PALMITOYLATION OF PULMONARY SURFACTANT PROTEIN SP-C IS CRITICAL FOR ITS FUNCTIONAL COOPERATION WITH SP-B TO SUSTAIN COMPRESSION/EXPANSION DYNAMICS IN CHOLESTEROL-CONTAINING SURFACTANT FILMS

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ABSTRACT

Recent data suggest that a functional cooperation between surfactant proteins SP-B and SP-C may be required to sustain a proper compression-expansion dynamics in the presence of physiological proportions of cholesterol. SP-C is a dually palmitoylated polypeptide of 4.2 kDa, but the role of acylation in SP-C activity is not completely understood. In the present work we have compared the behavior of native palmitoylated SP-C and recombinant non-palmitoylated versions of SP-C produced in bacteria to get a detailed insight into the importance of the palmitic chains to optimize interfacial performance of cholesterol-containing surfactant films. We found that palmitoylation of SP-C is not essential for the protein to promote rapid interfacial adsorption of phospholipids to equilibrium surface tensions (~22 mN/m), in the presence or absence of cholesterol. However, palmitoylation of SP-C is critical for cholesterol-containing films to reach surface tensions ≤1mN/m at the highest compression rates assessed in a captive bubble surfactometer, in the presence of SP-B. Interestingly, the ability of SP-C to facilitate reinsertion of phospholipids during expansion was not impaired to the same extent in the absence of palmitoylation, suggesting the existence of palmitoylation-dependent and -independent functions of the protein. We conclude that palmitoylation is key for the functional cooperation of SP-C with SP-B that enables cholesterol-containing surfactant films to reach very low tensions under compression, which could be particularly important in the design of clinical surfactants destined to replacement therapies in ARDS.

INTRODUCTION

Breathing and lung mechanics critically depend, on a molecular level, on a great variety of complex lipid/lipid and lipid/protein interactions that occur in pulmonary surfactant, a lipid/protein complex that lines the inner surface of the lung and functions to reduce the surface tension at the air/liquid interface in order to maintain alveolar stability, prevent alveolar collapse (atelectasis) and minimize the work of breathing (1, 2).The lack or dysfunction of an active surfactant results in severe respiratory disorders, such as Neonatal Respiratory Distress Syndrome (NRDS) (3) or Acute Respiratory Distress Syndrome (ARDS) that frequently accompanies lung injury and is often lethal (4).

In order to fulfill its function, pulmonary surfactant has been evolutionarily optimized to assemble a very specific and complex composition of lipid and protein components. About 80-85% of total surfactant weight are phospholipids, with about half of it being dipalmitoylphosphatidylcholine (DPPC), 5-10 % neutral lipids, mainly cholesterol, and 6-8% specific surfactant-associated proteins (5, 6). Despite the very complex composition that enables lung surfactant to function under variable physiological conditions and to respond to environmental challenges, DPPC, the major lipid species in lung surfactant, and the two small hydrophobic surfactant proteins SP-B and SP-C have emerged as the most critical components for a functional surfactant (7). DPPC is absolutely necessary for surfactant films to reach very low surface tensions ($\leq$1mN/m) upon compression. On the other hand, SP-B and SP-C have been shown to be required for efficient interfacial adsorption, film stability and respreading of the surfactant film upon expansion (8). The combination of these properties is pivotal for the dynamic behavior of surfactant films during repeated cycles of compression and expansion throughout breathing *in vivo* (9).

SP-C is the smallest of the surfactant-associated proteins. Mature SP-C consists of a C-terminal very hydrophobic alpha-helix and a dually palmitoylated N-terminal segment (10, 11). Although SP-C is not absolutely critical for survival, SP-C (-/-) mice develop progressive pneumonitis of different degrees depending on the genetic background (12, 13). *In vitro* data indicate that SP-C is required to maintain surfactant reservoirs attached to the interfacial film during states of high compression, such as those thought to occur at the end of expiration (14). These reservoirs could then reinsert rapidly under the critical participation of SP-B. The palmitoylation of SP-C has been proposed to be important for the stabilization of the surfactant film associated reservoirs (15, 16).

The presence of cholesterol in pulmonary surfactant, although constituting between 5 and 10% of total surfactant mass, has been regarded as detrimental for proper lung function. Hence, *in vitro* data suggest severe structural changes in surfactant films in the presence of high concentrations (20%) of cholesterol (17) and clinical surfactant substitutes do not usually contain cholesterol (5, 18). Furthermore, the presence of exacerbated levels of cholesterol has been implicated in the pathology of ARDS and other diseases (19, 20). Nevertheless, cholesterol has been shown to have important effects on the fluidity of surfactant mono- and bilayers (21), and to modulate molecular dynamics of phospholipids in surfactant complexes (22). In fact, cholesterol levels in surfactant are tightly controlled and are regulated in some species in response to temperature changes (23). Recent reports have shown that SP-B and SP-C can cooperate to counteract the deleterious effects of cholesterol on surfactant film stability during continuous cycling (24). Moreover, experimental data in support of a direct interaction between SP-C and cholesterol (25, 26) suggest an important functional role for these interactions in pulmonary surfactant structure and/or performance.

In the present work, we investigate the role of palmitoylation of SP-C for the cooperation with SP-B to stabilize cholesterol-containing surfactant films subjected to compression/expansion dynamics in a captive bubble surfactometer (CBS). By comparing native palmitoylated SP-C with recombinant (non-palmitoylated) versions of the protein, we show that the presence of the palmitic chains is critical for SP-C to cooperate with SP-B in this function. Only native palmitoylated SP-C enabled surfactant films containing cholesterol to reach very low surface tensions during continuous compression/expansion cycles, with little hysteresis. The present data, therefore, refine the present concept of how SP-B and SP-C might cooperate *in vivo* to enable proper functioning of cholesterol-containing pulmonary surfactant.

MATERIALS AND METHODS

Reagents. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)](POPG) and cholesterol were all from Avanti Polar Lipids (Alabaster, AL). Chloroform and methanol solvents, HPLC grade, were from Scharlau (Barcelona, Spain). High restriction grade thrombin was from Novagen. Native surfactant proteins SP-B and SP-C were isolated from minced porcine lungs as described elsewhere (27).

Recombinant protein purification. For the production of different recombinant SP-C versions, we essentially followed the procedures described by Lukovic et.al. (28). The protein had to be expressed as a fusion protein with a very soluble bacterial nuclease. A thrombin cleavage site (LVPR/GP), introduced between the nuclease and the amino acid sequence for mature SP-C, allowed the proteolysis of the fusion protein. After proteolytical processing, recombinant SP-C was recuperated by organic extraction and size exclusion chromatography on a Sephadex LH-20 column (Amersham Biosciences). Recombinant protein quality was controlled by SDS-PAGE, mass spectrometry and amino acid analysis. Table 1 summarizes and compares the sequences of the produced proteins. Due to the purification procedures that involve a thrombin digestion at an optimized cleavage site to yield recombinant SP-C from the fusion protein, most of the recombinant SP-C variants used here contain an N-terminal GP extension. Previously, we had not found significant structural or functional differences between recombinant forms bearing or not these extensions (28). Nevertheless, some of the experiments shown in the present study were also repeated with SP-C versions that do not contain the GP extension, and their behavior was again found to be completely comparable, as it will be described. Since final protein yield is much higher for the GP-containing SP-C versions, unless otherwise stated the recombinant protein used in the experiments was produced with this dipeptide-extension.

*Reconstitution of lipid and lipid/protein samples.*The standard lipid mixture used in this study to reconstitute pulmonary surfactant model suspensions contained DPPC/POPC/POPG (50/25/15, w/w/w). This mixture simulates the proportion of saturated/unsaturated and zwitterionic/anionic phospholipids in surfactant. The mixture was modified by adding different proportions of native or recombinant surfactant proteins, as described in each set of experiments. When indicated, the samples contained 5% cholesterol to mimic the physiological surfactant composition. Multilamellar suspensions were prepared by mixing the appropriate amount of protein and lipids in chloroform/methanol 2:1, drying the samples overnight under vacuum and resuspending them at 45ºC in the desired final volume of sample buffer (5 mMTris,150 mM HCl buffer, pH 7.0) for 1.5 hours and shaking. The lipid suspensions were generally prepared at a final concentration of 10mg/ml.

Circular Dichroism

Far-UV circular dichroism (CD) spectra were recorded as previously described (29) in a Jasco 715 spectropolarimeter equipped with a xenon lamp. All spectra were recorded in a 0.2 mL thermostated quartz cell with an optical path length of 0.1 cm. Molar ellipticity was calculated taking 110 as the mean molecular weight per residue. At least two different batches of each protein were analyzed, with comparable results. Estimation of the secondary structure content from the CD measurements was performed after deconvolution of the experimental spectra into four simple components (αhelix, β-sheet, turns and random coil) using the CDPro software package containing three commonly used programmes: SELCON3, CONTIN/LL and CDSSTR (30). This software allows the use of different sets of proteins, including membrane proteins (SMP50).

