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RESEARCH ARTICLE

Interaction of S-layer proteins of *Lactobacillus kefir* with model membranes and cells

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Abstract

In previous works, it was shown that S-layer proteins from *Lactobacillus kefir* were able to recrystallize and stabilize liposomes, this feature reveling a great potential for developing liposomal-based carriers. Despite previous studies on this subject are important milestones, a number of questions remain unanswered. In this context, the feasibility of S-layer proteins as a biomaterial for drug delivery was evaluated in this work. First, S-layer proteins were fully characterized by electron microscopy, 2D-electrophoresis, and anionic exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). Afterward, interactions of S-layer proteins with model lipid membranes were evaluated, showing that proteins adsorb to the lipid surface following a non-fickean or anomalous diffusion, when positively charged lipid were employed, suggesting that electrostatic interaction is a key factor in the recrystallization process on these proteins. Finally, the interaction of S-layer coated liposomes with Caco-2 cell line was assessed: First, cytotoxicity of formulations was tested showing no cytotoxic effects in S-layer coated vesicles. Second, by flow cytometry, it was observed an increased ability to transfer cargo molecules into Caco-2 cells from S-layer coated liposomes in comparison to control ones. All data put together, supports the idea that a combination of adhesive properties of S-layer proteins concomitant with higher stability of S-layer coated liposomes represents an exciting starting point in the development of new drug carriers.

Keywords

S-layer proteins, *Lactobacillus kefir*, liposomes, drug carriers

History

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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 usually are composed of a single protein or glycoprotein species (Gerbino et al., 2015; Ristl et al., 2011; Sleytr et al., 2007). S-layer proteins have the capability to recrystallize into isoporous monolayers in suspension, at liquid–surface interfaces, lipid structures and on solid supports. Due to their self-assembly ability, it was demonstrated that S-layer proteins possess a great potential for nanobiotechnological applications (Hynonen & Palva, 2013; Sleytr et al., 2007).

The S-layer seems to be a typical surface structure in several lactobacilli species (Hynonen & Palva, 2013; Jakava-Viljanen et al., 2002). Interest in lactobacilli S-layer has been reinforced by claiming and demonstrated probiotic properties for human and animal consumers (Bernardeau et al., 2008; Liu et al., 2011; Mastromarino et al., 2011; Mercenier et al., 2003). Although the biological functions of *Lactobacillus* S-layer proteins have not yet been completely elucidated, these proteins apparently are of importance, because a substantial part of the biosynthetic capacity of the bacterial cell is used for their synthesis (Uroić et al., 2016). Since an important property for a probiotic bacterium is the ability to adhere to and colonize host tissues to prevent colonization by pathogenic bacteria (Avall-Jaaskelainen et al., 2003; Golowczyc et al., 2007; Liu et al., 2011), characterization of the different lactobacilli S-layer proteins seems to be key to the selection of useful strains. Thus, the study of S-layer proteins of these safe bacteria has a special interest for further developments on the nanobiotechnological field.

Lactobacillus kefir is a lactic acid bacteria isolated from kefir grains or kefir milk (Garrote et al., 2001) and it was described that *L. kefir* carries S-layer proteins (Garrote et al., 2004). Golowczyc et al. (2007) showed that preincubation of salmonella cells with S-layer proteins from *L. kefir* leads to changes in the surface of salmonella, thus antagonizing invasion of cultured human enterocytes. Aslo it was recently reported that S-layer proteins from *L. kefir* strains were able to inhibit the damage induced by *Clostridium difficile* spent culture supernatant to Vero cells (Carasi et al., 2012). In addition, it was recently demonstrated that *L. kefir*i strains isolated from kefir grains stimulated the production of

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different ratios of pro/anti-inflammatory cytokines *in vitro*; the administration of *L. kefiri* CIDCA 8348 to mice not only downregulates expression of proinflammatory mediators, but also increases anti-inflammatory molecules in the gut immune system inductive and effector sites (Carasi et al., 2015). Another interesting feature of *L. kefir* S-layer proteins is the presence of glycan moieties. Moreover, the structural characteristic of these glycoproteins could have influence in determining the surface properties of whole bacterial cells (Mobili et al., 2009).

In previous works, we showed that S-layer proteins from *L. kefir* were able to cover and to stabilize liposomes composed of soybean lecithin, cholesterol and stearylamine (Hollmann et al., 2007). Furthermore, S-layer coated liposomes proved to be good candidates as a drug delivery system orally administered due to their increased stability against changes in pH, exposure to bile salts, and pancreatic extracts (Hollmann et al., 2007).

S-layer proteins were also able to decrease liposome spontaneous fusion, probably by the inclusion of bulky moieties protruding from the membrane (Hollmann et al., 2010a).

The high stability of S-layer-coated liposomes, combined with the probiotic and immunological functions described for S-layer proteins from *L. kefir*, reveal a broad application potential, particularly as carrier and/or for drug delivery (Avall-Jaaskelainen & Palva, 2005).

However, a structural and biochemical characterization of S-layer from *L. kefir* species will be needed in order to use these proteins in vaccine applications, as well a better knowledge of the crystallization process, especially from a thermodynamic perspective.

