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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