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Pulmonary surfactant protein SP-B is significantly more immunoreactive in anionic than in zwitterionic bilayers

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Abstract Binding of polyclonal and monoclonal antibodies, quantitated by enzyme-linked immunosorbent assay, to porcine SP-B reconstituted in different phospholipid bilayers has been used to assess differences in protein structure due to lipid–protein interactions. SP-B bound significantly more antibodies when it was reconstituted in bilayers made of anionic phospholipids (phosphatidic acid, cardiolipin, phosphatidylglycerol, phosphatidylinositol or phosphatidylserine) than in zwitterionic bilayers (phosphatidylcholine, phosphatidylcholine/cholesterol, or phosphatidylethanolamine) or in fatty acid micelles (made of salts of palmitic or stearic acids). These differences in immunoreactivity can be important in the development of quantitation methods for SP-B in clinical samples based on immunological techniques.

Key words: Protein epitope; Enzyme-linked immunosorbent assay; Membrane protein conformation; Surface activity; Lipid–protein interaction

1. Introduction

Total or partial deficiency of pulmonary surfactant protein SP-B results in impaired respiratory function [1,2] and severe respiratory failure that is lethal in cases of total lack of functional protein due to either genetic inactivation [3,4] or blockage of the protein by anti-SP-B antibodies [5,6]. The working hypothesis is that SP-B is essential for assembly, packing, secretion and restructuration in the airways of a surface active material, known as pulmonary surfactant, the main function of which is to reduce surface tension at the air/water interface of alveoli to very low values and so facilitate respiratory mechanics [7,8]. Surface activity resides principally in the phospholipid moieties of this complex and especially in a disaturated phosphatidylcholine (PC) species, dipalmitoylphosphatidylcholine (DPPC). Proteins such as SP-B and the hydrophobic lipopeptide SP-C facilitate transfer of the surface active phospholipids from the bulk of the aqueous alveolar lining into the interface where they form films able to achieve very low surface tension during exhalation [8,9]. Extensive analyses of lipid–protein interactions of SP-B in surfactant bilayers and monolayers have led to some comprehension of SP-B function from a molecular point of view [10], but we are still far from a full understanding of the essential role of the protein in the intracellular assembly and packing of surfactant bilayers. SP-B is thought to be necessary in vivo to promote insertion of phospholipids at the interface, an activity it promotes in vitro. A significant fraction of surfactant phospholipids, around 8–10% by weight, are negatively charged species at physiological pH, phosphatidylglycerol (PG) in most animals or phosphatidylinositol (PI) in some. Interactions of SP-B with anionic phospholipids in surfactant seem to participate in important processes in surfactant dynamics such as tubular myelin formation [11,12], interfacial adsorption [13,14], refining of surface films upon compression [15] or stabilization of compressed states of the films [16,17]. In the present work, we have explored structural features induced by negatively charged membranes into SP-B, analyzing possible differences in accessibility of the protein to polyclonal or monoclonal antibodies when it was reconstituted into either zwitterionic or anionic bilayers.

2. Materials and methods

2.1. Materials

Chloroform (Chl) and methanol (MeOH) were high performance liquid chromatography-grade solvents from Scharlau (Barcelona, Spain). Sephadex LH-20 and LH-60 chromatography gels were from Pharmacia (Uppsala, Sweden). Egg yolk PC, PG, phosphatidylethanolamine (PE) and phosphatidic acid (PA), bovine liver PI, bovine brain phosphatidylserine (PS), bovine heart cardiolipin (diphosphatidylglycerol, DPG), cholesterol (Ch), and palmitic (Pal) and stearic (Ste) acids were all from Sigma (St. Louis, MO, USA). DPPC and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Radiolabelled 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine (112 Ci/mol) was from Amersham International (Little Chalfont, Bucks, UK). Substrates for enzyme immunoassays, 3-dimethyl aminobenzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hidracine (MBTH), were also from Sigma. All other reagents and chemicals were from Merck (Darmstadt, Germany).

