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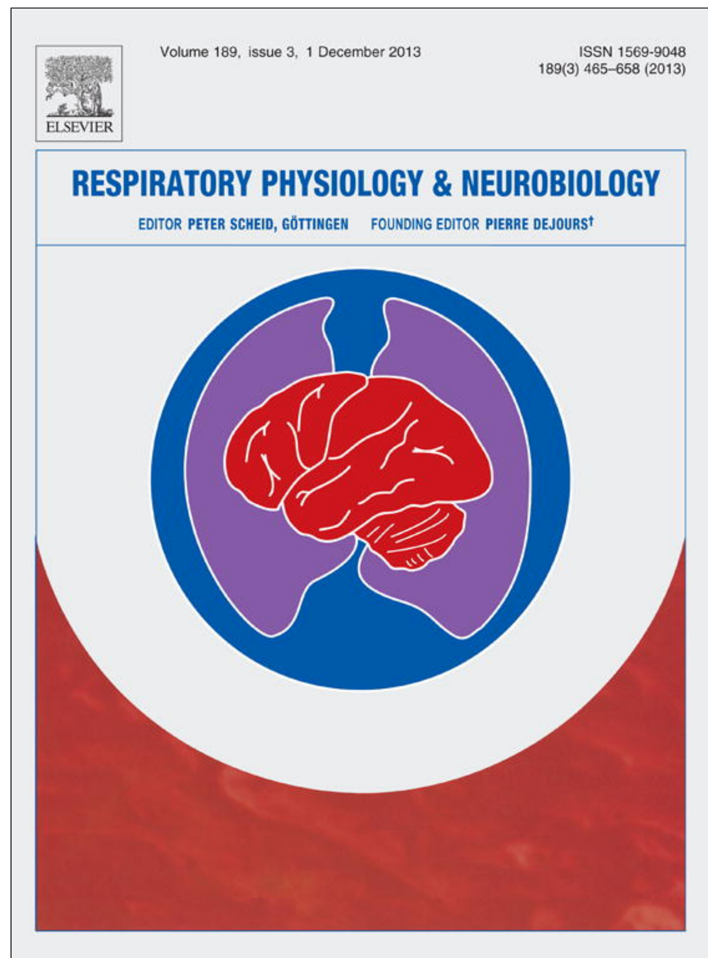

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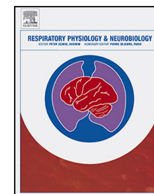

Effect of serum lipoproteins and cholesterol on an exogenous pulmonary surfactant. ESR analysis of structural changes and their relation with surfactant activity

María Martínez Sarrasague[a,*], Alejandra Cimato[a], Lidia Piehl[a], Fernando Brites[b], Graciela Facorro[a]

[a] Cátedra de Física, Departamento de Fisicomatemática, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Laboratorio de Lípidos y Lipoproteínas, Departamento de Bioquímica Clínica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

The study of structural changes in the surfactant may help to understand the mechanisms by which the surfactant is inactivated by serum. Here, we compared the *in vitro* effects of serum, albumin, lipoproteins (VLDL, LDL, HDL) and cholesterol on the dynamic and structural properties of surfactant suspensions by electronic spin resonance and surface tension measurements. Our results showed that albumin seems to be responsible for macrostructure disaggregation and increased rigidity in the hydrophobic region, but it did not affect surfactant activity. Fluidity in the polar area seems to be critical for proper physiological activity, and the changes induced by serum observed in this area would be generated by HDL or cholesterol, but the amount of cholesterol transferred by serum is not significant. Statistical analysis showed that surfactant activity correlated with the fluidity in the polar area but not with that in the hydrophobic region. We obtained strong evidence that among all the serum components tested, HDL is the one that causes the structural changes that compromise surfactant performance.

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1. Introduction

Pulmonary surfactant is a complex mixture of lipids and at least four specific proteins that form a surface-active film at the air–water interface of alveoli capable of reducing surface tension to near 0 mN/m (Creuwels et al., 1997). Rapid adsorption of pulmonary surfactant in the air-liquid interface is essential for normal breathing. Premature delivery can result in inadequate surfactant levels, a condition that can be reversed with transbronchial exogenous surfactant application (Clements and Avery, 1998; Poulain and Clements, 1995). While surfactant replacement has become a routine for the prevention and treatment of infant respiratory distress syndrome, exogenous surfactant therapy has proven to be less effective in acute lung injury and acute respiratory distress syndrome in adults. This failure may relate to a number of factors, among them plasma proteins leaking into the air spaces (Willson and Notter, 2011).