Captive bubble surfactometry. The surface activity of the model surfactant mixtures was assessed in a computer-controlled Captive Bubble Surfactometer (CBS) operated as described previously (24). This device, described in detail elsewhere (31, 32), allows a reliable and reproducible estimation of the surface tension of surfactant films subjected to compression/expansion dynamics. The CBS chamber was filled with ~1.5 mL of sample buffer (5 mM Tris, 150 mM NaCl, pH 7.0) containing 10% w/w sucrose (Sigma), in order to increase its density, and no surfactant. Once the surfactant sample is accurately measured and injected with a micropositioning device at the proximity of the bubble surface, surfactant suspensions

float and remain in close contact with the bubble upon injection (see supplementary Figure S1). The presence of sucrose does not affect surface activity of surfactant (33). The temperature was maintained at 37 ºC. A small air bubble (approx. 2mm in diameter, 50 μl) was introduced into the chamber after degassing the buffer solution and allowed to float up to the agarose ceiling. Then, ~0.5 μl of lipid or lipid/protein surfactant suspension (10 mg/ml) were deposited directly at the air/buffer interface of the bubble by means of a transparent capillary. The bubble was imaged with a video camera (Pulnix TM 7 CN) and recorded for later analysis. A 5 min adsorption (film formation) period followed the introduction of the surfactant model suspensions into the chamber during which the bubble was not manipulated and the change in surface tension was monitored (initial adsorption). The chamber was then sealed and the bubble was rapidly (1s) expanded to a volume of 0.15ml and changes in surface tension were monitored during 5min (post-expansion adsorption). As the size of the bubble is increased significantly, post-expansion adsorption also evaluates how efficiently a surplus of surfactant has maintained association with the interface, after initial injection, to refill the new opening surface. Then, quasi-static cycling was initiated, where the bubble size was first reduced and then enlarged in a stepwise fashion. Each step had two components: a 3 sec change in volume followed by a 4 sec delay where the chamber volume remained unchanged and the film was allowed to equilibrate. Under these conditions, interfacial structures could relax along the compression-expansion isotherms. Compression was always stopped when minimum surface tension was obtained, before the collapse of the film would lead to a variable level of non-reproducible overcompression. The collapse point at which overcompression can be prevented was judged visually, when the height of the bubble does not change upon further compression but the bubble shrinks in diameter instead. In the dynamic cycles, the bubble size was continuously compressed and expanded over the same volume range as during the quasi-static cycles for 20 cycles at a rate of 20 cycles/min (roughly in the range of the respiration rate of the lung). Supplementary Figure S2 illustrates and compares the waveform of area oscillation under quasi-static and dynamic conditions. For all imaged bubbles, volume, interfacial area, and surface tension were calculated using height and diameter of the bubble as previously described (24, 34). At least 4 independent experiments were performed for each surfactant model suspension, using at least two different batches of purified protein, with qualitatively comparable results. Data from adsorption experiments are presented as means ± s.d. Compression-expansion isotherms are representative experiments.

RESULTS

In order to get new insights into the role of the palmitoylation of the N-terminal segment of SP-C, we compared native palmitoylated SP-C purified from porcine lungs with two different recombinant versions of SP-C that were produced in *E.coli*. One of them reflects the wild-type sequence of human mature SP-C. Since bacteria lack the mammalian palmitoylation machinery, this recombinant wild-type SP-C (rSP-C CC) is isolated without palmitic chains and displays two free cysteines at positions equivalent to residues 5 and 6 in the wild type sequence (Table 1). Mass spectrometry and electrophoretic analysis confirm that this protein stably maintains the cysteines in their reduced form during storage and during the periods of time in which the present experiments were carried out. The other recombinant SP-C variant studied contained two phenylalanine residues substituting for the cysteines (rSP-C FF). The rationale for choosing this replacement was that in several carnivorous animals (e.g. dog, seals), one of the two cysteines at the N-terminus of SP-C is substituted by phenylalanine(35). It has been argued that this amino acid could mimic some properties of palmitoylcysteines due to the bulkiness of the aromatic ring and its propensity to insert within the membrane interface. rSPC FF has therefore been proposed as a potentially functional equivalent to wild-type SP-C in clinical preparations.

In a first series of experiments, we investigated how the presence or absence of palmitoylation would affect the surface activity of the protein. To this end, we analyzed the behavior in a captive bubble surfactometer (CBS) of DPPC/POPC/POPG (50/25/15, w/w/w) lipid suspensions containing or not 5% cholesterol, in the absence or presence of 1% of native or recombinant SP-Cs. Figure 1 shows the time-dependent reduction of surface tension upon adsorption of the different lipid or lipid/protein samples once deposed at the air/liquid interface of an air bubble (initial adsorption) or after expansion of the bubble and observation during 5 minutes (adsorption post-expansion). Initial adsorption includes association of injected complexes to the bubble surface and transfer of surface active molecules into the interface. Post-expansion adsorption to a bubble which has been considerably enlarged also evaluates the ability of the surplus of surfactant initially injected to form a surface-associated reservoir ready to refill the newly open interface. In the absence of protein, purely lipidic suspensions showed very limited interfacial adsorption to equilibrium tensions that were barely lower than 55 mN/m, regardless of the presence of cholesterol. In contrast, samples containing 2% of any of the SP-C forms exhibited, in the absence of cholesterol, a rapid, almost instantaneous drop of surface tension from ~70mN/m to below 30mN/m, close to the equilibrium surface tensions. Palmitoylation, therefore, does not seem to be required for SP-C to promote efficient interfacial adsorption in the absence of cholesterol, as there are no significant differences in this respect between native and recombinant variants. Surprisingly though, in the presence of 5% cholesterol, while surfactant films containing palmitoylated SP-C still readily reached low surface tension both right after sample deposition and after expansion, the presence of non-palmitoylated SP-C did not have the same effect and the respective lipid films were not able to reduce the surface tension as much as native SP-C (nSP-C). rSPC FF behaved similar to native SP-C, although the reduction in surface tension at initial adsorptions was somewhat slower. However, rSP-C CC performed worse both in initial and post-expansion adsorption, reaching surface tensions not lower than approximately 40mN/m. This would indicate that, in the presence of cholesterol, phenylalanines are able to mimic to a certain degree the effects of the palmitic chains in their role in adsorption, while the free cysteines are not.

We then explored the behavior during dynamic compression of surfactant films that contained palmitoylated or non-palmitoylated SP-C as the single protein component, in the presence or absence of cholesterol. Figure 2 shows quasi-static cycling of surfactant films containing either native or recombinant SP-C in the presence or absence of cholesterol. Controls were performed with films formed from pure lipid suspensions. In the absence of any protein, surfactant films cannot reach upon compression surface tensions lower than the equilibrium tension of ~22mN/m and show a very high maximum surface tension, indicating that the material is not efficiently re-spreading into the interface upon expansion. In the absence of cholesterol, 2% of native SP-C was sufficient to allow the surfactant film to reach surface tensions close to 0 mN/m with efficient respreading (Figure 2, upper panels), as revealed by the low maximal tensions. However, considerable compression/expansion hysteresis,

especially during the first two cycles, can be observed and reflects low stability of the compressed phases. Furthermore, a pronounced plateau at around 20mN/m is clearly visible before the compressed film reaches the lowest surface tensions. It has been proposed that quasi-static compression of surfactant films proceed through compression-driven three-dimensional transitions, likely facilitated by SP-C, before the film can reach a state capable of supporting the maximal surface pressures (minimal tensions) without collapsing (36). In this respect, recombinant versions of SP-C reproduce only partly the behavior of native SP-C. Samples containing rSP-C CC produce lower minimal tensions than pure lipidic samples but do not reach as low values as the samples containing native palmitoylated protein. This recombinant version of SP-C produced also maximal surface tensions that were intermediate between those reached by pure lipid and the low maximal tensions maintained by the native protein. The rSP-C FF variant, on the other hand, displays a behavior somehow closer to the native palmitoylated protein, as it allows reaching the lowest surface tensions while re-spreading is still impaired. These results are again in agreement with the proposed effect of phenylalanines in functionally replacing, at least in part, the palmitoylated cysteines.

In the presence of cholesterol, none of the SP-C versions tested could lead to a substantial reduction in surface tension upon compression compared to pure lipid films. Presence of the protein led to the appearance of large compression plateaus, at around 20 mN/m, which did not progress to a further reduction in tension as observed in the absence of cholesterol (Figure 2, bottom panels). The fluidizing effect of cholesterol may prevent the compression of the surfactant film to states where a further reduction of surface tension beyond the equilibrium tension is possible. It is noteworthy that native palmitoylated SP-C is significantly more efficient in promoting the respreading of the films upon expansion also in the presence of cholesterol, as can be seen in the considerably lower maximum surface tension reached in the presence of native SP-C.

For all samples, quasi-static compression-expansion cycles were followed by dynamic cycles, where the bubble was continuously compressed and expanded, without intermediate steps, at a relatively fast rate (20 cycles/min), thus simulating the regular inflation and deflation of the lung during breathing. Figure 3 shows dynamic cycling of the same surfactant films described in Figure 2. In the absence of cholesterol, all the films containing protein were able to reach very low surface tensions, irrespective of the SP-C version used (Figure 3, upper panels). The rapid compression/expansion rate used during dynamic cycling probably prevents reorganization of the film such as it occurs upon the step-wise compression and expansion applied in quasi-static cycles. This effect may explain why some of the interfacial films are able to reach lower surface tensions during rapid dynamic cycling but not under quasi-static conditions. A kinetic effect is probably also involved in the differences of the samples with respect to the reinsertion of lipids upon re-expansion. During rapid cycling there is not enough time for reinsertion, increasing the differences between proteins that promote re-spreading with different efficacies. Maximal tensions are higher for films cycled under dynamic conditions, with only native SP-C reducing significantly the maximal surface in the absence of cholesterol. Cholesterol has again a general impairing effect on the ability of the films to reach low surface tension during dynamic cycling, irrespective of the presence of SP-C (Figure 3, bottom panels).

Previous work of our group revealed a cooperative effect of SP-B and SP-C that could alleviate the deleterious effects of cholesterol on the surface activity of surfactant films (24). We therefore wanted to explore whether recombinant versions of SP-C could mimic native SP-C in this effect. First, we compared the behavior of samples containing 1% w/w SP-B as the only protein component, in the presence or absence of 5% w/w cholesterol. Measurements of initial and post-expansion adsorption showed that the presence of cholesterol impaired the ability of these samples to rapidly reach low surface tensions as compared to the samples that did not contain cholesterol (Figure 4). Inclusion of 2% (w/w) of SP-C, however, improved adsorption in the presence of cholesterol significantly, as shown in Figure 4, independently of the form of SP-C used. While recombinant non-palmitoylated SP-C is indistinguishable from native palmitoylated SP-C in their ability to promote adsorption in the presence of cholesterol, clear differences became apparent when the surfactant films were subjected to quasi-static and dynamic compression/expansion cycling. Quasi static cycles of surfactant films containing 5% (w/w) cholesterol, 1% (w/w) of native SP-B and 2% (w/w) of either native or recombinant SP-C are shown in Figure 5.

Cholesterol drastically impairs the ability of SP-B-containing surfactant films to reach very low surface tensions upon compression. Strikingly, the presence of palmitoylated SP-C counteracts this deleterious effect of cholesterol and the behavior of the film resembles very much that of the films without cholesterol (Figure 5, left panel), confirming recently published results (24).However, in the absence of the protein-attached palmitic chains, in both of the recombinant versions of SP-C, this effect is lost and cholesterol-containing films including non-palmitoylated recombinant forms of SP-C are unable to reach surface tensions below ~20mN/m. Interestingly, the ability of SP-C to facilitate respreading of the films upon expansion, as can be inferred from the maximum surface tensions reached upon cycling, is retained in the presence of recombinant non-palmitoylated SP-C, both in the case of rSP-C CC and rSP-C FF. The palmitic chains therefore do not seem to be relevant for this function of SP-C, at least in the presence of SP-B (see Discussion). Note that rSP-C FF is slightly more efficient in lowering the maximum surface tension, supporting the role of the phenylalanines in partly mimicking the palmitoylcysteines.