2.2. Protein and lipid–protein samples

Surfactant proteins SP-B and SP-C were isolated from minced por-

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Abbreviations: Ch, cholesterol; Chl, chloroform; DMAB, dimethyl aminobenzoic acid; DPG, bovine heart diphosphatidylglycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-glycerol; ECL, enzyme chemiluminescence; ELISA, enzyme-linked immunosorbent assay; LPC, 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine; MBTH, 3-methyl-2-benzothiazolinone hydrazine; MeOH, methanol; PAGE, polyacrylamide gel electrophoresis; PA, egg yolk phosphatidic acid; Pal, palmitic acid; PC, egg yolk phosphatidylcholine; PE, egg yolk phosphatidylethanolamine; PI, bovine liver phosphatidylinositol; PG, egg yolk phosphatidylglycerol; PS, bovine brain phosphatidylserine; SDS, sodium dodecyl sulfate; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; Ste, stearic acid

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cine lungs by an adaptation of the method of Curstedt et al. [18], which is described elsewhere [19]. SP-A was purified from porcine lung lavages as described [20]. Purity of protein preparations was routinely checked after isolation by SDS–PAGE and matrix-assisted laser desorption ionization-time of flight mass spectrometry, and the protein concentration determined by amino acid analysis. Solutions of purified SP-B and SP-C in Chl/MeOH (2:1, v/v) and SP-A in buffer Tris–HCl 5 mM pH 7.4 were stored at −20°C until use. To reconstitute SP-B in phospholipid bilayers, the proper amounts of protein and the given lipid were first mixed as Chl/MeOH (2:1, v/v) solutions and evaporated to dryness under a N_2 stream. Afterwards, 50 mM HEPES buffer pH 7 containing 150 mM NaCl was added and the samples were allowed to hydrate for 1 h at a temperature above the phase transition temperature of the corresponding lipid, namely at room temperature (PC, PE, PG, PI, PS, PA, DPG, PC/Ch) or 50°C (DPPC, DPPG), whereupon they were dispersed by extensive sonication with a Branson UPS-2000 tip-sonifier to form lipid–protein vesicles. The conditions selected for sonication produce unilamellar vesicles of 130–160 nm with a polydispersity index of around 0.2 in different phospholipids, as determined by quasi-elastic light scattering in an Autosizer IIc Photon Correlation Spectrometer (Malvern Instruments) [20–22].

2.3. Antibodies

The anti-SP-B polyclonal antiserum used in the present study was raised in New Zealand white rabbits by employing five intramuscular inoculations scheduled every 7 days. A 100 μg dose of SP-B reconstituted in micelles of lysophosphatidylcholine (LPC, 2:1 lipid to protein molar ratio by weight) in complete Freund's adjuvant was used for the first inoculation, and 70 μg of protein in LPC micelles in incomplete adjuvant was used in the subsequent doses. The inoculated rabbit was bled 10 days after the last injection. The anti-SP-B polyclonal antiserum obtained following this protocol recognized SP-B but not SP-A nor SP-C when it was used in Western blots.

Anti-SP-B monoclonal antibody 8B5E, recognizing an epitope of human SP-B [23], was a generous gift of Dr. Yasuhiro Suzuki (University of Kyoto, Japan).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The ELISA method used in this work was carried out as follows. The wells of the plates (immunoplates Polysorp F96, Nunc A/S, Roskilde, Denmark) were filled with 100 μl each of lipid or lipid–protein suspensions prepared as described above and incubated at 4°C overnight. In a control experiment, the wells of the plates were coated by evaporation at 37°C of a methanolic SP-B solution. After protein, lipid or lipid–protein coating, the wells of the microtiter plates were washed six times with a solution of 0.05% (v/v) Tween-20 and blocked for 1 h at 37°C with 100 μl each of 5% dried skim milk in phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.6 mM KCl, pH 7.4). After washing six times with 0.05% Tween-20, an aliquot of 100 μl of the primary antibody (1/500 dilution of the polyclonal antiserum in PBS containing 0.05% Tween-20) was added to each well and the plate was incubated for 1 h at 37°C. The plates were then washed again six times with 0.05% (v/v) Tween-20, and incubated for 1 h at 37°C with the secondary antibody, peroxidase-labelled anti-rabbit IgG antibody (Amersham International, Buckinghamshire, UK), that had been diluted 1/500 in PBS containing 0.05% Tween-20. After washing six times, the plates were developed by adding 200 μl per well of the detection solution (80.6 mM DMAB, 1.56 mM MBTH, H_2O_2 0.0075% in 0.1 M phosphate buffer pH 7). The reaction was stopped by the addition of 50 μl of 1.5 M H_2SO_4. Results were assessed spectrophotometrically at 570 nm in a Digiscan 340T microtiter reader (Asys Hitech Gmbh, Austria). Negative controls were always run, using an anti-SP-A polyclonal antiserum [24] as primary antibody to discount non-specific signals.