Understanding the mechanism by which serum proteins inactivate the surfactant is essential for formulating effective therapeutic preparations for these pathologies (Ochs et al., 2006; Matthay and Zemans, 2011). Among the proteins involved, albumin, hemoglobin and fibrinogen or fibrin monomers show strong surfactant inhibitory properties (Gregory et al., 1991; Günther et al., 2001; Lu et al., 2000; Seeger et al., 1985; William Taeusch et al., 1999). Several studies have shown that albumin, the most abundant serum protein, decreases the rapid adsorption of exogenous pulmonary surfactant (EPS) to an air–water interface to form surface-active films, but there are speculations about its action as an effective inhibitor of exogenous surfactants (Bummer et al., 1994; Otsubo and Takei, 2002; Warriner et al., 2002). Our previous studies have shown that surfactant inactivation by serum could not be attributed to the presence of serum albumin because, despite generating more inactive surfactant subtype, albumin did not affect the surface tension at any of the concentrations tested (0–20 mg/ml). We thus concluded that the disaggregation of the surfactant macrostructure would not be enough to generate EPS inactivation and that a serum component other than albumin would be interacting with EPS, producing its loss of activity (Martínez Sarrasague et al., 2012).

Among serum components, cholesterol (Cho) is widely recognized as a substance that alters the structure and surface activity of the surfactant (Blanco and Pérez-Gil, 2007; Daniels and Orgeig,

* Corresponding author at: Junín 956, 1113 Buenos Aires, Argentina.
Tel.: +54 11 49648201; fax: +54 11 49648204.
E-mail addresses: marga@ffyb.uba.ar, mmartinezsarrasague@yahoo.com.ar (M. Martínez Sarrasague).

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2003). Although cholesterol accounts for the main fraction of neutral lipids in pulmonary surfactant, how this molecule impacts the lateral structure and its role in surface dynamics are unclear (Diemel et al., 2002; Discher et al., 2002; Hook et al., 1984; Keating et al., 2007; Lange and Steck, 1985; Markart et al., 2007).

On the other hand, it is known that fluidity is a major factor determining the surfactant ability to spread at the air-cell interface. Electron Spin Resonance (ESR) is one of the most useful tools to study the bilayer fluidity and the interactions of different spin-labeled lipids with proteins (Budai et al., 2003, 2004; de Sousa Neto et al., 2009; Hubbell and McConnell, 1971; Lange and Steck, 1985; Marsh and Horvath, 1998). Previously, we have found that one or more serum components interact with phospholipids, and generate an increased rigidity of the bilayer in both the hydrophobic core and in the proximity to the polar region. The increased rigidity in the hydrophobic area could be due to albumin, whereas some other serum component would be responsible for the decrease in fluidity in the polar region. We have also demonstrated that the increased rigidity in the hydrophobic area does not affect surfactant activity, while the fluidity in the polar region would be critical for proper physiological activity (Martínez Sarrasague et al., 2012).

The aim of the present study was to find out which serum component/s is/are responsible for the changes observed in the polar region and the lack of surfactant activity. To achieve this objective, we focused on evaluating the *in vitro* effects of human serum lipoproteins (VLDL, LDL, HDL) and cholesterol on the dynamic and structural properties of surfactant suspensions and their respective heavy subtypes. We chose 5-doxyl stearic acid (5DSA) and 16-doxyl stearic acid (16DSA) as spin probes, as the radical in the 5- or 16-position of the alkyl chain can determine local motional profiles near the polar head group (5DSA) or at the end of the hydrophobic chain of the lipid (16DSA) (Nisreen et al., 2012). The analysis by ESR of the interaction of lipoproteins or cholesterol with the surfactant might help to understand the nature of these interactions and to establish *structure–function* relations.

2. Materials and methods

2.1. Exogenous pulmonary surfactant (EPS)

Prosurf is an active pharmaceutical ingredient (API) produced at industrial scale in Argentina (Nialtec S.A., Buenos Aires, Argentina). This API has been used by the pharmaceutical industry (GeMePe S.A. and Laboratorios Richet S.A., Buenos Aires, Argentina) for the elaboration of therapeutic surfactants. Prosurf is a sterile chloroform solution that contains lipids and proteins extracted by means of bronchoalveolar lavage from bovine lungs, with slightly hypertonic solution (Hager and De Paoli, 2001). Prosurf is composed of: phospholipids (PL) 94.8%; dipalmitoylphosphatidylcholine (DPPC) 46% of total phospholipids; cholesterol 4.4% and proteins (SP-B, SP-C) 0.8%. Chloroform was evaporated at low pressure and below 40 °C; the pellet was resuspended in sterile saline solution (0.9% NaCl) at 50 °C, obtaining a final phospholipid concentration of 30 mg/ml. This final suspension, fractionated in sterile vials, constitutes the exogenous pulmonary surfactant (EPS).

2.2. Exogenous proteins

Human serum was obtained from healthy adult donors. A pool of this human serum was used and the concentrations of its main components were: total proteins 6.8 g/dl, albumin 3.7 g/dl, gamma globulin 0.9 g/dl, total cholesterol 150 mg/dl, HDL with 54 mg/dl of Cho, LDL with 91 mg/dl of Cho, VLDL with 5 mg/dl of Cho, triglycerides 80 mg/dl and phospholipids175 mg/dl.

Lipoproteins were obtained from human serum and separated by ultracentrifugation. Bovine serum albumin (BSA) and cholesterol were purchased from Sigma–Aldrich.