Figure 6 shows dynamic cycling of the same surfactant films as in the quasi-static cycles shown in Figure 5. Native SP-C is very efficient to cooperate with SP-B in lowering surface tension in the presence of cholesterol upon minimal compression, while producing compression-expansion isotherms with very little hysteresis and very low maximum surface tension. However, rSP-C CC is hardly able to fulfill the same function. Films containing cholesterol and this non-palmitoylated protein reach rapidly 20 mN/m upon compression but do not progress further to lower pressures, presumably because in the absence of palmitoylation the protein is not efficient in preventing the relaxing structural transitions suffered by the fluid cholesterol-containing films upon compression. Interestingly, rSP-C FF is able to mimic the behavior of the native protein to some extent but only in the first cycle. The phenyl rings can probably support some anchoring of SP-C, but not to the same degree as the palmitoyl groups that are able to penetrate deep into the lipid film. It should be noted that the expansion/compression isotherms during dynamic cycling of films containing rSP-C FF showed a certain variance, in some cases being even more similar to the isotherms in the presence of the palmitoylated protein, confirming that this version of SP-C can potentially mimic the behavior of native SP-C, although only in part (see supplementary Figure S3 that shows four independent experiments).

To discard that the inefficiency of the recombinant SP-C forms to mimic the behavior of native SP-C could be due to the dipeptide extension present at the N-terminal end of all the recombinant protein versions studied, we produced and characterized a recombinant variant of SP-C bearing phenylalanines and lacking the GP extension (see compared sequences in Table 1). As summarized in the supplementary figure S4, the behavior of this variant lacking the GP extension was completely analogous to that of the rSP-C FF form so far described. It restores interfacial adsorption of SP-B-containing samples in the presence of cholesterol, and maintains good re-spreading properties during film expansion, while it shows itself inefficient to restore the ability of the films to produce very low surface tension upon compression of the lipid-protein films.

As a complementary control, we also performed the cycling experiments described above, for films simultaneously containing SP-B and one of the different forms of SP-C, in the absence of cholesterol (Figure 7). Both the quasi-static and the dynamic behavior of lipid films containing SP-B and native palmitoylated SP-C resembled the results shown in Figure 5 and 6, obtained in the presence of cholesterol, which were characterized by minimal surface tensions close to 0 mN/m attained with low compression rates ($\leq$ 20%), little compression-expansion hysteresis and low maximum surface tensions. In the absence of cholesterol, however, we could also observe a different effect of the recombinant versions of SP-C on the behavior of the lipid films during quasi-static and dynamic cycling. As shown in the four lower panels of Figure 7, the interfacial films containing recombinant proteins were also able to reach very low surface tensions, just like in the presence of the native protein. Especially in dynamic cycles though, considerable differences could be observed in terms of hysteresis and maximum surface tensions, which were both significantly impaired in the presence of recombinant SP-C forms compared with the behavior of films containing the palmitoylated native version of SP-C. These results suggest that in films containing the recombinant proteins that lack palmitic chains, and in spite of the presence of SP-

B, some of the material that is excluded during compression is not efficiently re-spreading into the air/liquid interface when cycling is fast.

To check whether the differences detected in surface behavior between native and recombinant forms of SP-C could be due to significant differences on the structure of the proteins once reconstituted in membranes, in the absence or presence of cholesterol, we carried out a conformational analysis of the proteins in the two lipid systems studied, by means of far-UV CD spectroscopy. Figure 8 compares the CD spectra of SP-C and rSP-C reconstituted in DPPC/POPC/POPG membranes in the absence or presence of 5% (w/w, with respect to phospholipids) cholesterol. All the spectra had morphology consistent with a mainly α-helical conformation of the two proteins in the two studied environments, discarding that production/manipulation of the proteins, affected or not by the palmitoylation level and/or the exposure to cholesterol, could cause a shift of the typical helical conformation of SP-C to various degrees of β-sheet aggregates (37). Interestingly, the presence of cholesterol in the membranes increased the negative ellipticity of the CD spectra of the two proteins, which is indicative of an increase/stabilization of their α-helical content. Determination, from the CD spectra, of the content of the different types of secondary structure in the two proteins (see supplementary Table 1) revealed that SP-C had 58±2% and 61±1% α-helix in the absence and in the presence of cholesterol, respectively, while rSP-C contains 61±3% α-helix in the absence of cholesterol and 70±5% in its presence. Differences in helical percent between the two proteins or between the conformation of the proteins in the absence and in the presence of cholesterol are not significant. However, the presence of cholesterol seems to introduce a consistent similar trend towards an increase in helical content in the two proteins, as it was recently revealed for the native protein using FTIR spectroscopy (26).

DISCUSSION

The two hydrophobic surfactant proteins SP-B and SP-C serve complementary purposes in the function of pulmonary surfactant, as has been shown both *in vitro* (38) and *in vivo* (9). Recently, our group demonstrated a functional cooperation between the two proteins in mediating surfactant film stability in the presence of cholesterol (24). The role of cholesterol in pulmonary surfactant has remained elusive and is vigorously debated. Cholesterol is a critical component of many membrane systems, where it modulates the fluidity, the structure, the stability, as well as the permeability to water of mono- and bilayers (21, 39, 40). In native pulmonary surfactant preparations, cholesterol is critical to sustain segregation of fluid ordered and disordered phases (41) and it influences phospholipid mobility and dynamics (22) with possible consequences for surfactant film stability. Although surfactant films must be rigid enough to support high surface pressures, especially at the most compressed states, they must also be sufficiently dynamic to cope with the continuous compression/expansion cycles that occur during breathing. While the high content in saturated lipids (mainly DPPC) is pivotal for the mechanical stability of pulmonary surfactant, cholesterol could improve the dynamic behavior of the lipid films. In heterothermic animals, the proportion of cholesterol in pulmonary surfactant increases when the body temperature decreases, highlighting its physiological importance (23). Nevertheless, cholesterol is considered detrimental to surfactant function based on *in vitro* data, where it impairs the surface activity of surfactant films (25, 42). An exacerbated proportion of cholesterol in surfactant has been associated with ARDS (43), a condition with a fatality of 30-40% and 150,000 cases per year in the US (44). For these reasons, cholesterol is removed from all clinically used surfactants (5).

Recent data suggest that SP-C could have an important role to sustain surfactant function in the presence of cholesterol (24). In most animals SP-C is dually palmitoylated at cysteine residues 5 and 6. Protein palmitoylation has been reported to be an important factor to promote interaction of some proteins with cholesterol-enriched raft-like domains in membranes (45). We therefore wondered whether palmitoylation of SP-C could be also essential for the protein to interact with cholesterol-rich lipid domains and so to promote stability of cholesterol-containing surfactant films under compression/expansion dynamics. Previously, palmitoylation of SP-C had been primarily linked to the structural integrity of mature SP-C (46, 47), but also to a role in maintaining the mechanical stability of surfactant films (15, 16, 48). These studies, however, relied on model systems that did not include cholesterol and varied greatly in the sources of SP-C (natural SP-C, SP-C analogs or chemically depalmitoylated SP-C) and the methods of analysis. In the present study we compared both native palmitoylated and recombinant non-palmitoylated versions of SP-C, where the structure of SP-C is not altered by chemical treatment during depalmitoylation.

The present data indicate that in cholesterol-free samples palmitoylation of SP-C is not essential to promote interfacial adsorption of phospholipids, an activity strictly required to facilitate rapid and efficient formation of surface active films, but probably also required for facilitating rapid re-extension of surfactant along the interface during the periods of expansion. These results agree with previous data showing that under equilibrium conditions deacylated SP-C is equally active in facilitating the insertion of phospholipids into interfacial films (15, 16). In contrast, in the presence of cholesterol, palmitoylated SP-C showed clearly better interfacial adsorption activity than the non-palmitoylated recombinant protein, an activity that could be mimicked by replacing palmitoylated cysteines with phenylalanines. During compression/expansion cycles, the lack of palmitoylation resulted in an increased maximum surface tension and a reduced ability of the corresponding films to reach very low surface tensions, particularly in the quasi-static regime. These results illustrate why a synthetic surfactant preparation containing recombinant SP-C as the single protein additive could be efficient enough to be useful in respiratory therapies (49, 50), particulary if using SP-C forms bearing phenylalanines, in lipid mixtures lacking cholesterol. The situation could be less favourable in the presence of the sterol. However, the surface behavior of surfactant systems containing SP-C or SP-C analogues alone as the only protein components is clearly inferior to surfactants containing also SP-B.

The differences between native and recombinant SP-C in equilibrium adsorption that were seen in the presence of cholesterol were not observed when SP-B was also present in the surfactant samples (seen in Figure 4). Therefore, palmitic chains are apparently not essential for SP-C to cooperate with SP-B in promoting interfacial adsorption of cholesterol-containing lipid-protein samples. However, important differences between native and recombinant versions of SP-C were again observed during compression of SP-B/SP-C based surfactant films. Native palmitoylated SP-C counteracts the deleterious effects of cholesterol in compression/expansion dynamics of SP-B-containing films. In the absence of palmitic chains, however, recombinant SP-C forms are not completely efficient in preventing the negative effects of cholesterol. Still, the recombinant versions of SP-C are able to fulfill some functions in cooperation with SP-B. Figure 5 shows how both recombinant versions of SP-C can limit maximum surface tensions to values very similar to those obtained for native SP-C, although they are unable to improve minimum surface tensions. We conclude that the reinsertion of phospholipids into the lipid film during expansion that is facilitated by SP-C does not depend on the palmitic chains when SP-B is also present.