The aim of the present work was to gain an understanding of structural and chemical properties of *L. kefir* S-layer proteins, and study in depth the interaction of S-layer proteins with lipid membranes. In this context, the use of lipid monolayer to follow the kinetic of protein insertion in the monolayers revealed to be a useful tool specially to track the protein–lipid interaction in a dynamic perspective (Disalvo et al., 2013a; Hollmann et al., 2010b, 2016).

Finally, as a first stage to evaluate the feasibility of this new kind of membranes (lipid membrane-stabilized by S-layer proteins) we studied some applications related to oral drug delivery using Caco-2 cells, a well-characterized intestinal *in vitro* model.

Methods

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

L. kefir JCM 5818 was grown to mid-log phase in 250 ml of MRS broth (Biokar Diagnostics, Beauvais, France) at 37 °C, harvested by centrifugation (5000 *g*, 15 min, 4 °C), and washed twice with physiologic solution. The S-layer proteins were extracted with lithium chloride solution (5 M LiCl) (Carlo Erba, Milan, Italy) at 20 °C for 1 h. LiCl-extracted S-layer proteins were dialyzed 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. (1996) protocol was carried out to eliminate large S-layer protein aggregates. The S-layer protein content of the clear supernatant was evaluated by SDS-PAGE (12.5%), showed a single protein band with an apparent molecular mass of 61 kDa as published in previous works (Garrote et al., 2004; Hollmann et al., 2007). S-layer protein concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, CA).

Electron microscopy (EM) by freeze-fracture

For freeze-fracture preparations, whole cells 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, Hillsboro, OR) transmission electron microscope (TEM) at 120 kV.

Isoelectric point characterization

Samples were solubilized directly in 2D buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, and 0.5% pH 7–11 NL carrier ampholytes) and spiked with 3 µg glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from rabbit muscle, Sigma, St. Louis, MO) and 65 mM DTT. Samples (100 µl) were loaded, by anodic cup loading, onto a pH 7–11 non-linear, 11 cm Immobiline DryStrip (GE Healthcare, NJ, USA) gel which had been hydrated in 2D sample buffer containing 1.2% (v/v) 2-hydroxethyldisulfide. Gels were run in a step-wise voltage gradient: 0–300 V/2 h; 300–500 V/2 h; 500–1000 V/2 h; 1000–4000 V/5 h followed by 4000 V/3 h and then maintained at 500 V. Total volt hours (V/h) ranged between 25 and 30,000 V/h. Focused proteins from individual gel strips were then separated by SDS-PAGE, using a 10% or 12% gel with a 29:1 acrylamide:bis-acrylamide ratio.

The distance migrated along the IEF strip from the loading point (anodic, pH 7 end) was measured as a percentage of the total gel-strip length (11 cm) and the p*I* calculated from an idealized pH 7–11 non-linear migration reference graph (GE Healthcare, UK).

Chemical characterization of S-layer proteins

Protein glycosylation was determined by SDS-PAGE on a gel stained with periodic acid/Schiff reagent using a commercial kit GelCode Glycoprotein Staining Kit (Pierce Biotechnology, Rockford, IL). In order to characterize the S-layer glycans, S-layer proteins were treated with TFA 2N and the S-layer glycans residues were recovered and analyzed by anionic exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). A DX-300 Dionex BioLC system with pulse amperometric detector was used. Analysis was performed in a Carbopack PA-10 column equipped with a PA-10 pre-column using an isocratic gradient elution of 200 mM NaOH. Flow: 1 ml/min.

Characterization of S-layer protein-lipid attachment

Changes in the surface pressure of lipid monolayers induced by injection of S-layer proteins were measured in a Kibron Langmuir–Blodgett trough, at constant temperature (25 ± 0.5 °C). The surface of the buffer solution contained

in a Teflon trough of fixed area was exhaustively cleaned by surface aspiration. Then, a chloroform solution of lipids was spread on this surface, to reach surface pressures between 20 and 40 mN/m. S-layer proteins were injected in the subphase and the changes of surface pressure were recorded until a constant value was reached. The surface pressure of the air–water interface upon injecting the largest concentration of each peptide was 18 mN/m. For this reason, the lowest initial surface pressure of the lipid monolayers before the addition of the S-layer proteins to the subphase was above that value. In this condition, the changes in surface pressure observed upon the injection of the peptide can be ascribed to an effect of the peptide on the monolayer interfacial tension.

Pressure data obtained were fitted to the following equation:

$$\theta = \frac{\Delta\Pi}{\Delta\Pi\,\max} = \frac{[S-\text{layer}]^n}{kd + [S-\text{layer}]^n} \quad (1)$$

where θ is the degree of coverage, $\Delta\Pi$ is the surface pressure shift, [S-layer] is the S-layer protein concentration, n the heterogeneity parameter describing the width of energy distribution and k_d the dissociation constant.

The kinetics of protein penetration was followed using:

$$\Delta\Pi = kxt^r \quad (2)$$

from where the constants k and *r,* relaxation coefficient, were evaluated (Crank, 1975).