2.3. Samples

EPS was diluted with saline solution (0.9% NaCl) to a final PL concentration of 10 mg/ml. EPS was added with serum to obtain a sample with 10 mg/ml of PL and 20 mg/ml of total serum proteins. The different serum proteins (BSA, LDL, VLDL, and HDL) were added to EPS separately. A sufficient amount of each protein was added to EPS to obtain a sample with 10 mg/ml of PL and a final protein concentration equal to that of each protein in the sample of EPS added with serum. All the samples were incubated for 20 min at 37 °C.

Taking into account that serum diluted to a final protein concentration of 20 mg/ml brings 0.5 mg/ml of cholesterol and that Prosurf has an average cholesterol concentration of 0.7 mg/ml (PL concentration 10 mg/ml), adequate aliquots of cholesterol chloroform solution were added to Prosurf before solvent was evaporated, to obtain EPS with 0.5 mg/ml of added cholesterol (final cholesterol concentration 1.2 mg/ml). EPS (10 mg/ml of PL) without added proteins or cholesterol was used as control.

2.4. Chemicals

The spin derivatives of stearic acid, 5DSA and 16DSA, were purchased from Sigma.

2.5. Chemical determinations

Phospholipids and protein concentrations were measured by the Stewart (1980) and Lowry et al. (1951) methods, respectively. Cholesterol was determined by the enzymatic method (Allain et al., 1974).

All the reagents were of analytical grade.

2.6. Heavy and light subtypes

Two surfactant subtypes, with different physiological capabilities, may coexist. They were separated by centrifugation at 10,000 × g for 20 min at room temperature. The supernatants containing the light subtype (inactive) were separated, and the pellets with the heavy subtype (active) were washed and resuspended to initial volume with saline solution (0.9% NaCl) (Gross et al., 2000; Veldhuizen et al., 1993). The percentage of each subtype was estimated as: (PL concentration in the fraction/PL concentration in the non-fractionated EPS) × 100, measured by chemical determination as described above.

2.7. Electronic spin resonance (ESR) spectroscopy

2.7.1. ESR samples

EPS samples were labeled with 5DSA or 16DSA as previously described (Martínez Sarrasague et al., 2012). Briefly, an adequate quantity of the spin probe in ethanolic solution was dried onto the sides of the incubation tubes under a stream of N_2 gas. Samples were added and incubated with the spin probe for 10 min at room temperature. The final concentration of the spin probe was 1.74 μM. Each sample was then placed into a capillary tube, and each capillary was placed into a quartz ESR sample tube and centered in a rectangular microwave cavity for ESR measurements.

2.7.2. ESR measurements

ESR measurements were performed using a Bruker EMX-Plus, X-band spectrometer (Germany) as previously described

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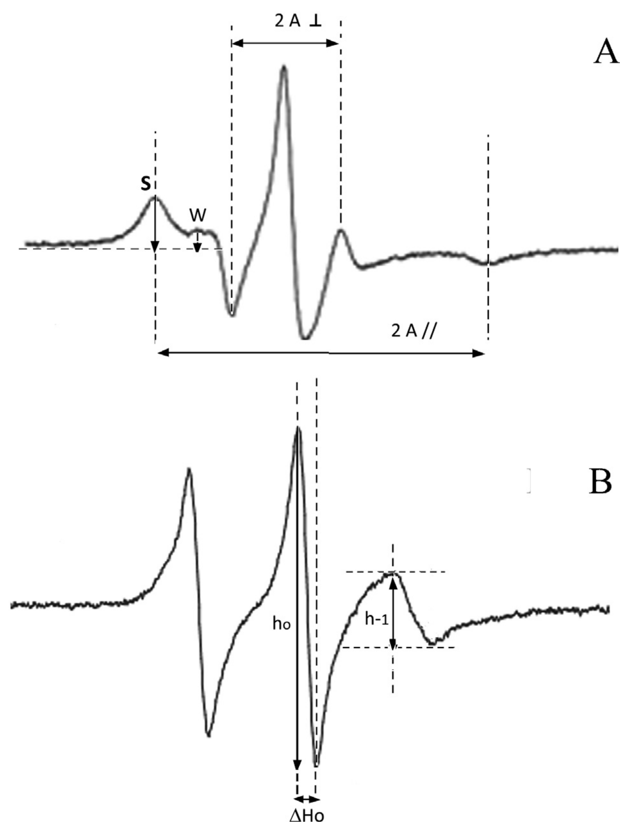

Fig. 1. ESR spectra of EPS labeled with 5DSA (A) and with 16DSA (B). (A) Separation, in Gauss, of the outermost ($2A_{\parallel}$) and innermost ($2A_{\perp}$) peaks of the ESR spectra. The arrows show the amplitude of two spectral components of the low-field peaks. These components are commonly called strongly (*S*) and weakly (*W*) immobilized ones. (B) Width of the central peak (ΔH_0), and the amplitude of the central peak h_0, and high field peak h_{-1} of the ESR spectra.