It is interesting to observe that the presence of SP-C has different effects on the lipid films in the presence or absence of SP-B. On the one hand, comparing Figure 3 and Figure 7, the presence of native SP-C stabilizes lipid films without cholesterol to a greater extent if SP-B is also present. On the other hand, as can be seen in quasi-static cycles, in the absence of SP-B, recombinant versions of SP-C are not able to lower maximum surface tension, while in the presence of SP-B maximum surface tension is not much higher than in the presence of native SP-C. It is possible that the activity of SP-B alters the properties of surfactant films, for instance enriching the interfacial film in saturated lipids (51, 52), or providing additional mechanical stability through extensive superficial lipid-protein interactions (53, 54). This could explain certain discrepancies with the recombinant versions of SP-C in the presence or absence of SP-B.

A last remarkable feature revealed by the present results is that recombinant forms of SP-C, but not the native palmitoylated protein, could somehow affect negatively the activity of SP-B. Incorporation of native SP-C does not essentially affect the behavior of SP-B-containing films subjected to quasi-static or dynamic compression-expansion cycling (compare upper panels in Figure 7 with the left panel in Figures 5 and 6). However, incorporation of the two recombinant SP-C forms tested here impairs substantially the compression/expansion dynamics of SP-B-containing films, which behave practically as in the absence of SP-B (compare Figure 7 with the upper panels in Figure 3). This feature might be important in the context of designing new surfactant preparations. As said, recombinant non-palmitoylated SP-C variants seem to be useful as primary additives in surfactant preparations for therapeutic use, although they would be still far less optimized than surfactant preparations containing both native SP-B and SP-C proteins. The design and production of future surfactant preparations that could combine analogues of both SP-B and SP-C will require a detailed study confirming that the analogues of the two proteins do really cooperate and are not actually counteracting under physiologically meaningful dynamic constraints.

Differences in activity of native palmitoylated and non-palmitoylated recombinant SP-C forms are not due to significant differences in the conformation of the protein in membranes, as confirmed by CD spectroscopy. Both proteins have a similar conformation as prepared and reconstituted in our experiments, and they both are similarly influenced by the presence of cholesterol. As it was also determined for native SP-C using FTIR (26), presence of cholesterol could increase the amount and/or stability of the α-helical conformation, probably as a consequence of the increase in thickness of the membranes caused by the presence of cholesterol. Other data (55) also support the concept that native and recombinant SP-C interact similarly with membranes containing cholesterol, suggesting that the differences observed in this study with respect to surface behavior are only manifested once the lipid-protein complexes are subjected to high lateral pressures during compression of the interface.

Figure 9 shows a model that illustrates how palmitoylation could facilitate SP-C-promoted stabilization of compressed states in cholesterol-containing surfactant films. Previous studies have shown that the N-terminal segment of SP-C is able to interact with model membranes even in the absence of palmitic chains (56), but that palmitoylation is crucial for SP-C to remain associated with lipid films subjected to high compression (57). At the most compressed states, surfactant films likely segregate

regions highly enriched in DPPC, which would have properties of a liquid-condensed phase in the absence of cholesterol, or more similar to a liquid-ordered phase in the presence of cholesterol. Condensed DPPC-rich phases could behave like a solid film while, in the presence of cholesterol, liquid-ordered regions would have a variable degree of fluidity. The partial fluidity of liquid-ordered regions could impart a highly dynamic character (22) particularly favorable to facilitate dynamics of the film upon compression/expansion cycling, but it would make the film more instable at the high pressures than an actual solid phase. We propose that SP-C-promoted association of surfactant bilayers with highly packed liquid-ordered regions would provide additional stability to the multilayered interfacial film at the end of expiration, and that palmitoylation would be required for SP-C to sustain association with ordered phases compressed to their most condensed state, precluding their out-of-plane relaxation (33). In the absence of palmitoylation, SP-C could still associate with bilayers and monolayers, and therefore contribute to promote lipid transfer, but could not maintain association with the maximally compressed states of the interfacial film, which would then become intrinsically instable and could partially collapse before reaching the maximal pressures (minimal tensions). Lack of palmitoylation could be only partly compensated by the introduction of aromatic residues, because the side chains of aromatic amino acids such as phenylalanines show high affinity for cholesterol-containing membrane phases (58). The difference between the effects of palmitoylated and non-palmitoylated SP-C could be more evident in the presence of cholesterol because, in its absence, the solid character of DPPC-enriched patches could provide enough stability even in the absence of tight bilayer-monolayer contacts.

Overall, the present results suggest that optimization of new clinical surfactants may require the defined combination of proper versions of surfactant proteins with adequate proportions of the lipid components of the surfactant preparations. Non-palmitoylated versions of SP-C produced in bacteria may be useful as a basis to produce relatively simple surfactant preparations, with good surface activity under basic conditions. However, surfactants containing deacylated SP-C may not be efficient enough under more demanding conditions, such as those potentially existing in the airways of patients suffering of ARDS or other pathologies, where an excess of cholesterol may constitute an insuperable challenge.

ACKNOWLEDGEMENTS

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Table 1.
Sequence of native and recombinant SP-C variants

nSP-C	native pSP-C	LRIP**CC**PVNLKRLLVVVVVVVLVVVVIVGALLMGL
rSP-C CC	recomb.hSP-C, free Cys	GPFGIP**CC**PVHLKRLLIVVVVVVLIVVVIVGALLMGL
rSP-C FF	recomb.hSP-C, Phe substitution	GPFGIP**FF**PVHLKRLLIVVVVVVLIVVVIVGALLMGL
rSP-C FF (no GP)	recomb.hSP-C, Phe substitution, no GP	FGIP**FF**PVHLKRLLIVVVVVVLIVVVIVGALLMGL

The conserved cysteine residues are in bold and palmitoylatedcysteines are highlighted with a black box. The C-terminal alpha-helical segment of SP-C is boxed in grey. Note the N-terminal GP dipeptide extension (underlined). A recombinant SP-C version without GP extension was also produced as a control (see text and supplementary Figure S2 for details).

FIGURE LEGENDS

Figure 1.
Initial and post-expansion adsorption in the presence of SP-C.
The effect of native and recombinant SP-C on the interfacial adsorption kinetics of lipid suspensions composed of DPPC/POPC/POPG (50/25/15, w/w/w) was measured in the presence (upper panels) or absence (lower panels) of 5% w/w cholesterol. Left: decrease of surface tension over time during the first 5 min after deposition of ~0.5 μl of the different lipid or lipid/protein samples (10 mg/ml).Right: decrease in surface tension monitored during 5 min after expansion of the bubble from a volume of ~ 0.045 to 0.15 ml.. Data are means ± s.d. after averaging data from three independent experiments.

Figure2.
Quasi-static compression/expansion isotherms in the presence of SP-C.
Films formed from DPPC/POPC/POPG (50/25/15, w/w/w) suspensions in the absence (upper panels) or presence (lower panels) of 5% (w/w) cholesterol and containing no protein (left), or 2% (w/w) of native SP-C (central left), rSP-C CC (central right) or SP-C FF (right) were subjected to quasi-static cycling. The surface tension vs. relative area is plotted for the four consecutive step-wise compression-expansion isotherms.

Figure 3.
Dynamic compression/expansion isotherms in the presence of SP-C.
Films formed from DPPC/POPC/POPG (50/25/15, w/w/w) suspensions in the absence (upper panels) or presence (lower panels) of 5% (w/w) cholesterol and containing no protein (left), or 2% (w/w) of native SP-C (central left), rSP-C CC (central right) or rSP-C FF (right) were subjected to dynamic cycling at a rate of 20 cycles/min. The surface tension vs. relative area is plotted for the first, 10th and 20th cycle of continuous compression-expansion isotherms.

Figure 4.
Initial and post-expansion adsorption in the presence of cholesterol, SP-B and SP-C.
The effect of native and recombinant SP-C on the interfacial adsorption kinetics of lipid suspensions composed of DPPC/POPC/POPG (50/25/15/10, w/w/w) plus 1% (w/w) porcine SP-B was evaluated in the presence of 5% w/w cholesterol. Left panel: Initial adsorption (recorded during 5 min after deposition of the respective lipid samples).Right panel: post-expansion adsorption(decrease in surface tension during 5 min after expansion of the bubble to a volume of 0.15 ml). The samples, with 1% (w/w) of SP-B, contained no cholesterol, 5% (w/w) cholesterol, or 5% (w/w) cholesterol plus 2% (w/w) native SP-C, rSP-C CC or rSP-C FF. Data are means ± s.d. after averaging data from three independent experiments.

Figure 5.
Quasi-static cycling and SP-B/SP-C cooperation in cholesterol-containing surfactant films.
Films formed from DPPC/POPC/POPG (50/25/15, w/w/w) suspensions containing 1% w/w SP-B and no cholesterol (top left) or containing 5%(w/w) cholesterol (right panels) were subjected to quasi-static cycling in the absence of SP-C (top), or in the presence of 2% (w/w) of native SP-C (upper center), rSP-C CC (lower center) or SP-C FF (bottom). The surface tension vs. relative area is plotted for the first, second, third and fourth cycle of four consecutive step-wise compression-expansion isotherms.

Figure 6.
Dynamic cycling and SP-B/SP-C cooperation in cholesterol-containing surfactant films.
Films formed from DPPC/POPC/POPG (50/25/15, w/w/w) suspensions containing 1% w/w SP-B and no cholesterol (top left) or containing 5% (w/w) cholesterol (right panels) were subjected to dynamic cycling

in the absence of SP-C (top), or in the presence of 2% (w/w) of native SP-C (upper center), rSP-C CC (lower center) or rSP-C FF (bottom).The surface tension vs. relative area is plotted for the first, 10th and 20th cycle of continuous compression-expansion isotherms.

Figure 7.
Effect of SP-C on the quasi static and dynamic compression-expansion isotherms of SP-B-containing films in the absence of cholesterol.
Quasi-static (left panels) and dynamic (right panels) cycles of films formed from DPPC/POPC/POPG (50/25/15, w/w/w)s uspensions in the absence of cholesterol were obtained in the presence of 1% (w/w) of SP-B plus 2% (w/w) of native SP-C (top), rSP-C CC (center) or SP-C FF (bottom). For the quasi-static cycling, the surface tension vs. relative area is plotted for the first, second, third and fourth cycle of four consecutive step-wise compression-expansion isotherms. For the dynamic cycling, the surface tension vs. relative area is plotted for the first, 10th and 20th cycle of continuous compression-expansion isotherms.

Figure 8.
Secondary structure of native and recombinant forms of SP-C in membranes, in the absence or presence of cholesterol.
Far-UV CD spectra of native (left panel) or recombinant (right panel) SP-C reconstituted in membranes made of DPPC/POPC/POPG (50/25/15, w/w/w) in the absence (continuous line) or in the presence (dashed line) of 5% (w/w with respect to phospholipid) cholesterol.