Regression analyses of the pressure curves were performed with Graph pad® (GraphPad Software, Inc. CA), by minimizing the root mean square error between the experimental data rate and the model equation.

Liposomes preparation

Positively charged liposomes were prepared from a mixture composed of 625 nmoles of soybean lecithin, 312 nm cholesterol and 62.5 nm stearylamine (Sigma, St. Louis, MO). Lipids were dissolved in 2 ml chloroform and evaporated under nitrogen flow. The dry lipid film was rehydrated by the addition of 1 ml PBS pH 7.0 and loosened from the glass wall during agitation above the transition temperature of SA (45 °C) for 1 h. For uptake assays, calcein-loaded liposomes were prepared by rehydrating the lipid film with PBS pH 7.0 supplemented with 10 mM calcein (Molecular Probes, Eugene, OR). The non-entrapped probe was removed from the external media after two centrifugations at 5600 *g* for 5 min, and washed with PBS pH 7.0.

S-layer coated liposomes

Liposomes prepared with and without probe as described above were coated by incubation of 1000 nmol of total lipids with 0.65 μmol of S-layer proteins from *L. kefir* during 150 min under agitation at 22 °C. These protein/lipid ratios were chosen based on previous works to obtain completely coated liposomes (Hollmann et al., 2007, 2010a). As a control of completely coated, liposomes Zeta potential measures were done as described previously (Hollmann et al., 2007).

Dynamic light scattering of large unilamellar vesicles in the presence of S-layer proteins

Dynamic light scattering experiments were carried out on a Malvern Zetasizer Nano ZS (Malvern, UK) with a backscattering detection at 173°, equipped with a He–Ne laser ($\lambda = 632.8$ nm), at 37 °C, using disposable polystyrene cells. The LUV suspensions were diluted to the desired final concentration and then filtered using a syringe filter with 0.45 μm pore size (Whatman, Florham Park, NJ). Lipid concentration was kept constant at 50 μM and the S-layer protein from *L. kefir* concentration was 238 nM. The samples were left equilibrating for 15 min at 37 °C. Normalized intensity autocorrelation functions were analyzed using the CONTIN method (Provencher, 1982), yielding a distribution of diffusion coefficients (D). The measured D is used for the calculation of the hydrodynamic diameter (D_H) through the Stokes–Einstein relationship:

$$D = \frac{kT}{3\pi\eta D_H} \quad (3)$$

where k is the Boltzmann constant, T the absolute temperature, and η the medium viscosity. A set of 15 measurements (∼13 runs each) for the liposomes in the absence and presence of S-layer proteins was conducted to calculate the D_H value. The D_H of the sample was obtained from the peak with the highest scattered light intensity (i.e. the mode) in light scattering intensity distributions.

Cell culture conditions

Human colon adenocarcinoma Caco-2 cell line (Zweibaum et al., 1983) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM, HyClone, UT) containing 25 mM-glucose, 1.0 mM sodium pyruvate, 15% heat-inactivated (56 °C, 30 min) fetal bovine serum (Gibco, NY), 1% nonessential amino acids, 100 U of penicillin G/ml, 100 mg streptomycin sulfate/ml, and 0.25 mg amphotericin B/ml (Sigma, St. Louis, MO). All measurements were performed using cells between passage 38 and 42.

Cytotoxicity assays

The effect of S-layer coated and control liposomes on the viability of Caco-2 cells (14-days post-confluent) was determined with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) conversion assay, based on the reduction of the tetrazolium salt, MTT, to a crystalline blue formazan by cellular oxidoreductases in viable cells. After 4 h incubation of cells in liposomes suspensions, cells were washed twice with phenol red-free DMEM and incubated at 37 °C with DMEM containing 0.5 mg MTT/ml in a humid atmosphere with 5% CO_2 for 3 h. Media were removed from test cultures and ethanol (200 μl) was added to each well to solubilize the formazan. Plates were then shaken for 5 min to ensure complete solubilization and the optical density (OD) was measured at 540 nm in a microtiter plate reader (Rayto Elisa reader, P.R. China)

Uptake assays

For flow cytometric experiments and microscopic examination, Caco-2 cells in polypropylene 6-well plates (14-days post-confluent) were washed twice with PBS. Subsequently, cells were incubated 1 h with complete DMEM containing calcein loaded coated or control liposomes (0.85 mg total lipids/ml). Removal of unbound vesicles was accomplished by washing twice with PBS. As a control, cells were incubated with empty liposomes. The FACS analysis was taken to acquire quantitative information in terms of uptake. Becton Dickinson FACScan (Becton–Dickinson Immunocytometry Systems, USA) was used to perform flow cytometry analysis. 20 000 cells were measured for each analysis. The vesicle uptake monitored by calcein fluorescence was stored in a histogram mode on a logarithmic scale. From the obtained graphs, the percentage of fluorescent positive cells and the mean fluorescence intensity (fluorescence per cell, in arbitrary units; MFI) were calculated. The calcein fluorescence reflected the intracellular dequenching of the dye and was analyzed at excitation and emission wavelengths of 488 nm and 520 nm, respectively. The fluorescence intensity of loaded vesicles in untreated control cells was set to 100% for inhibition quantification, whereas the cellular uptake of empty vesicles containing no probe served as blank for the evaluation of the basic autofluorescent level.