(Martínez Sarrasague et al., 2012). All ESR experiments were performed at 20 °C. Instrumental parameters were as follows: sweep width 100 Gauss, center field 4380 Gauss, time constant 5.12 ms, conversion time 5.12 ms, modulation amplitude 0.75 Gauss, modulation frequency 50 kHz, resolution 1024 points, microwave power 10 mW and microwave frequency 9.7 GHz.

2.7.3. Determination of ESR parameters

For 5DSA spectra, the hyperfine splitting tensors parallel and perpendicular with respect to the perpendicular direction of the membrane plane ($A_{\parallel}$ and $A_{\perp}$, respectively) were estimated by the separation in gauss of the outermost ($2A_{\parallel}$) and innermost ($2A_{\perp}$) peaks of the ESR spectra (Fig. 1A). The order parameter (*S*) is given by the ratio between the spectral anisotropy in the membranes ($A_{\parallel}$–$A_{\perp}$) and the maximum anisotropy obtained in a rigidly oriented system (defined by A_{xx}, A_{yy} and A_{zz}, the principal values of the spin label tensor) and can be calculated from the ESR spectrum (Martínez Sarrasague et al., 2012). Ordered phases, such as the gel or ordered liquid crystal phase, are characterized by values of *S* that approach 1, while the more fluid phases are characterized by *S* values that are significantly lower than 1. An increase in the *S* value is understood as a decrease in membrane fluidity.

The dynamic properties of the probe are related to the signal width (ΔH_0) and were estimated by calculating the rotational correlation time (τ). This empirical parameter can be calculated from the ESR spectrum of 16DSA (Fig. 1B) incorporated into EPS by the equation given by Keith and Snipes (1974) and Morse et al. (1979), as previously described (Martínez Sarrasague et al., 2012). In this case, τ is the parameter that can be used to measure the motion of the phospholipid acyl chains near the hydrophobic end. An increase in the τ value is understood as a decrease in the motional freedom of the probe in the hydrophobic area, due to an increase in the microviscosity of the environment.

The ESR spectra of spin-labeled phospholipid bilayers are generally characterized by the coexistence of two spectral components with very different states of probe mobility. These components are commonly called strongly (*S*) and weakly (*W*) immobilized ones, and are associated with restricted and less restricted nitroxide motion. The *S*/*W* ratio of the low-field peaks represents the population ratio of the spin label in the two motional states (Fig. 1A). Although this ratio is empirical, it provides a convenient method to compare the 5DSA spectra in different environments (Hayes and Jost, 1976).

2.8. Surface activity measurements

Surface activity was measured with a pulsating bubble surfactometer (Electronetics, Buffalo, NY, USA), as described by Enhorning (1977). Briefly, 36 μl of EPS suspension was instilled into the sample chamber of the surfactometer at 37 °C. A bubble communicating with ambient air was created in the surfactant suspension and the surfactant was allowed to adsorb to the air/liquid interface for 10 s. After this time, the bubble was pulsated at 20 oscillations per minutes between a minimum radius of 0.4 mm and a maximum radius of 0.55 mm.

2.8.1. Surface tension (ST)

This parameter represents the tendency of liquids to reduce their exposed surface to the smallest possible area. To determine ST, the pressure across the bubble was measured by a pressure transducer and the ST calculated using the La Place equation: $P = 2\,ST/r$, where *P* is the inflating pressure, and *r* is the radius of the bubble. The minimum value of ST at 200 cycles was determined. Each sample was measured five times, and the results are expressed as the mean ± SD. For the analysis of the results, a ST limit value of 5 mN/m was considered for a proper surfactant activity.

2.8.2. Percentage reduction in bubble surface area (ΔA_{10})

The percentage reduction in bubble surface area (SA) from its maximum value to that required for the surface tension to reach a value of 10 mN/m (ΔA_{10}) was calculated after 100 cycles of bubble cycling. ΔA_{10} is an indicator of dynamic film compressibility. Films with low compressibility cause a large decrease in ST with a relatively small decrease in SA. If the ST of the surfactant suspension does not reach 10 mN/m, then the actual, although unmeasured, ΔA_{10} values should be >47% because that is the difference in SA between the maximum and the minimum bubble areas in the Electronetics pulsating bubble surfactometer.

2.9. Experimental data acquisition and statistical analysis

All measurements were repeated with several independent surfactant batches that showed similar qualitative behavior. The results shown are the average of at least five separate experiments. Data are expressed as the mean ± SD.

Statistical analyses were performed using repeated measures analysis of variance (ANOVA), and comparisons between pairs of groups were made using the Bonferroni test. The linear relation between two variables was determined using the Pearson correlation test.

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Table 1
Correlation time, order parameter, S/W ratio, surface tension (ST) and percentage reduction in bubble surface area (ΔA_{10}) of the whole EPS (10 mg/ml of PL), and heavy fractions from: control EPS and EPS added with serum, VLDL, LDL, HDL, albumin (BSA) or cholesterol.