Figure 9.
Model for the role of palmitoylation of SP-C in surfactant film stability.

Figure 1

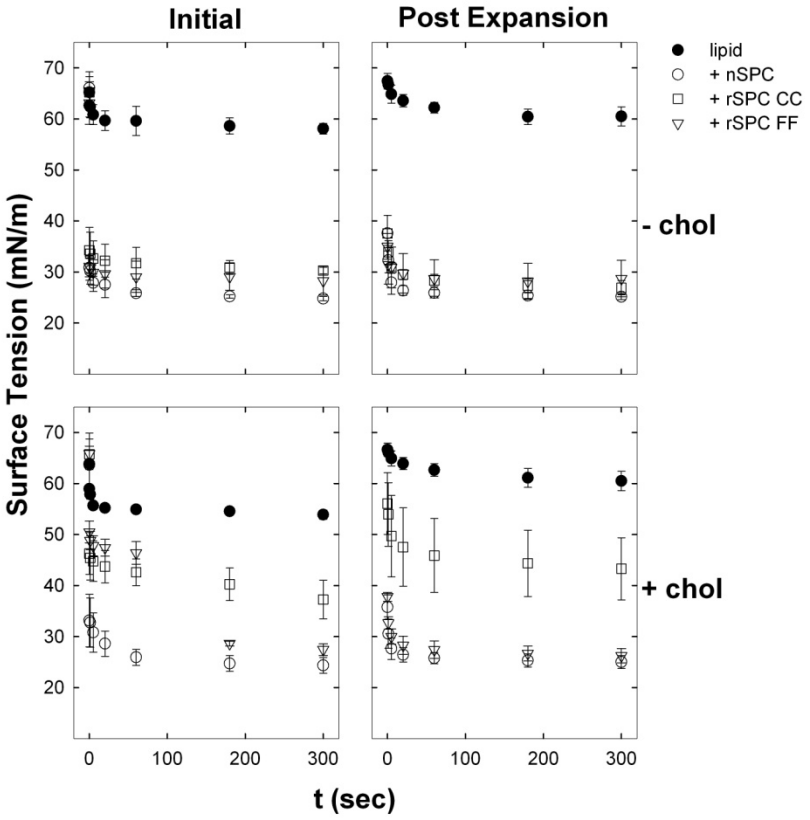

Initial
Post Expansion
lipid
+ nSPC
+ rSPC CC
+ rSPC FF
- chol
+ chol
Surface Tension (mN/m)
t (sec)
70
60
50
40
30
20
0
100
200
300

Figure 2

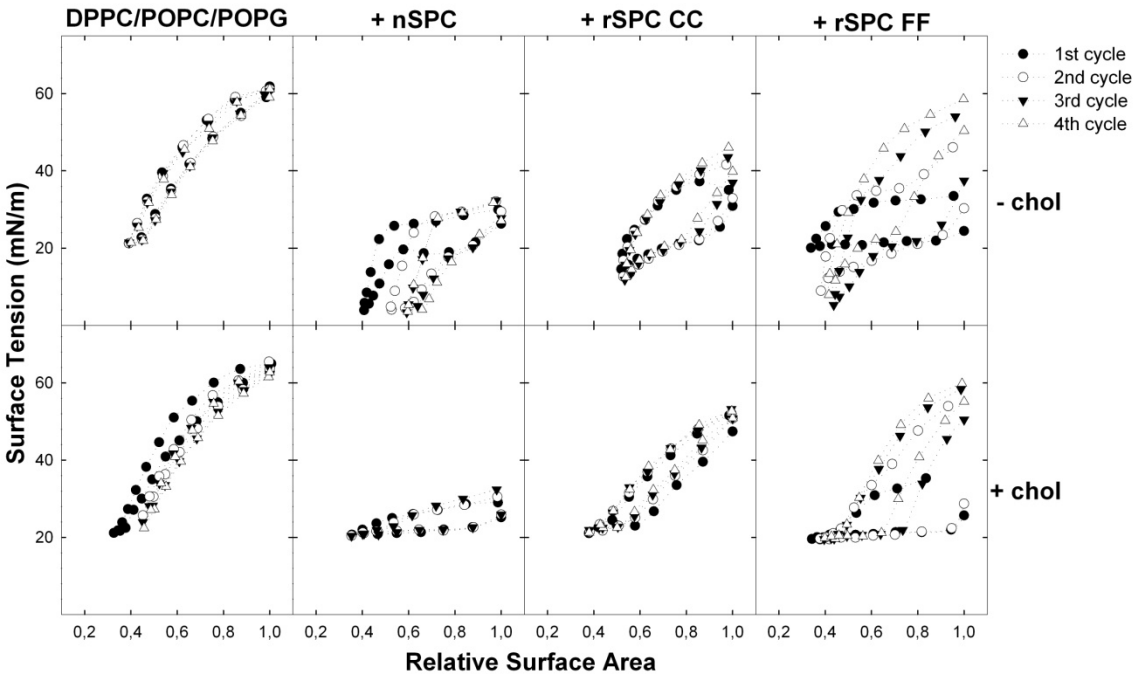

DPPC/POPC/POPG
+ nSPC
+ rSPC CC
+ rSPC FF
1st cycle
2nd cycle
3rd cycle
4th cycle
- chol
+ chol
Surface Tension (mN/m)
Relative Surface Area
20
40
60
0,2
0,4
0,6
0,8
1,0

Figure 3

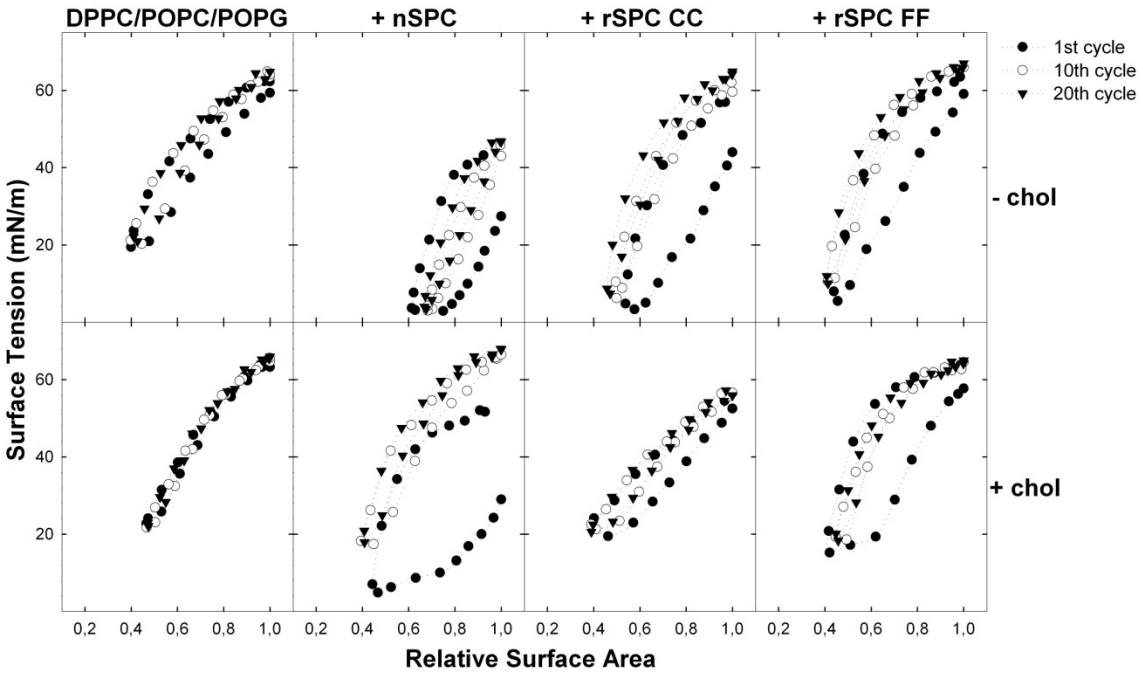

DPPC/POPC/POPG
+ nSPC
+ rSPC CC
+ rSPC FF
1st cycle
10th cycle
20th cycle
- chol
+ chol
Surface Tension (mN/m)
Relative Surface Area
20
40
60
0,2
0,4
0,6
0,8
1,0

Figure 4

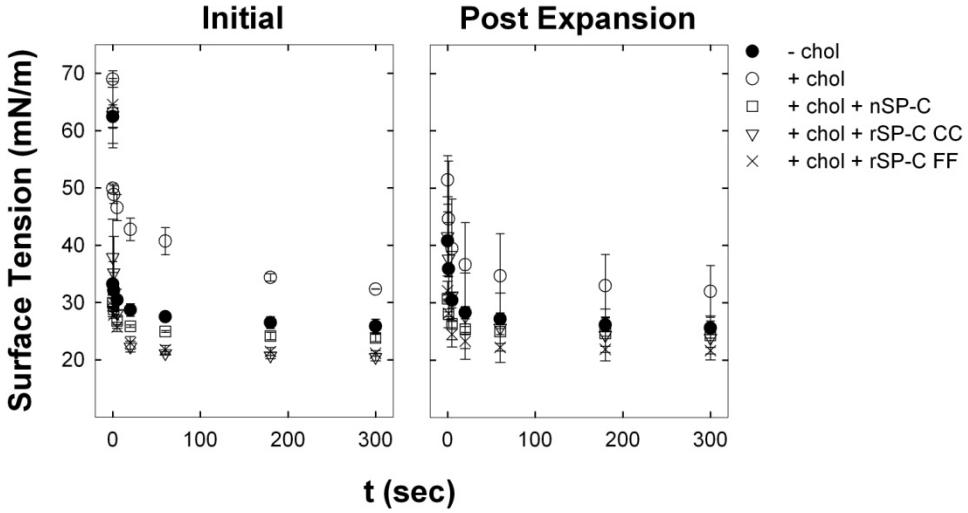

Initial
Post Expansion
Surface Tension (mN/m)
70
60
50
40
30
20
0
100
200
300
t (sec)
- chol
+ chol
+ chol + nSP-C
+ chol + rSP-C CC
+ chol + rSP-C FF