Reproducibility of results

All determinations were the average of three independent replicate assays. Data are shown as mean values. The statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA).

Results

S-layer characterization

Because the ability of these kinds of proteins to form a regular array is a key factor in its latter ability for stabilizing of the supramolecular structures as liposomes and due the lack until now of a proper micrographic characterization of S-layer proteins of *L. kefir*, in the present study the characterization of S-layer protein arrays from *L. kefir* was started by means of EM micrographs using freeze-etched preparations. As shown in Figure 1, a regular bidimensional arrangement was observed on the bacterial cell surfaces. This arrangement of proteinaceous subunits, characteristic of S-layer proteins, was evident in *L. kefir* intact washed cells showing oblique lattice symmetry ($p2$) with an angle between the base vectors of approximately 80°

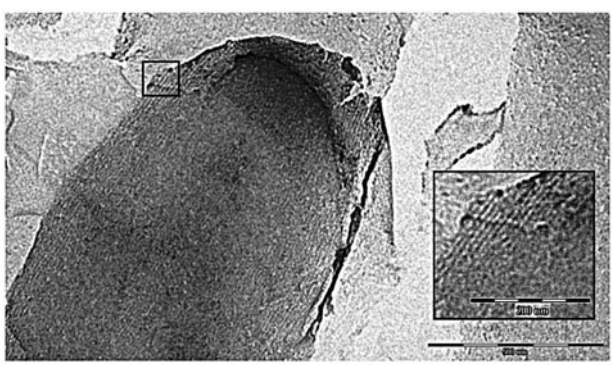

Figure 1. Transmission electron micrograph of freeze-etched preparation of entire cell of *L. kefir*.

Following microscopic characterization and as charges play an important role on protein adsorption toward lipid membranes (Hollmann et al., 2007, 2010a),2D gel electrophoresis of S-layer extracted proteins from *L. kefir* was made in order to estimate its isoelectric point (*pI*), giving a *pI* value around 9.4, corresponding to a highly basic protein (Table 1).

Finally, in order to confirm and quantify the presence of sugar residues on S-layer proteins, a chromatographic determination by HPAEC-PAD was made to verify the presence of a glycoprotein in the preparation. The quantities and types of monosaccharide released via acid hydrolysis of the S-layer protein revealed that glycans are composed of glucose and mannose in the relative abundances of ∼1:1. Taking into account the molecular mass of each protein unit, 1.4% w/w of glycosylation was determined, probably corresponding to five molecules of glucose and five molecules of mannose (Table 1).

S-layer lipid interactions

Assembling surface-exposed domains and topographic motifs in a highly precise and nanopatterned regularity, S-layer technology offers significant advantages to nanovehicles including improved stability and antifouling characteristics which are particularly important when exposed to biological environment (Ucisik et al., 2013). In this context the characterization of proper assembly of the proteins in the lipids is a key factor in the technological features of this new drug delivery candidates. The ability of S-layer proteins from *L. kefir* to recrystallize on lipid interfaces was already demonstrated (Hollmann et al., 2007, 2010a). However, to gain knowledge of the nature of the interactions, we studied the adsorption of the S-layer protein from *L. kefir* to the lipid monolayers. Experiments with monolayers have the unique advantage that the arrangement and packing of the molecules can be easily measured and controlled and the energetics can be studied via surface tension measurements (Brezesinski & Möhwald, 2003; Reis et al., 2009).

As expected, S-layer proteins were able to increase the surface pressure of a lipid monolayer composed of soybean lecithin, cholesterol and stearylamine (Figure 2A). In order to get an insight on the peptide insertion mechanism, we analyze the surface pressure data using the Langmuir isotherm, described in Methods (Equation (1)), giving a n coefficient of 1.8, indicating that S-layer interactions do not follow a

Table 1. S-layer proteins monosaccharide residue characterization.

Sample	Monosaccharide residue	Retention time	% (w/w)	SD[a]
L. kefir JCM 5818	Glucose	10.45	1.4	0.01
	Mannose	11.30	1.4	0.04

[a]SD: standard deviation.

Monosaccharide quantification, by HPAEC-PAD, of gylacan moieties in S-layer proteins from *L. kefir*. Data represent the averages of three independent measurements.

