).

In summary, *L. brevis* and *L. kefir* S-layer proteins are able to modulate the lipid order in the bilayer. At 45 °C, the increase in anisotropy is parallel to the increase in GP values, denoting that the effect at the interface affects the chain mobility (Figs. 4 and 6). This increase of rigidity can be explained by the lower hydration of the interface and hence a lower polarity as shown by Laurdan. The electrostatic interactions at the SA in the liposome surface are reduced by the S-layer, thus promoting a partial dehydration at the head group region and a concomitant increase in chain packing. The correlated effects observed by the probes located at different depths indicate that the changes at the interface propagate into the hydrophobic zone by increasing the packing of the lipid chains. Taking into account data from other S-layer proteins we conclude that the protein probably does not interact directly with the lipid alkyl chains but affects the chains via coupling to the lipid head groups [31,32]. This leads to a higher dipolar relaxation in this region of the membrane. The effect of protein on charge neutralization, anisotropy and GP is concentration dependent. The results obtained in this work are in good correlation with reports for other S-layer proteins from *Bacillus* members, suggesting that there are at least two to three contact points between the lipid film and the attached S-layer protein. Therefore, only a few lipid molecules are anchored via their head groups to protein domains on the S-layer lattice, whereas the remaining lipid molecules diffuse freely in the membrane between the pillars consisting of anchored lipid molecules. Because of its widely retained fluid characteristic,

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this nano-patterned type of lipid membrane is also referred to as "semifluid membrane" [17]. It may be possible that, the adsorbed S-layer proteins will inhibit liposome aggregations and/or fusion increasing its stability and avoiding leakage by the decrease in permeability. S-layer proteins adsorbed to the lipid vesicles can be a permeability barrier of non electrolytes in addition to the lipid bilayer.

In addition, the initial step of fusion implies a close apposition of bilayers so that membrane contact can occur [33]. The presence of S-layer proteins on the liposomes surface, however, may counteract this close approach hindering the mixing of lipids from labeled and unlabeled liposomes [34]

4. Conclusions

As was reported previously S-layer proteins isolated from GRAS lactobacilli are able to stabilize positively charged liposomes against different condition [14]. This effect can now be explained by the concourse of different changes promoted by the proteins on the lipid bilayer. An increase in the bilayer packing due to charge neutralization decreases in the acyl chain mobility and hence permeability. In addition, S-layer proteins were able to decrease liposome fusion, probably to the inclusion of bulky moieties protruding from the membrane, giving place to steric hindrance for surface-surface interaction.

The higher stability of S-layer-coated liposomes, combined with the adhesion and immunogenic functions of S-layer proteins, and the possibility for immobilizing or entrapping biologically active molecules [32] reveal a broad application potential, particularly as carrier and/or drug delivery [17].

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