	Whole EPS	Heavy fractions						
		Control EPS	EPS + SERUM	EPS + VLDL	EPS + LDL	EPS + HDL	EPS + BSA	EPS + CHO
Correlation time τ (ns)	0.40 ± 0.01	0.36 ± 0.02	0.35 ± 0.09	0.35 ± 0.03	0.38 ± 0.03	0.38 ± 0.03	0.41 ± 0.11	0.39 ± 0.04
Order parameter S	0.619 ± 0.021	0.593 ± 0.009	0.607 ± 0.018	0.590 ± 0.008	0.597 ± 0.013	0.596 ± 0.015	0.599 ± 0.023	0.632 ± 0.008*
S/W ratio	2.09 ± 0.10	2.22 ± 0.20	2.19 ± 0.15	2.40 ± 0.08	2.09 ± 0.06	2.25 ± 0.11	1.94 ± 0.21	1.98 ± 0.10
ST (mN/m)	2.2 ± 1.8	4.5 ± 2.2	5.3 ± 3.2	4.0 ± 1.5	4.3 ± 2.1	4.8 ± 1.7	4.4 ± 1.9	5.8 ± 2.8
ΔA_{10} (%)	37.2 ± 3.3	34.5 ± 3.0	41.7 ± 5.8	34.8 ± 8.0	36.9 ± 3.7	36.8 ± 3.0	34.8 ± 4.5	42.5 ± 5.4

* Statistically significant differences compared to control EPS: $p < 0.01$.

3. Results

3.1. Heavy and light surfactant subtypes

We have previously found that the presence of serum or albumin causes disaggregation of the surfactant structures, inducing the transformation of the active into the inactive subtype. To evaluate if lipoproteins or cholesterol cause similar effects in the macrostructure of the surfactant, we measured the amounts of the heavy and light subtypes obtained in the presence of each type of lipoprotein or cholesterol by the chemical method. The addition of serum or BSA to the surfactant similarly decreased ($p < 0.01$) the amount of the heavy subtype. In contrast, the addition of cholesterol did not change the heavy/light subtype ratio significantly. No significant changes were observed in this ratio by the addition of any of the lipoproteins tested (Fig. 2).

3.2. ESR spectral analysis

The ESR spectrum yields information about the molecular environment of the spin probe (Fig. 1). The rotational correlation time (τ) calculated from the ESR spectrum of 16DSA incorporated into EPS reflects the motion of the PL acyl chain near the hydrophobic area. On the other hand, the ESR spectra of 5DSA incorporated into the EPS bilayer show an anisotropic motion, indicating that the probe movement is highly restrained. The order parameter (S) calculated from these spectra reflects the rotational freedom of PL close to the polar head groups in the layer. An increased S can be associated with a decrease in bilayer fluidity (Mat Met 2.7.3).

3.2.1. Structural changes in the hydrophobic area

Fig. 3 shows the τ value obtained from the spectra of EPS labeled with 16DSA, with and without exogenous proteins or cholesterol. The τ value increased in the presence of serum or albumin ($p < 0.01$). The increase in this parameter caused by the addition of albumin (10 mg/ml) was similar to that caused by the addition of serum (SP 20 mg/ml). High values of τ can be understood as a slower movement of the probe in the hydrophobic core due to a decrease in the fluidity of the environment. None of the lipoproteins tested changed this parameter significantly. Neither did cholesterol.

In all cases, the τ values of the heavy fractions obtained from the samples added with serum, albumin, lipoproteins or cholesterol showed no significant differences respect to the heavy fraction of the control. Further, the spectra of these fractions showed τ values similar to those of the whole EPS (Table 1).

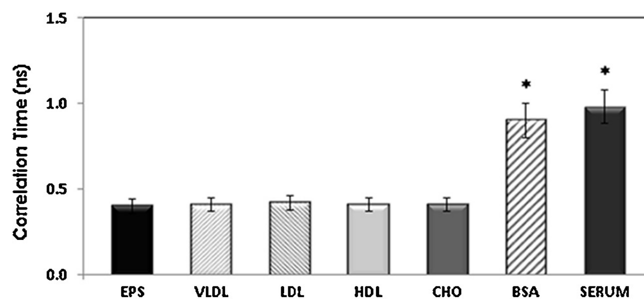

Fig. 3. Rotational correlation time (τ) obtained from the spectra of EPS (10 mg/ml of PL) labeled with 16DSA, with and without exogenous proteins or cholesterol. EPS was added with VLDL, LDL, HDL, cholesterol, albumin (BSA) or serum. The surfactant without added protein or cholesterol was used as control. Data are presented as the mean ± SD. Statistically significant increase compared to control, $^*p < 0.01$.