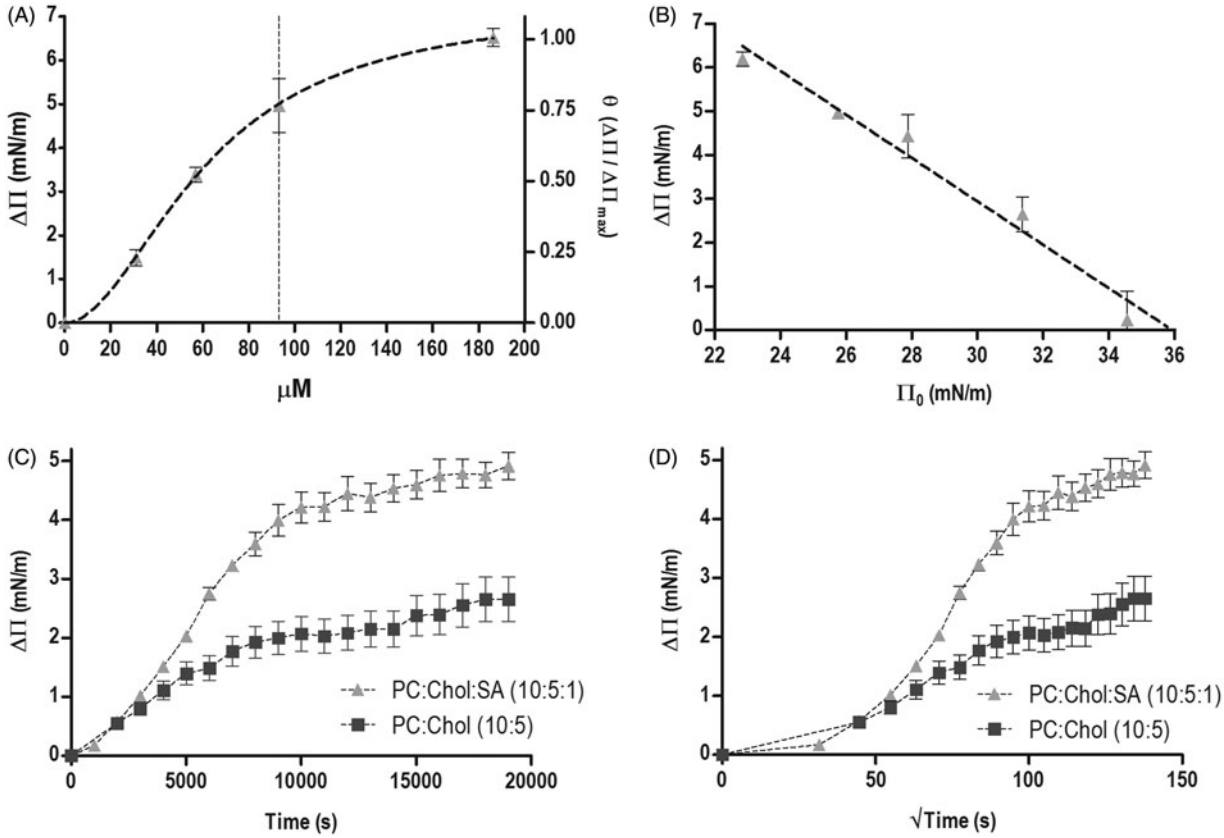

Figure 2. Interaction of S-layer proteins from *L. kefir* with lipid monolayers. (A) Changes in the surface pressure of monolayers made from phosphatidylcholine, cholesterol, and stearylamine in the molar relation of 10:5:1 expressed as ΔΠ (left axis) or degree of coverage θ (right axis) as a function of the protein concentration. (B) Changes in surface pressure as a function of Π_0 after addition of S-layer protein to reach a final concentration of 0.95 μM (dashed line on panel A). Variation of surface pressure on lipid monolayers after addition of S-layer protein to reach a final concentration of 0.95 μM as a function of time, (C) or time$^{1/2}$ (D). Initial surface pressure for curves in A, C and D was 21.5 mN/m. Each point is the average of at least triplicates of independent samples.

Langmuir adsorption behavior (n coefficient ≈ 1) (Figure 2A), in good agreement with previous results obtained by Zeta potential on liposomes (Hollmann et al., 2010a). When the effect of the condensation state of the monolayer was analyzed by changing the initial surface pressure (Π_0) we found that ΔΠ decreases linearly with increasing Π_0, being an exclusion surface pressure, determined by extrapolating the regression of the surface pressure (ΔΠ) versus Π_0 plots to the *xx* axis, of 35.9 mN/m (Figure 2b). This limiting surface pressure, defined as the maximum initial surface pressure above which the perturbation of the monolayer no longer occurs, is a measure of the membrane-penetrating power of the molecule (van Klompenburg et al., 1998).

Another advantage of the monolayer system as a lipid membrane model is that allows following the kinetic behavior in lipid–protein interactions. In this context, variations of the surface pressure as a function of time, after the protein injection, were recorded using a fixed protein concentration (Figure 2C). The kinetic pattern shows a deviation of linearity when it was plotted as a function of time$^{1/2}$ (Figure 2D). For a better comparison between the different deviations in the kinetic analysis, a relaxation coefficient (r), defined according to Equation (2), was obtained to characterize the protein adsorption kinetics (Table 2). The r coefficient value obtained, 0.66 ± 0.03, was far from 0.5, denoting an anomalous or non-Fickean diffusional process (Disalvo et al., 2013b; Hollmann et al., 2010b). However, if we deplete the stearylamine, a positively charged lipid, beside of a significant reduction of ΔΠ value (Figure 2), also the kinetic behavior changes to a fickean one with an r value of 0.52 ± 0.01 significantly lower than the ones obtained with stearylamine (p values $= 0.0163$, non-parametric t-test).