The detection limit of this ELISA assay, under the conditions of our experiments, was in the range 10–20 ng SP-B/ml (limit values were 8 ng/ml for SP-B in PG bilayers and 25 ng/ml for SP-B in PC bilayers). The coefficient of interassay analytical variation was 7–8% in all the assayed lipids (7 ± 3% for SP-B in PC vesicles, calculated from 19 independent determinations of samples containing 25–500 ng SP-B/ml; 8 ± 3% for SP-B in PG vesicles, from 14 independent samples in the range 8–250 ng SP-B/ml).

2.5. Statistical analysis

The experiments in this study have been repeated at least three times, in each case using protein isolated from different surfactant batches, and the results were qualitatively similar. Data plotted in the figures represent the mean of triplicate determinations ± S.D. from a single protein batch. Student's *t*-test was used to analyze the difference between the means. Differences with a P value of < 0.05 were considered statistically significant ($^{**}P < 0.01$ and $^{*}P < 0.05$ vs. control).

3. Results

Fig. 1 compares immunoreactivity to anti-SP-B antibodies of porcine SP-B reconstituted in zwitterionic (PC, DPPC) or anionic (PG, DPPG) bilayers with that of the protein in the absence of lipids. In general terms, when the protein was embedded in membranes of PC, DPPC or DPPG its epitopes were less recognized by polyclonal antibodies than in the absence of lipids, in the range of 1–100 ng SP-B per well. This fact suggests that some of the epitopes are at least partly shielded in the lipid–protein complexes. When a monoclonal anti-SP-B antibody was employed (Fig. 1, right panel) SP-B immunoreactivity was similar in the absence and in the presence of PC, DPPC or DPPG, suggesting that the epitope