3.2.2. Structural changes in the polar region

Fig. 4 shows the order parameter (S) obtained from the spectra of EPS labeled with 5DSA, with and without exogenous proteins or cholesterol. In concordance with our previous results (Martínez Sarrasague et al., 2012), both EPS added with serum and that added with cholesterol showed an increased S value ($p < 0.01$), being the increase in samples with serum higher than that in samples with cholesterol. The S value did not change significantly with the addition of albumin, and among the lipoproteins tested, only HDL caused an increase in S ($p < 0.01$). No significant difference was

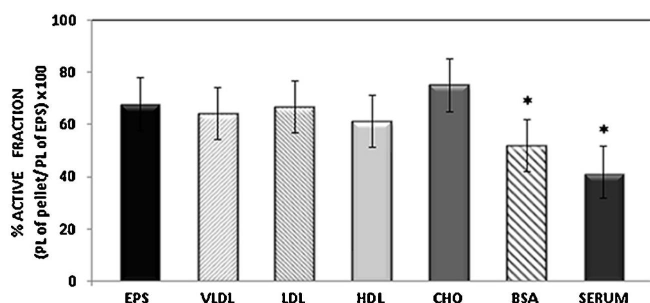

Fig. 2. Percentage of the active subtype obtained from EPS added with different serum components. The amount of heavy fraction was determined by the chemical method, and is expressed as (PL pellet/PL whole EPS) × 100. EPS (10 mg/ml of PL) was mixed with VLDL, LDL, HDL, cholesterol, albumin (BSA) or serum. The surfactant without exogenous protein or cholesterol was used as control. Data are represented as the mean ± SD. Statistically significant differences compared to control, $^*p < 0.01$.

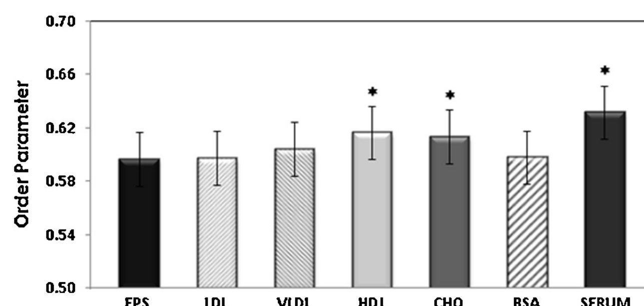

Fig. 4. Order parameter (S) obtained from the spectra of EPS (10 mg/ml of PL) labeled with 5DSA, with and without exogenous proteins or cholesterol. EPS was added with VLDL, LDL, HDL, cholesterol, albumin (BSA) or serum. The surfactant without added protein, or cholesterol was used as control. Data are represented as the mean ± SD. Statistically significant increase compared to control, $^*p < 0.01$.

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Table 2
Surface tension (ST) and percentage reduction in bubble surface area (ΔA_{10}) of the surfactant (10 mg/ml of PL) added with serum, BSA, Cho or lipoprotein (VLDL, LDL, HDL). ST was measured with a pulsating bubble surfactometer at 37 °C.

	Control EPS	EPS + serum	EPS + VLDL	EPS + LDL	EPS + HDL	EPS + BSA	EPS + CHO
ST (mN/m)	2.2 ± 1.8	20.0 ± 5.7*	2.0 ± 0.9	3.3 ± 2.1	8.5 ± 2.9*	3.5 ± 1.9	10.5 ± 5.1*
ΔA_{10} (%)	37.2 ± 3.3	>47(*)	35.5 ± 9.0	38.4 ± 4.6	43.6 ± 3.0*	37.7 ± 3.9	40.0 ± 4.7*

* Statistically significant differences compared to control: $p < 0.01$.

observed between the increase in S generated by serum and that generated with HDL.

Similarly to that observed with τ, the S of the heavy fractions obtained from the samples added with serum or HDL showed no significant differences respect to the heavy fraction of the control or the whole EPS (Table 1). The order parameter value of the heavy fractions obtained from EPS added with cholesterol was higher than that of the heavy fraction of the control ($p < 0.01$).

3.2.3. Bilayer organization

A common feature of almost all lung surfactants and model mixtures is the coexistence of a semi-crystalline fluid-ordered phase and a fluid-disordered phase (Alonso et al., 2004). The spin probe incorporated into these phases has different rotational motion and consequently yields a spectrum with differences at the low-field peak (see Fig. 1A). The increase in S/W ratio could be understood as an increase in the fluid-ordered phase where the spin probe is more immobilized. Fig. 5 shows the S/W ratio obtained from the 5DSA spectra of EPS with and without exogenous proteins or cholesterol. The addition of BSA decreased the S/W ratio ($p < 0.05$), whereas the addition of LDL, HDL or VLDL increased it ($p < 0.01$; $p < 0.05$; $p < 0.01$, respectively). On the other hand, neither cholesterol nor serum changed this parameter significantly. In all the cases, the S/W ratio of heavy fractions obtained from samples added with proteins showed no significant differences respect to the heavy fraction of the control (Table 1).

3.3. EPS surface properties

Table 2 shows that the addition of serum or cholesterol to EPS increased ST and ΔA_{10}, reaching values higher than those compatible with biological activity. Among the different lipoproteins tested, only HDL increased these parameters, causing surfactant inactivation. In contrast, when BSA was added to EPS, ΔA_{10} and ST did not change significantly.