Table 2. S-layer proteins – lipid interactions characterization.

		S-layer from *L. kefir* JCM 5818
n from Langmuir equation	PC:Chol:SA	1.8
Cut off (mN/m)	PC:Chol:SA	35.9 ± 0.3
Slope (m)	PC:Chol:SA	-0.496 ± 0.050
Relaxation coefficient (r)	PC:Chol:SA	0.66 ± 0.06
	PC:Chol	0.52 ± 0.03

Parameters obtained from the fitting of the pressure data of lipid monolayers assays (Figures 2 and 3) using Equations (1) and (2).

S-layer coated liposomes

As an indication that liposomes were properly coated with S-layer proteins Zeta potential measures were carried out prior to cell interactions assays. After 150 min of incubation it was observed an inversion of Zeta potential values of liposomes from 80 mV to around −15 mV, which correspond with completely coated liposomes (Hollmann et al., 2007, 2010a). In a previous work by using freeze etching microscopy, we showed that s-layer coated liposomes retain their spherical

morphology (Hollmann et al., 2010a). In order to a better characterization of the liposomes after being coated with S-layer proteins, and taking advantage of the know sphericity, dynamic light scattering assays were done. The size distributions obtained for the LUV show a low polydispersity, with a 160 ± 5 nm average hydrodynamic diameter. Upon addition of S-layer protein, the hydrodynamic size of the LUV was increased 21 nm until 181 ± 6 nm (Figure 3). This significant (p values $= 0.0096$, non-parametric t-test), increase in the size could be easily explained by polymerization of the S-layer proteins onto the lipid bilayer as previously shown by electron microscopy (Hollmann et al., 2010a).

S-layer coated liposomes cells interactions

Finally, the interactions of S-layer coated liposomes with cells were characterized. The cytotoxic effect of control and S-layer coated liposomes on Caco-2 cell line was studied by MTT assay. The results showed that coated liposomes did not reduce the viability of Caco-2 cells in any of the concentrations tested (Figure 4). On the other hand, Caco-2 viability was significantly reduced upon incubation with the higher concentrations tested of control liposomes. At 3.3 mg total lipids/ml, the reduction of cell viability was $18.2 \pm 21\%$ and $30.4 \pm 10.9\%$ for 5.0 mg lipids/ml (Figure 4).

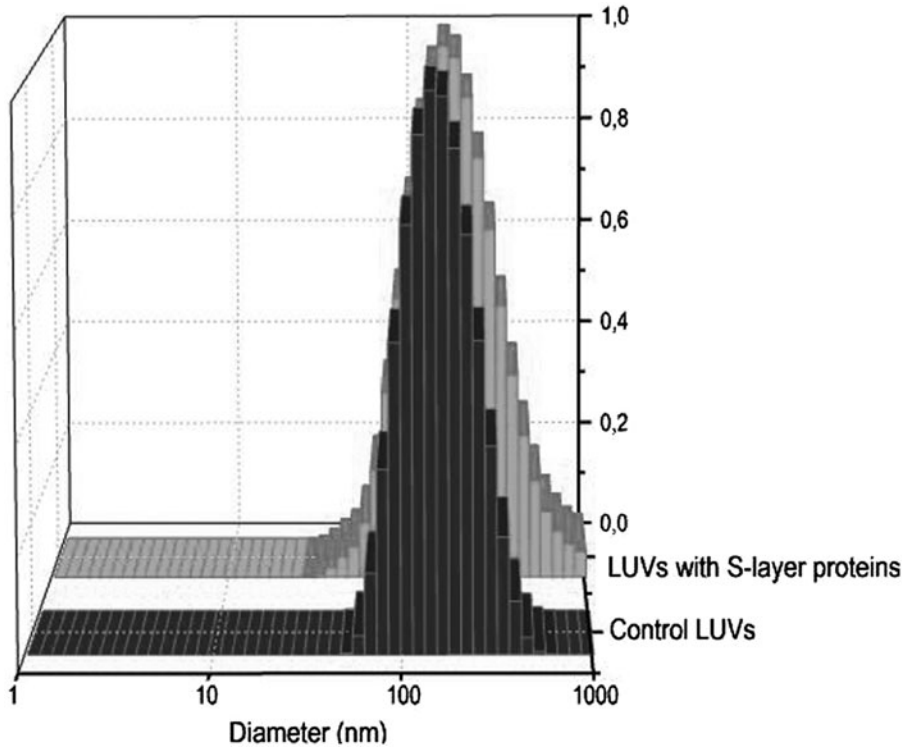

Figure 3. Size distributions of large unilamellar vesicles in the presence and absence of S-layer proteins.