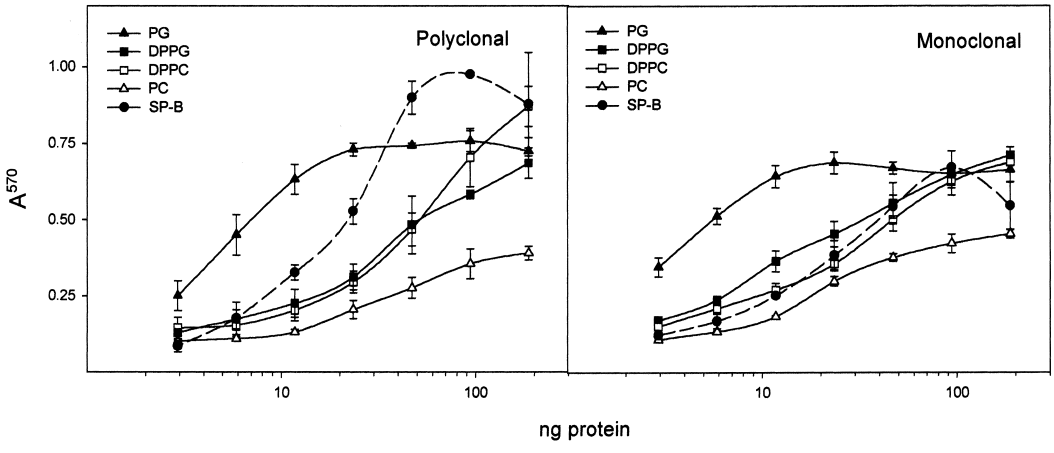

Fig. 1. Binding curve of polyclonal (left panel) and monoclonal (right panel) primary anti-SP-B antibodies to porcine SP-B in the absence of lipids (dashed lines) and reconstituted in phospholipid bilayers (solid lines) of different composition. SP-B protein to lipid ratio in the bilayers was 1:20 by weight. Error bars represent standard deviation after averaging three different experiments. Those bars not shown are within the symbol sizes.

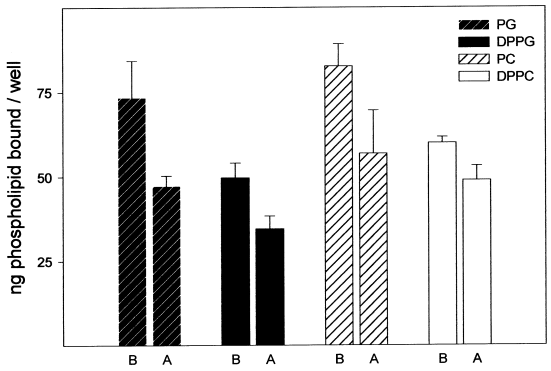

Fig. 2. Phospholipid bound to ELISA plates coated with 50 ng of SP-B in phospholipid vesicles (1:20 protein to lipid weight ratio), before (B) and after (A) carrying out all the washing steps of the ELISA procedure. Absorbed phospholipid amounts were calculated by including in the lipid–protein samples a ^{14}C-radiolabelled DPPC trace. Error bars represent standard deviation after averaging three different experiments.

recognized by the antibody 8B5E is located in the exposed parts of SP-B in the lipid–protein complexes. In DPPG bilayers, SP-B always showed higher binding of antibodies than in bilayers made of PC or DPPC, although the differences were small. Interestingly, when SP-B was reconstituted in vesicles of egg yolk PG, it bound much more of both antibodies when tested either in the absence or presence of the other lipids, suggesting that these negative membranes could promote higher exposure of protein epitopes. The results were very similar when comparing lipid–protein samples reconstituted at 1/10 and 1/20 SP-B protein to lipid ratio by weight (not shown). Coating of ELISA wells with lipid vesicles, in the absence of protein, did not produce any antibody binding to any of the lipids tested, discounting artefacts due to direct binding of anti-SP-B antibodies to these membranes. To ensure that differences detected in antibody binding were not caused by a different efficiency of the protein/lipid systems to adsorb to the plates during the coating procedure, we quantitated the amount of material actually adsorbed onto the wells by including a trace of ^{14}C-labelled DPPC in the different lipid–protein complexes. At different steps during the coating and washing procedures, the wells were extensively washed with Chl/MeOH (2:1, v/v) to recuperate all the adsorbed lipids and quantitated by measuring ^{14}C in a scintillation counter. Fig. 2 shows that there are some differences in the amount of material bound initially to ELISA plates, depending on the lipid used to reconstitute the protein. The amount of DPPC or DPPG vesicles initially adsorbed onto the plates was about 30% lower than that of PC or PG vesicles, suggesting that the membranes can enhance sample/support interactions. However, the amount of material which remained attached to the plates after the washing steps was not significantly different among samples containing PC, DPPC, PG or DPPG, confirming that the different amounts of antibodies bound to the SP-B/lipid samples are most likely explained as a consequence of differences in the availability of SP-B epitopes in the different membranes.