The heavy fractions of all the samples tested showed ΔA_{10} and ST values not significantly different from those of the heavy subtype control (Table 1).

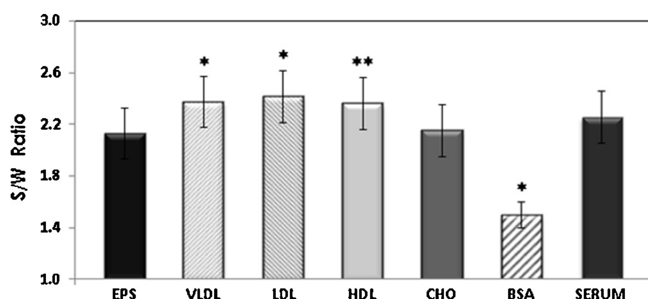

Fig. 5. S/W ratio obtained from 5DSA spectra of EPS (10 mg/ml) with and without exogenous proteins or cholesterol. EPS was added with VLDL, LDL, HDL, cholesterol, albumin (BSA) or serum. The surfactant without added protein, or cholesterol was used as control. Data are presented as the mean ± SD. Statistically significant differences compared to control, $^{**}p < 0.05$ and $^{*}p < 0.01$.

All the supernatants showed ΔA_{10} values higher than 47% and ST higher than 18 mN/m, confirming that the light fraction is devoid of surfactant activity.

3.4. Statistical correlations

To confirm the relations between the spectroscopic parameters evaluated and surfactant activity found in our previous paper, linear regression studies were carried out for EPS added with serum, BSA, cholesterol, lipoproteins and EPS control (Table 3). The increase in S was associated directly with an increase in ST and with an increase in ΔA_{10}. On the other hand, the fluidity in the hydrophobic area, the fluid-ordered phase/fluid disordered phase ratio (S/W ratio) and the amount of heavy subtype did not correlate with the surface tension parameters.

4. Discussion

It is widely known that the serum shows strong inhibitory properties on the surfactant and causes changes in its structure, related to its inactivation process (Greene et al., 1999; Griese, 1999; Günther et al., 2001; Nag et al., 2007; Schmidt et al., 2001). The present study was designed with the aim to find out which serum component/s is/are responsible for the structural changes, and their relation with surfactant activity. To achieve this goal, we analyzed the changes caused by different fractions of the serum in the surfactant macrostructure and its bilayer organization, in an attempt to understand the mechanisms by which the surfactant is absorbed at the air–liquid interface and could be inactivated by serum components.

In several types of lung injury, the surfactant macrostructure is affected by an increase in the ratio between small and large surfactant aggregates (Maitra et al., 2002; Ueda et al., 1994; Veldhuizen et al., 1996). We have previously reported that both serum and albumin produce the disaggregation of surfactant structures, increasing the quantity of the inactive subtype, but found that this effect is not enough to cause surfactant inactivation (Martínez Sarrasague et al., 2011). The results obtained in the present study confirm this hypothesis and allow us to conclude that the disaggregation of the macrostructure of the surfactant, generated by the addition of serum, can be attributed to albumin since neither lipoproteins nor cholesterol affected the heavy/light subtypes ratio.

In our previous study (Martínez Sarrasague et al., 2012), we found that serum interacts with the surfactant phospholipids,

Table 3
Pearson test for statistical correlation between structure (Variable 1) and activity (Variable 2).

Structure–activity		
Statistical correlation		
Variable 1	Variable 2	Pearson correlation
Order parameter	Surface tension	r = 0.941 (p < 0.001)
S/W ratio	Surface tension	No correlation
Correlation time	Surface tension	No correlation
Amount of heavy fraction	Surface tension	No correlation
Order parameter	Spreading (ΔA10)	r = 0.835 (p < 0.001)

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generating an increased rigidity of the bilayer in both the hydrophobic core and in the proximity to the polar region. Among the different serum components tested, only albumin modified the fluidity in the hydrophobic area, and did it very similarly to that observed by the addition of serum. So, we argued that the increased rigidity in this area could be due to the albumin present in it. To confirm this assumption, in the present work we tested other serum components and we found that neither lipoproteins nor cholesterol altered the fluidity in the hydrophobic area. Thus, albumin would be the only responsible for changes in this area generated by serum.

On the other hand, both the addition of cholesterol and HDL caused alterations in the polar zone order. Cholesterol is widely recognized as a substance that alters the structure and surface activity of the surfactant (de la Serna et al., 2004; Diemel et al., 2002; Discher et al., 2002; Keating et al., 2007). So, it could be thought that serum cholesterol is the main responsible for the changes induced by serum in the polar region of the bilayer. The results obtained in this study do not agree with this hypothesis. First, samples of EPS added with cholesterol (equivalent to the one that would be obtained if the serum transferred all its cholesterol to the surfactant) showed lower rigidity in the polar region than EPS added with serum. Second, the heavy fractions obtained from EPS added with serum did not show a significant increase in the rigidity whereas the heavy fractions from EPS added with cholesterol did. Finally, samples of EPS added with cholesterol showed higher values of surface tension than those consistent with adequate surface activity. So, if all the serum cholesterol had been transferred, the heavy fractions from samples added with serum should show no surface activity; however, when the serum was removed, the heavy fractions recovered normal surface tension values. This demonstrates that the amount of cholesterol transferred by serum would not be sufficient to generate the changes observed in the polar region of the surfactant. Therefore, it is reasonable to conclude that HDL would be responsible for the increased rigidity of this area. Moreover, the increase in the order parameter obtained in the presence of HDL was not significantly different from that obtained in the presence of serum.