Figure 5

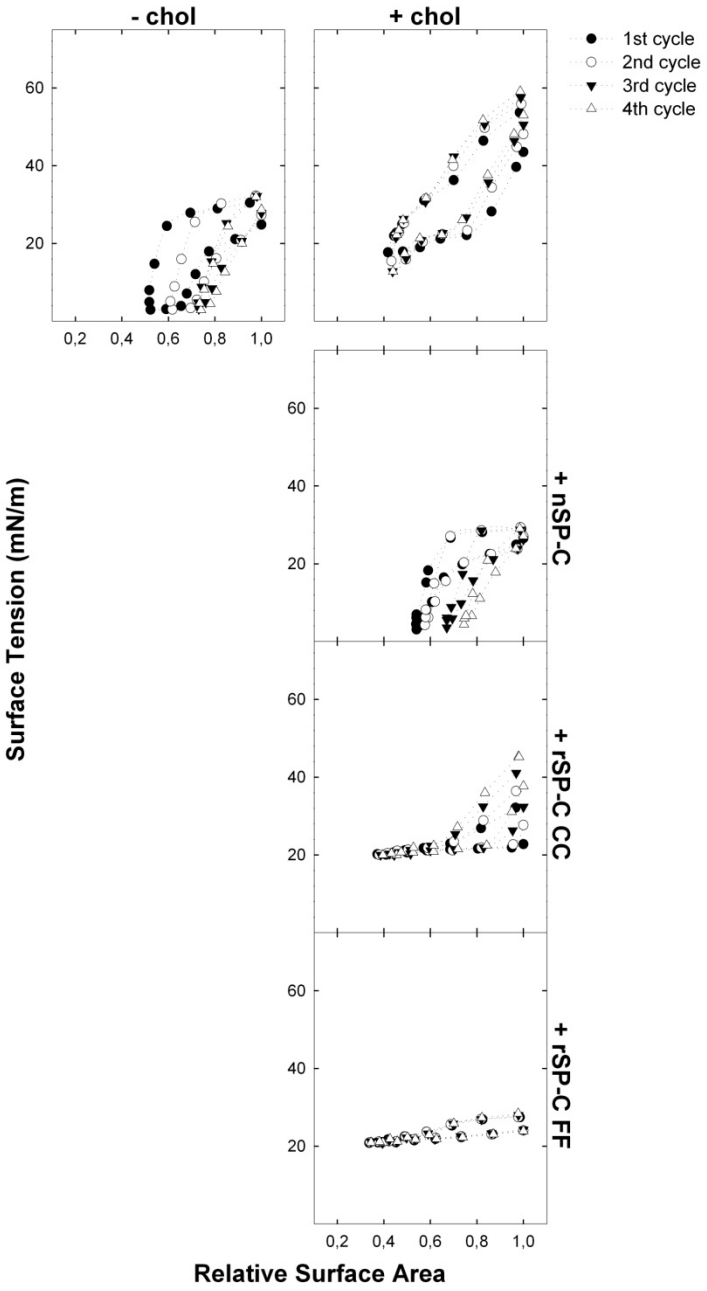

- chol
+ chol
1st cycle
2nd cycle
3rd cycle
4th cycle
Surface Tension (mN/m)
Relative Surface Area
+ nSP-C
+ rSP-C CC
+ rSP-C FF
60
40
20
0,2
0,4
0,6
0,8
1,0

Figure 6

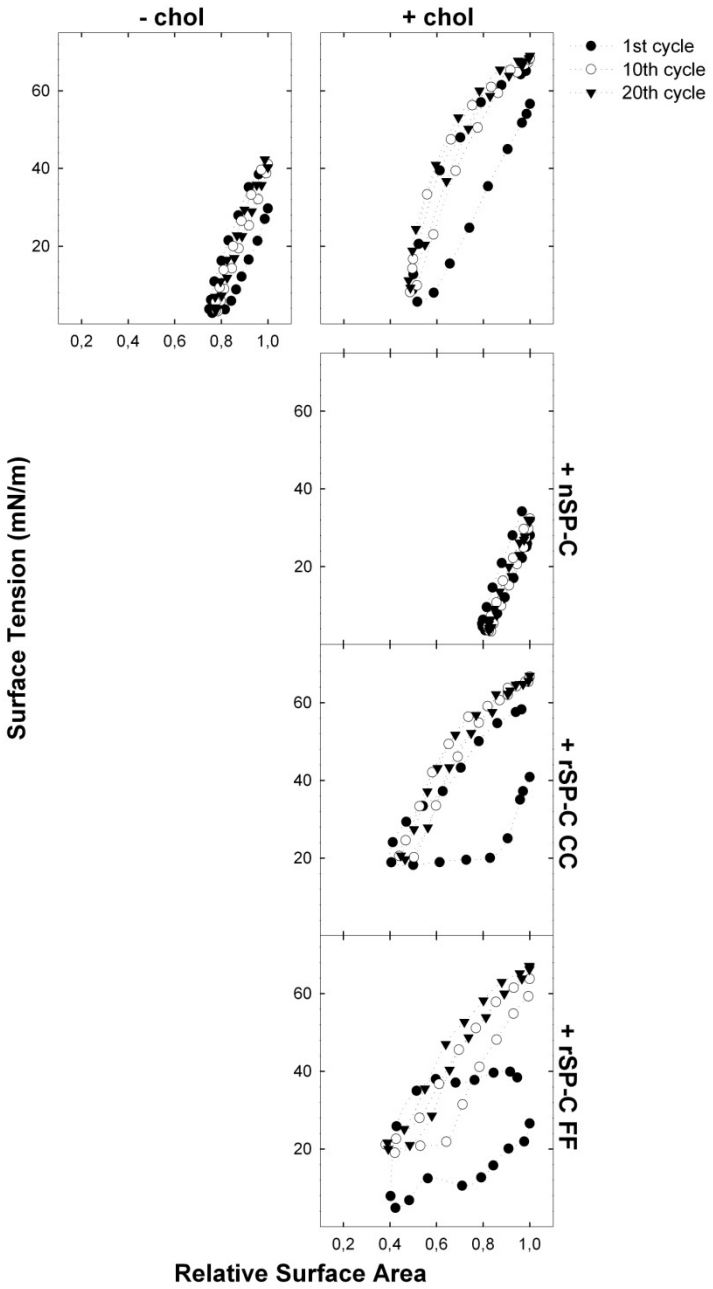

- chol
+ chol
1st cycle
10th cycle
20th cycle
Surface Tension (mN/m)
+ nSP-C
+ rSP-C CC
+ rSP-C FF
Relative Surface Area

Figure 7

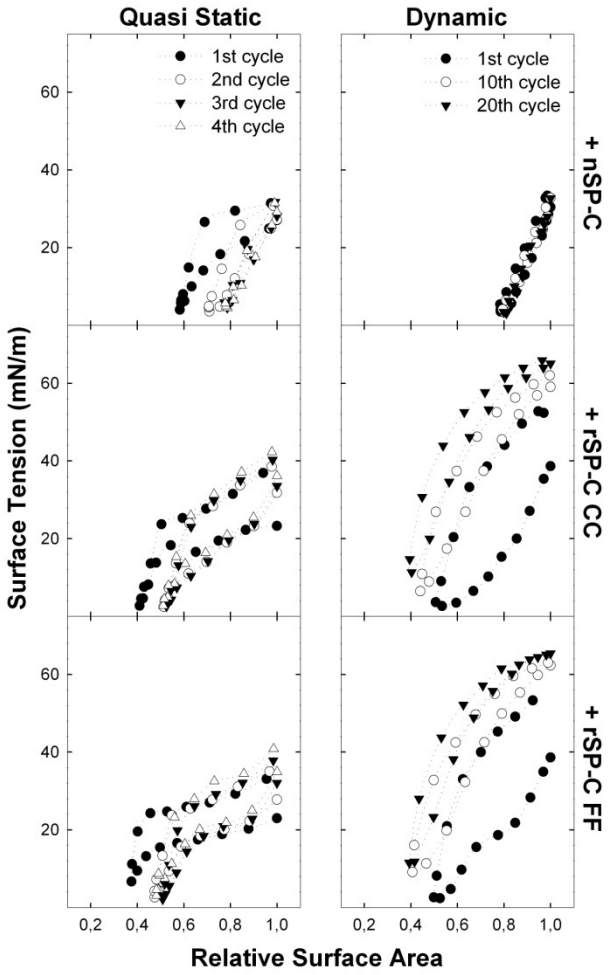

Quasi Static
Dynamic
1st cycle
2nd cycle
3rd cycle
4th cycle
1st cycle
10th cycle
20th cycle
+ nSP-C
+ rSP-C CC
+ rSP-C FF
Surface Tension (mN/m)
Relative Surface Area
20
40
60
0,2
0,4
0,6
0,8
1,0