To determine if the greater exposure of SP-B epitopes in PG vesicles is a specific feature shown by SP-B in membranes containing PG or a general effect caused by negatively charged membranes, we have tested and compared recognition of SP-B by antibodies after reconstitution of the protein in bilayers made of different zwitterionic (PC, PE, PC/Ch 7% by weight) or anionic (PG, PI, PA, PS, DPG) phospholipids or in fatty acid anionic micelles (made of salts of Pal or Ste acids). Fig. 3 shows that SP-B is more immunoreactive in all the anionic membranes tested than in zwitterionic bilayers or in anionic micelles such as those formed by the fatty acids. Quantitative examination of the amount of antibodies bound to SP-B reconstituted in the different lipids, in the linear portion of the ELISA curve (Fig. 3c), indicated that the phospholipid enhancement of SP-B immunoreactivity followed the sequence

$$PS \approx PI \approx PG \gg DPG > PA > PE \approx PC \approx PC/Ch \approx Pal \approx Ste$$

Statistical analysis of the differences in immunoreactivity (Fig. 4) indicated that SP-B is significantly more immunoreactive in all the anionic bilayers than in zwitterionic PC membranes.

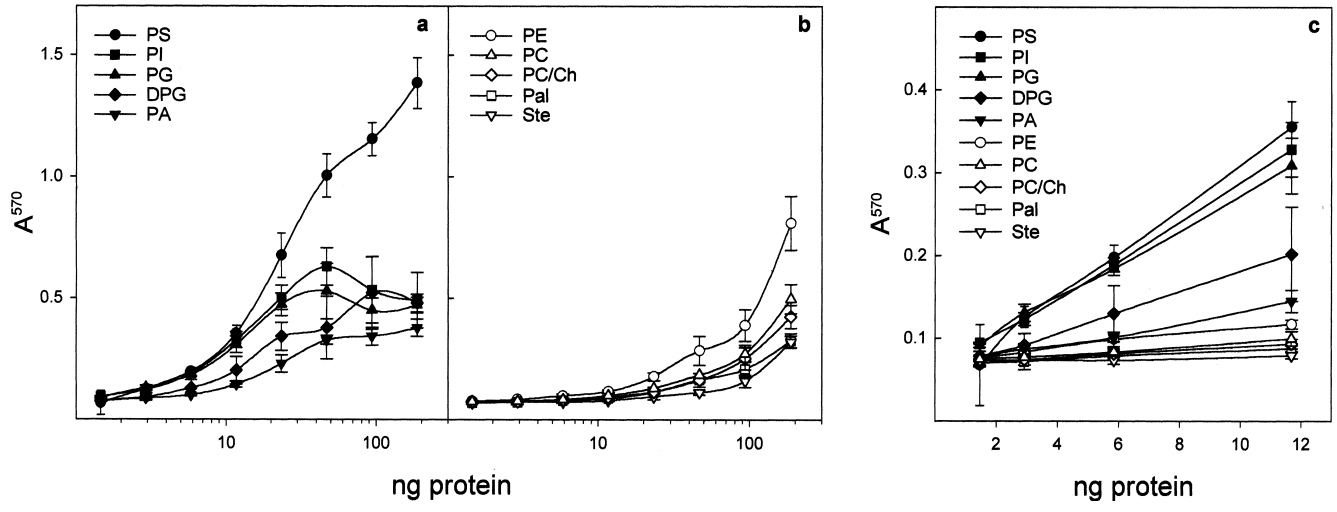

Fig. 3. Binding curves of polyclonal anti-SP-B antibodies to porcine SP-B reconstituted in anionic (a) or zwitterionic (b) phospholipids or in fatty acid micelles (also in b). c: Linear rescaling of plots at lower protein densities. Protein to lipid ratio was 1:20 by weight in all the samples. Error bars represent standard deviation after averaging three different experiments. Those bars not shown are within the symbol sizes.