Furthermore, among all the serum components tested individually, only HDL and cholesterol modified the surface properties of the surfactant. So, considering that the EPS added with serum, cholesterol or HDL showed an altered dynamic surface tension and compressibility (ΔA_{10}), as well as an increased rigidity in the polar region, we conclude that the fluidity in the polar region would be critical for proper physiological activity. This last assumption is supported by the correlation between the results of the order parameter and the tension activity parameters obtained by statistical analysis. In addition, albumin, LDL or VLDL generated no changes in surface tension or in S, confirming the link between the fluidity of the polar region of the lipid layer and the surfactant activity.

On the other hand, albumin did not alter the dynamic surface tension or compressibility but led to an increase in the rigidity in the hydrophobic zone of the bilayer. So, it could be assumed that the increased rigidity in this region does not affect surfactant activity. Correlation studies confirmed this assumption.

The mechanical properties of an interfacial film are determined by their physical state. Bilayers composed of DPPC exhibit two phase transitions induced by temperature: a highly ordered, relatively immobile gel phase, exhibiting neither lateral nor rotational mobility (L_β), and a fluid disordered-like phase (L_α). These two phases are defined by the strength of the attractive Van der Waals interactions, and determine the fluidity of the bilayer (Casals and Cañadas, 2012). Several studies have evidenced that these lipid phases coexist in the surfactant (Nag et al., 2002; Scherfeld et al., 2003).

The decrease in the S/W ratio in the EPS added with albumin indicates that this protein interacts with the surfactant, generating a larger proportion of fluid-like disordered phase. This further evidences that albumin would not be responsible for the inactivation of the surfactant, because the increase in fluid-disordered-like phases would allow better spreading and proper dynamic surface behavior (de la Serna et al., 2004; Dhar et al., 2012). On the other hand, all the lipoproteins generated an increased proportion of fluid ordered regions. These two opposite effects produced by albumin and lipoproteins could explain why the S/W ratio is not affected in samples of EPS added with serum. Lipoproteins would offset the decrease in S/W ratio caused by albumin. However, our results do not allow correlating the S/W ratio with the dynamic surface tension and compressibility. Although all lipoproteins increased the proportion of fluid-ordered phase, neither LDL nor VLDL caused loss of surface activity. The only lipoprotein that altered the surface properties was HDL, but this inhibitory effect could not be attributed to a higher amount of ordered domains. Statistical analyses confirmed this assumption. Therefore, as discussed above, the lack of surface activity correlates only with the increased rigidity in the polar region, and among lipoproteins, only HDL caused this effect.

We have previously demonstrated that surfactant inactivation by serum is due to a physical interaction with phospholipids of the surfactant, an effect that is reversed when serum or albumin are removed, as all heavy fractions obtained from the surfactant added with serum or albumin show no significant differences regarding the respective heavy fraction of the control or the whole EPS (Martínez Sarrasague et al., 2012). In this work, we study the structure and surface activity of the heavy fractions and we obtained similar reversible results with all the lipoproteins assayed (Table 1).

Our results clearly indicate that changes in the lipid order in the polar region, where surfactant proteins are anchored (Ding et al., 2001; Korlach et al., 1999), would affect the essential role that they have in surface properties. The fluidity in the hydrophobic zone seems to be less important since the surfactant does not act as a bilayer but becomes a monolayer to expand-positioned at the air-liquid interface.

5. Conclusions

In summary, our results show that:

(a) the disaggregation of the macrostructure of the surfactant, generated by the addition of serum, can be attributed to albumin since this effect is not caused by lipoproteins or cholesterol;
(b) the fluidity in the polar region would be critical for proper physiological activity while the increased rigidity in the hydrophobic area would not affect surfactant activity;
(c) the amount of cholesterol transferred by serum would not be enough to generate the changes observed in the polar region of the surfactant and its subsequent inactivation;
(d) the proportion of fluid-disordered/fluid-ordered phases does not correlate with surface activity;
(e) changes in fluidity of the polar region, essential for proper surfactant performance, would be generated by HDL.

We thus obtained strong evidence that among all the serum components tested, HDL is the one that causes structural changes in the surfactant bilayer phase, which compromise the surfactant performance. Our results contribute to a better understanding of the structure–function relations in an exogenous pulmonary surfactant which may help in the design and production of more efficient clinical surfactants. Further studies will be carried out to find out if the changes in the fluidity of the bilayer could cause structural alterations in the surfactant proteins.

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