Figure 8

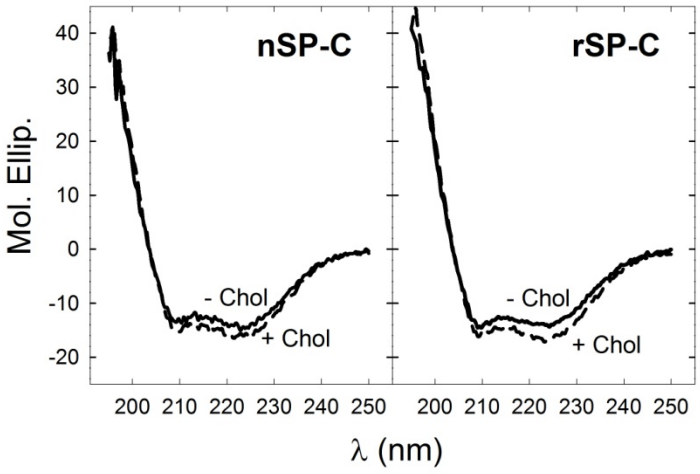
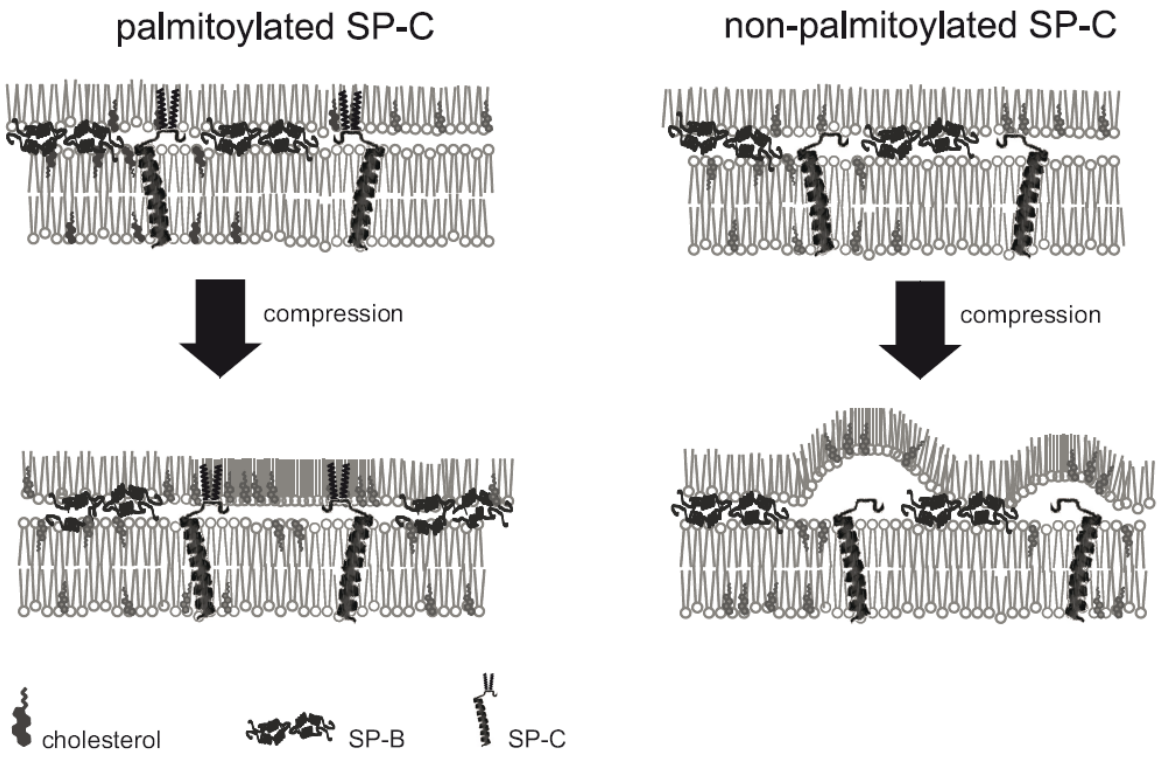

nSP-C
rSP-C
Mol. Ellip.
40
30
20
10
0
-10
-20
- Chol
+ Chol
- Chol
+ Chol
200 210 220 230 240 250
200 210 220 230 240 250
λ (nm)

Figure 9


palmitoylated SP-C
non-palmitoylated SP-C
compression
compression
cholesterol
SP-B
SP-C

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Supporting Material
PALMITOYLATION OF PULMONARY SURFACTANT PROTEIN SP-C IS CRITICAL FOR ITS
Florian Baumgart, Olga L. Ospina, Ismael Mingarro, Ignacio Rodriguez-Crespo, and Jesus Perez-Gil

Supplementary Table 1.
Sequence of native and recombinant SP-C variants

nSP-C	native pSP-C	LRIP**CC**PVNLKRLLVVVVVVVLVVVVIVGALLMGL
rSP-C CC	recomb.hSP-C, free Cys	GPFGIP**CC**PVHLKRLLIVVVVVVLIVVVIVGALLMGL
rSP-C FF	recomb.hSP-C, Phe substitution	GPFGIP**FF**PVHLKRLLIVVVVVVLIVVVIVGALLMGL
rSP-C FF (no GP)	recomb.hSP-C, Phe substitution, no GP	FGIP**FF**PVHLKRLLIVVVVVVLIVVVIVGALLMGL

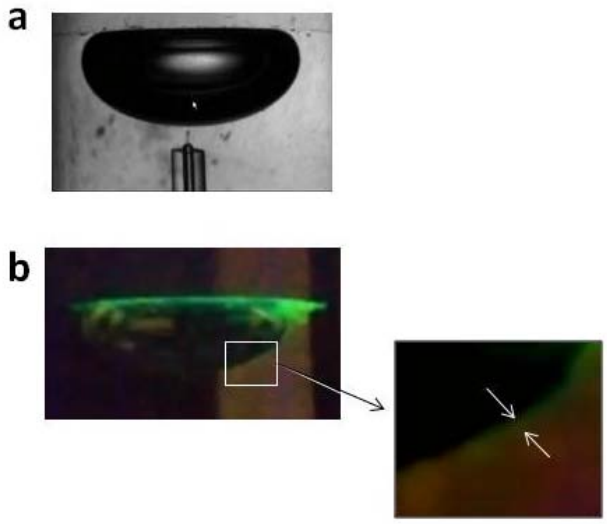
The conserved cysteine residues are in bold and palmitoylated cysteines are highlighted with a black box. The C-terminal alpha-helical segment of SP-C is boxed in grey. Note the N-terminal GP dipeptide extension (underlined). A recombinant SP-C version without GP extension was also produced as a control (see text and supplementary Figure S4 for details).

Supplementary Table 2

Secondary structure of native and recombinant forms of SP-C reconstituted in membranes in the presence or absence of cholesterol, as determined from far-UV CD spectra

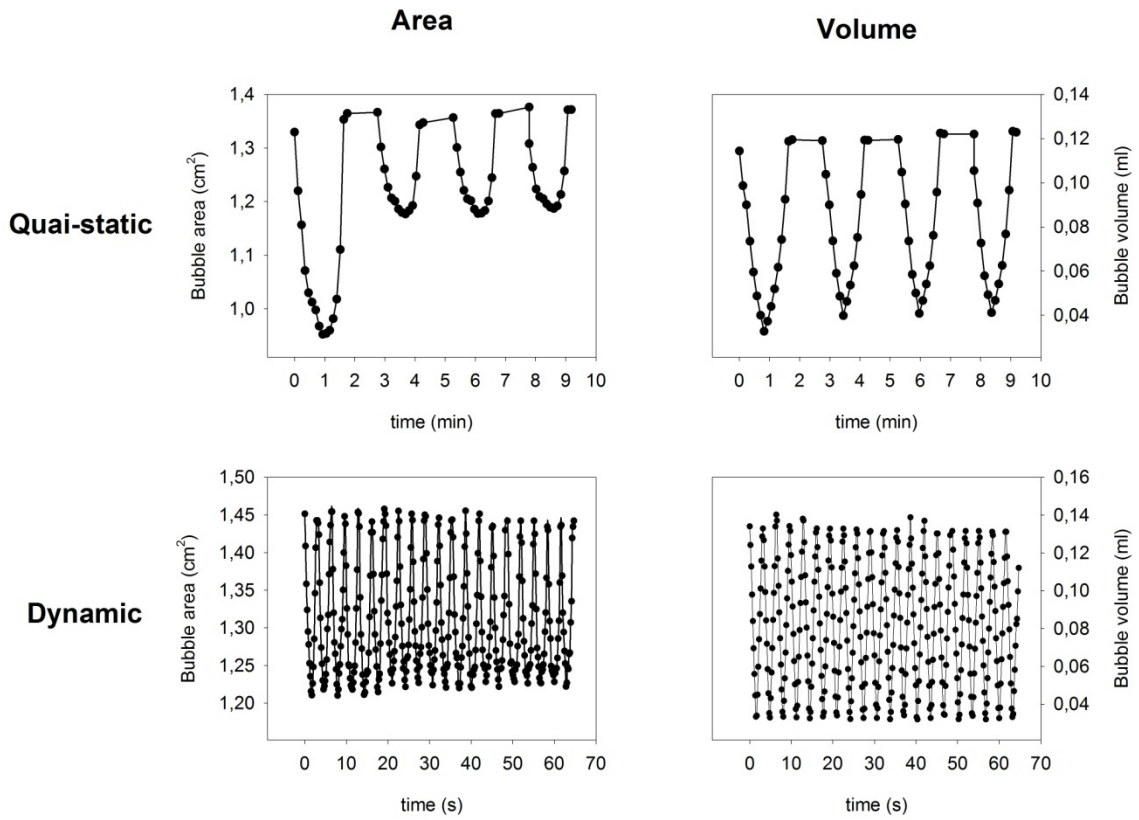
	% secondary structure			
	α	β	t	r
SP-C				
DPPC/POPC/POPG	58 ± 2	9 ± 2	12 ± 1	21 ± 3
DPPC/POPC/POPG/Chol	62 ± 1	6 ± 1	12 ± 1	20 ± 2
rSP-C				
DPPC/POPC/POPG	61 ± 3	7 ± 2	12 ± 1	20 ± 3
DPPC/POPC/POPG/Chol	70 ± 5	5 ± 1	10 ± 1	15 ± 4

α: α-helix; β: β-sheet; t: turns; r: random coil

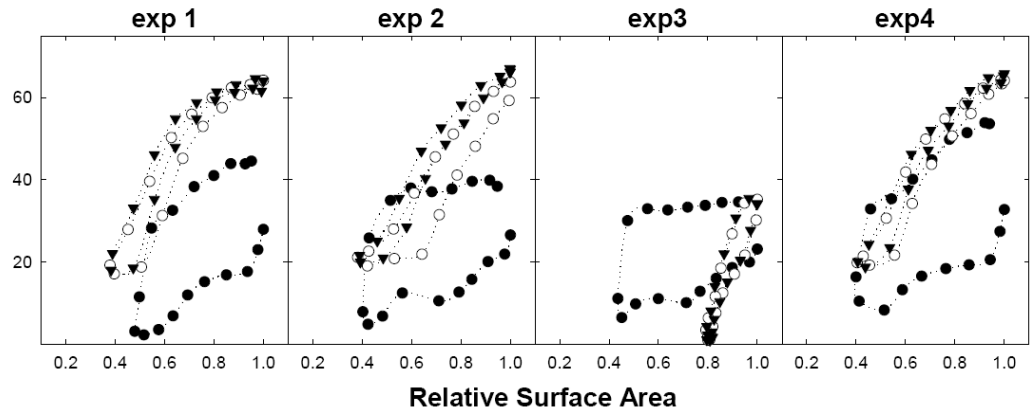


Suplementary Figure S1
Application of surfactant as a layer surrounding the bubble at the captive bubble surfactometer (CBS)