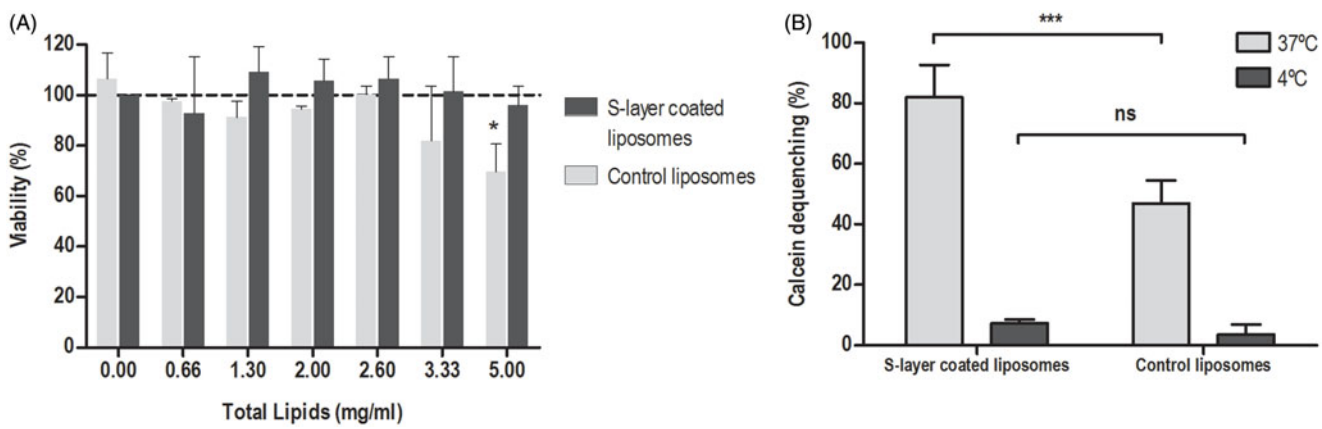

Figure 4. Interaction of S-layer coated liposomes with cells. (A) Cytotoxicity of control and S-layer coated liposomes on Caco-2 cells upon 4 h incubation, measured by MTT assay. Data represent the averages of three independent measurements. Error bars indicate standard deviations of the mean values. *: $p < 0.05$, 2 Way ANOVA followed by a Bonferroni post-test for multiple comparisons. (B) Quantification of the effect of temperature on cell internalization of control and S-layer coated liposomes analyzed by flow cytometry. Data represent the averages of three independent measurements. Error bars indicate standard deviations of the mean values. ***: $p < 0.001$, ns: Not significant, 1-Way ANOVA followed by a Dunnett post-test for multiple comparisons versus control column [Control liposomes].

In order to evaluate the ability of liposomes to transfer a cargo to Caco-2 cells, the fluorescent dye calcein was encapsulated in liposomes, prior to being coated with S-layer proteins, and by flow cytometry studies calcein fluorescence dequenching was recorded. The amount of lipid used for these tests (0.85 mg/ml) was lower than the concentration at which any cytotoxicity effects on control liposomes appeared. As a control of the experiment, micrographs of Caco-2 monolayers, as well as Caco-2 liposomes incubated cells were obtained in order to evaluate the integrity of monolayer cells during the experiment (data not shown).

As pointed above, the transference of cargo molecules from the liposomes to the cell cytoplasm was quantified as a calcein dequenching, as a result of dilution of the probe into the cells. It was observed that both liposomal preparations (coated and control) were able to transfer fluorescent probes to the cells (M1 region on histograms given by equipment, Figure 4(A) and Supplementary Figure 1). However, S-layer coated liposomes were able to transfer around 40% more fluorescent molecules than control ones (Figure 4B). In order to infer between fusion and endocytotic pathway in the uptake of vesicles, the effect of temperature on cargo transfer was also studied. It was previously demonstrated that most endocitytotic pathways are inhibited at 4 °C (Di Marzio et al., 2008; Kessner et al., 2001). Histograms on Supplementary Figure 1(C,D) show that uptake of both types of vesicles was strongly inhibited at 4 °C with respect to the same assay carried out at 37 °C. As it was expected from histograms, when transference at 4 °C was quantified, less than 10% of cargo molecules transfer were found for both liposomal preparations, without significant differences between each population (Figure 4).

Discussion

As food-grade and potentially probiotic organisms, lactobacilli are excellent candidates for health-related applications like live oral vaccines, where their ability to survive in the gastrointestinal tract could be utilized and their S-layer proteins could be used as carriers of antigens or other medically important molecules, possibly in combination with immunostimulatory or adhesive molecules (Hynonen & Palva, 2013). In the present work, we carried out a biophysical and biochemical characterization of the S-layer proteins from *L. kefir* as well as its interaction with lipid membranes and finally we tested by using Caco-2 cell lines the feasibility of using liposomes coated with S-layer proteins as a potential oral drug carrier.

The chemical characterization of S-layer proteins from *L. kefir* showed an oblique p2 symmetry on the cell surface, in good agreement with previously reported for *L. brevis* (Jakava-Viljanen et al., 2002) and *L. buchneri* (Messner et al., 1997), *Lactobacillus* species closely related to *L. kefir*. Furthermore, if we compare with the previous result obtained by recrystallization of proteins from S-layer of *L. kefir* on liposomes we could observe the same oblique pattern (Hollmann et al., 2010a). The highly basic *pI* found for this protein is also in good correlation with previous reports for another S-layer proteins from lactobacilli, with *pI* values between 9.35 and 10.4 (Avall-Jaaskelainen & Palva, 2005).