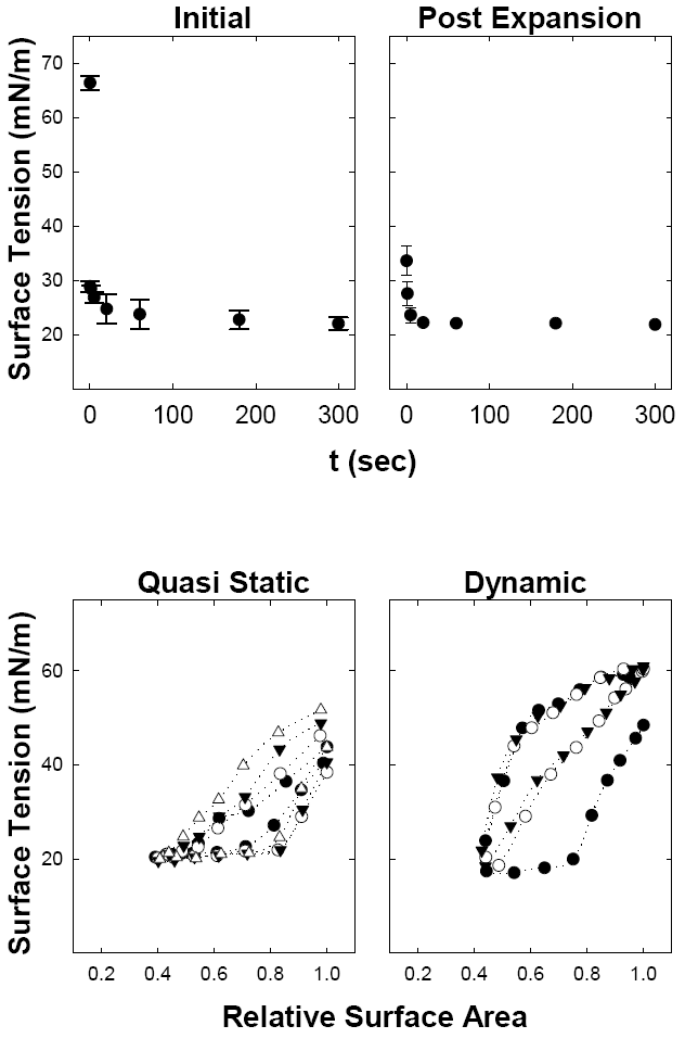
a) Surfactant samples are injected at the vicinity of the surface of the captive bubble by using a micropositioning system and a micro capillary tube that allows accurate measurement of surfactant volumes at the 100 nL range and precise and repetitive positioning.
b) Injection of a sample of surfactant doped with 1% of the fluorescent probe BODIPY-PC allows observation of the layer of surfactant that remains associated with the bubble surface, without diffusing away into the sucrose-containing highly dense solution filling the CBS chamber.



Supplementary Figure S2.
Comparison of the waveform of area (left panels) and volume (right panels) oscillation in a typical captive bubble experiment, operated under quasi-static (upper panels) or dynamic (lower panels) conditions
Time-dependent variations of bubble area and volume during step-wise or continuous cycling have been calculated from recorded images. In the quasi-static experiment, compression is stopped in each cycle when reaching minimal surface tension, just before getting overcompression. In the first quasi-static cycle, surfactant films require larger compression to reach minimal tension than in the subsequent cycles, once the films have initiated compression-driven reorganization. Notice the very different timescale of oscillations when comparing quasi-static and dynamic regimes.

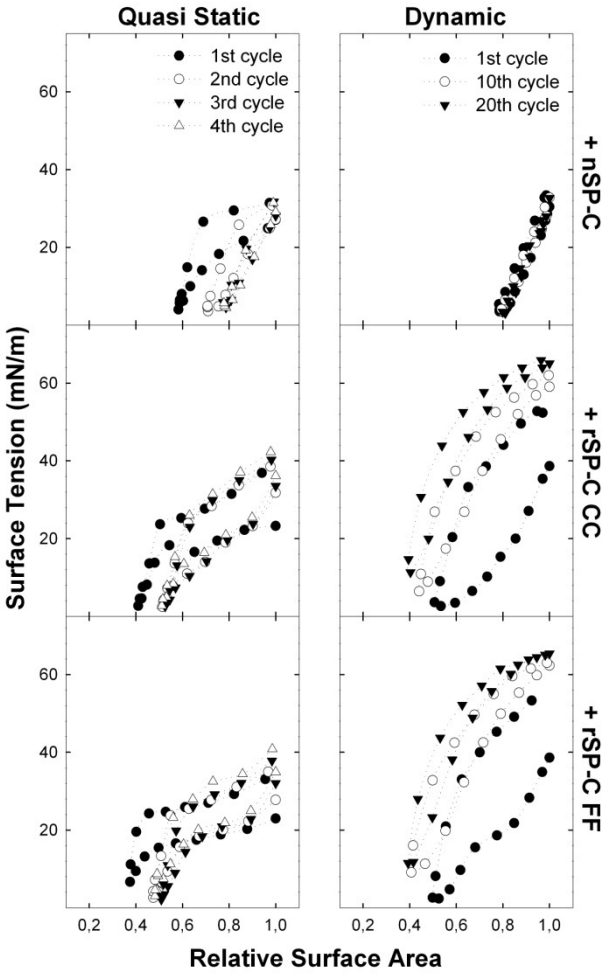


Supplementary Figure S3.
rSP-C FF can lower the surface tension to < 5 mN/m.
In about 25% of the experiments, the presence of 2% (w/w) rSP-C FF improved compression/expansion dynamics of films formed from DPPC/POPC/POPG (50/25/15, w/w/w) suspensions containing 5% (w/w) cholesterol and 1% (w/w) of porcine SP-B. Isotherms from four independent experiments are shown. Surface tension was plotted vs. relative area for the first (closed circles), 10th (open circles) and 20th (closed triangles) cycle upon continuous compression/expansion cycling of the bubble volume at 20 cycles/min.

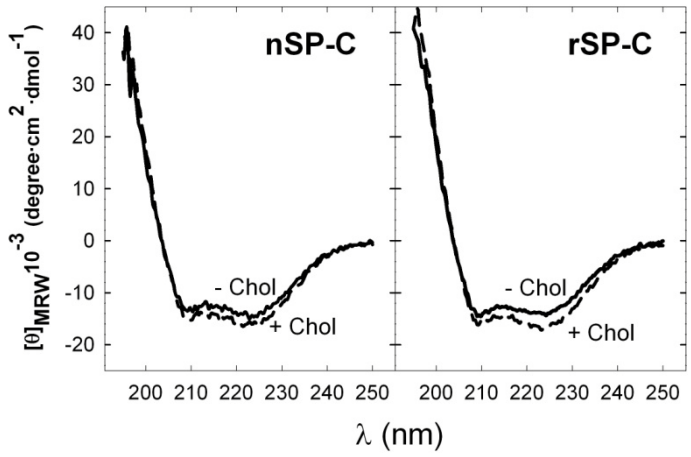


Supplementary Figure S4.
Adsorption and compression/expansion isotherms of films containing rSP-C FF (without GP N-terminal extension).
Films were formed upon adsorption of DPPC/POPC/POPG (50/25/15, w/w/w) suspensions containing 5% (w/w) cholesterol, 1% (w/w) of SP-B and 2% (w/w) of a recombinant variant of rSP-C FF that did not contain the N-terminal GP extension (see Materials and Methods and Table 1). Top panels show initial (left) and post-expansion (right) adsorption isotherms. Adsorption data are means +/- s.d. of three idependent experiments. Bottom panels show representative quasi-static (left) and dynamic (right) compression/expansion cycles. For the quasi-static cycling, the surface tension vs. relative area is plotted for the first (closed circles), second (open circles), third (closed triangles) and fourth (open triangles) cycle of four consecutive step-wise compression-expansion isotherms. For the dynamic cycling, the surface tension vs. relative area is plotted for the first (closed circles), 10th (open circles) and 20th (closed triangles) cycle upon continuous compression/expansion cycling of the bubble volume.

Comparison with the isotherms represented in Figures 4, 5 and 6 reveals no significant differences between rSP-C FF and rSP-C FF (noGP).



Supplementary Figure S5.
Effect of SP-C on the quasi static and dynamic compression-expansion isotherms of SP-B-containing films in the absence of cholesterol.
Quasi-static (left panels) and dynamic (right panels) cycles of films formed from DPPC/POPC/POPG (50/25/15, w/w/w) suspensions in the absence of cholesterol were obtained in the presence of 1% (w/w) of SP-B plus 2% (w/w) of native SP-C (top), rSP-C CC (center) or SP-C FF (bottom). For the quasi-static cycling, the surface tension vs. relative area is plotted for the first, second, third and fourth cycle of four consecutive step-wise compression-expansion isotherms. For the dynamic cycling, the surface tension vs. relative area is plotted for the first, 10th and 20th cycle of continuous compression-expansion isotherms.



Supplementary Figure S6.
Secondary structure of native and recombinant forms of SP-C in membranes, in the absence or presence of cholesterol.
Far-UV CD spectra of native (left panel) or recombinant (right panel) SP-C reconstituted in membranes made of DPPC/POPC/POPG (50/25/15, w/w/w) in the absence (continuous line) or in the presence (dashed line) of 5% (w/w with respect to phospholipid) cholesterol. Dichroism is presented as molar ellipticity per residue.

Circular Dichroism
Far-UV circular dichroism (CD) spectra were recorded as previously described (1) in a Jasco 715 spectropolarimeter equipped with a xenon lamp. All spectra were recorded in a 0.2 mL thermostated quartz cell with an optical path length of 0.1 cm. Molar ellipticity was calculated taking 110 as the mean molecular weight per residue. At least two different batches of each protein were analyzed, with comparable results. Estimation of the secondary structure content from the CD measurements was performed after deconvolution of the experimental spectra into four simple components (αhelix, β-sheet, turns and random coil) using the CDPro software package containing three commonly used programmes: SELCON3, CONTIN/LL and CDSSTR (2). This software allows the use of different sets of proteins, including membrane proteins (SMP50).

1. Serrano, A. G., M. Ryan, T. E. Weaver, and J. Perez-Gil. 2006. Critical structure-function determinants within the N-terminal region of pulmonary surfactant protein SP-B. Biophys J 90:238-249.
2. Sreerama, N., and R. W. Woody. 2004. On the analysis of membrane protein circular dichroism spectra. Protein Sci 13:100-112.