Glycosylation represents the most common post-translational modification in S-layer proteins and it has been demonstrated in many archaeal and bacterial species. Although, most of S-layers from lactobacilli appear to be non-glycosylated (Avall-Jaaskelainen & Palva, 2005), it was previously shown that S-layer proteins from *L kefir* were glycosylated (Mobili et al., 2009). When glycosidic moieties were characterized we found ten glycan residues for each protein unit, with a total percent of glycosylation of about 3% of weight, in a good correlation with previously reported from related species (Messner et al., 1997; Ristl et al., 2011). The present of glycosylation adds significantly to the potential functional spectrum of S-layer proteins (Messner et al., 1997). Mobili et al. (2009) have proposed that glycosylation on *L. kefir* could be related to the aggregation ability of these bacteria. Furthermore, the presence of glycan groups seems to be related to a higher affinity of these proteins for lipid membranes (Hollmann et al., 2010a).

When lipid interactions were evaluated, a departure from a Langmuir adsorption behavior was found when positively charged lipids were included in the monolayer. This behavior implies a non-independent adsorption sites, probably related to the protein–protein interactions in the crystallization process. The kinetics results were employed in order to characterize the diffusional process by obtaining the relaxation coefficient (r). A physical interpretation of r coefficient can be discussed in terms of normal and anomalous diffusion of the protein through the membrane. The linearity of the amount of substance adsorbed by a material with the square root of time assumes that the system responds to a linear gradient of concentration across the material in a steady state (Disalvo et al., 2015; Sanabria et al., 2007). When the diffusion behavior cannot be described adequately by Fick's law, anomalous or non-Fickean diffusion occurs. The difference is that the material where S-layer attaches (means lipid membrane) responds rapidly to changes in that last condition. Anomalous effects may be directly related to changes in the surface concentration that may account for changes in the solubility and diffusional mobility (Crank, 1975; Disalvo et al., 2015). In our context, the anomalous diffusion, present when positively charged lipid were added to the monolayer, suggests a synergistic effect due to a combination of electrostatic forces of interaction between lipid and proteins plus the interaction between proteins during the recrystallization process. This result confirms, that charges on the membrane are a key factor to the protein recrystallization. In other words, in the absence of charges the proteins still are able to adsorb to the membrane but from the pressure data, we could speculate that this adsorption does not let the formation of S-layer crystal structure. These findings are important factors in order to design liposome-based drug delivery systems, as a proper uniform recrystallization of protein on the surface of liposomes is a key factor in the resulting stability of the formulation (Hollmann et al., 2007).

As a first step to evaluate the possible application of this membrane supported by S-layer proteins, Caco-2 interaction experiments were done using liposomes coated with S-layer proteins. Caco-2 cells have been widely used as an *in*

vitro model to evaluate the intestinal epithelium transport of hydrophilic macromolecules, because the cell line expresses several characteristics of differentiated cells of the normal human small intestine (Awortwe et al., 2014; Volpe, 2011). Cytotoxic effects found for control liposomes are probably associated with the presence of stearylamine which, as many cationic lipids, have some toxic effects on different cell cultures (Cortesi et al., 1996; Lv et al., 2006; Yun et al., 2016). The absence of cytotoxicity on S-layer coated liposomes, which also have stearylamine, could be related to the titration of the charges of this lipid in the recrystallization process of the proteins on liposomes surface, the net charge exposed on the liposome surface shifts from 80 mV to −15 mV, indicating charge neutralization after S-layer incubation. It was previously described that Zeta potential of cationic vesicles dominates the cytotoxicity (Liang & Chou, 2009). Furthermore, recently it was shown that by changing the Zeta potential of the emulsions from positive to negative, S-layer lattice protected the cell from oxidative stress and cell membrane damage and, therewith, decreased the cytotoxicity (Ucisik et al., 2013).

Finally, we found that S-layer coated liposomes were able to transfer almost twice molecules than controls ones. The natural adhesive properties of S-layer proteins of lactobacilli to different epithelial cells (Avall-Jaaskelainen & Palva, 2005; Mobili et al., 2009; Schneitz et al., 1993), probably could promote the internalization, explaining the result found in the present study.

Although the internalization mechanisms require further study, the fact that no significant fluoresce transference were observed at 4 °C seems to indicate that an active process should be involved.

Results presented in this study represents the first insight concerning the interaction of S-layer coated liposomes with cells, revealing that S-layer liposomes may hold a great promise in developing a delivery system for drugs. Beside further experiment should be made in order to fully characterize this new kind of vehicle, our data confirm that the nanopatterned S-layer envelope did not only coat and modify the surface of the liposomes, but also transferred its characteristic features, thereby improving its biocompatibility and adhesive properties. These results, concomitant with higher stability of S-layer coated liposomes, and the fact that proteins are obtained from a GRAS (Generally Recognized as Safe) bacteria represent an exciting starting point in the development of new liposomal-based drug carriers. Furthermore, these findings offer a rational basis for the design of improved new carrier since they suggest that maximizing efficiency requires finding the proper balance of liposomal stability and cargo transfer ability.

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Declaration of interest

The authors report no declarations of interest.

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