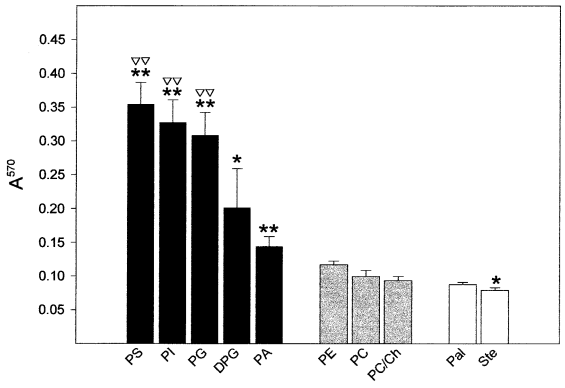

Fig. 4. Analysis of the differences in binding of polyclonal anti-SP-B antiserum to 12 ng of porcine SP-B reconstituted in anionic (black bars) or zwitterionic (gray bars) bilayers or in fatty acid micelles (white bars). Significance was assessed of all the samples with respect to the immune response of the protein in PC membranes (asterisks) and of those in anionic phospholipids with respect to the protein in PA (triangles). Protein to lipid ratio was 1:20 by weight in all the samples. Error bars represent standard deviation after averaging three different experiments. Significance was determined by applying a *t*-test. **, $\triangledown\triangledown P < 0.01$ and $*P < 0.05$ vs. control.

SP-B also bound significantly more antibodies in PG, PI or PS bilayers than in PA membranes. We also detected a statistically significant lower binding of anti-SP-B antibodies to the protein in stearate micelles, indicating a presumably unfavorable exposure of the protein epitopes in that environment.

4. Discussion

The results shown indicate that in anionic bilayers, pulmonary surfactant protein SP-B assumes structural features which lead to enhanced recognition by antibodies, in comparison to protein included in zwitterionic bilayers. This effect could originate in a different conformation of the protein being assumed as a consequence of electrostatic protein–lipid interactions, which would then expose epitopes to a greater extent. Alternatively, a higher SP-B antibody binding in negatively charged membranes could be caused by a more favorable geometry of the lipid–protein complexes, for instance a lower extent of penetration, and therefore less shielding, of the SP-B protein into those bilayers as they are formed. Several lines of evidence suggest that SP-B really has a more extended conformation in negatively charged membranes. As determined by scanning calorimetry [25], the number of phospholipid molecules perturbed per SP-B monomer upon interaction with DPPC bilayers was lower, around 50, than those perturbed upon interaction with DPPG membranes, close to 70. On the other hand, SP-B has a substantially slower rotational diffusion coefficient in DPPG than in DPPC bilayers, which can only be explained by considering that the conformation of the protein in anionic membranes gives rise to an apparently larger rotation radius [26]. We have previously determined that the extent of penetration of SP-B into DPPC bilayers depends on the method used to reconstitute lipid–protein complexes [27]. A similar study gives a comparable penetration of SP-B into PC and PG bilayers, at least with respect to the region of the protein where the single tryptophan residue is located (results not shown). Whatever its origin, the conformation/disposition of SP-B should be considered in the context of selective interactions of the protein with the anionic phospholipids in pulmonary surfactant. SP-B has shown a relative selectivity for interaction with negatively charged as opposed to zwitterionic phospholipid species [28], which is specially pronounced toward PG, the most abundant anionic phospholipid in surfactants from many animal species. Several lines of evidence suggest that electrostatic interactions between SP-B and anionic phospholipids may be important in processes occurring in surfactant metabolism. In vitro both SP-B and PG are necessary components to reconstitute tubular myelin, a unique structure observed in extracellular surfactant in vivo [11,12]. SP-B promotes removal of non-DPPC components from surfactant monolayers during compression/expansion cycling. This process is important to enrich the films during compression in the highly tensoactive species, DPPC. This refinement has been observed in the presence but not in the absence of PG [15], although the possible effect of other anionic phospholipids was not tested. A mechanism has recently been proposed by which selective association of SP-B with anionic phospholipids in the surfactant monolayer would lead to formation of three-dimensional protein–lipid aggregates excluded from the surface, via a compression-driven anionic phospholipid-induced conformational change in the protein [29]. Such a conformational transition might produce greater exposure of epitopes as the data of the present study suggest. Presence of anionic species in surfactant monolayers is also necessary for SP-B to sustain high pressures in compressed films, an effect that may be important to ensure stabilization of the respiratory surface until the compression of the lungs is completed [17,30]. Some of these activities could be mediated by specific lipid–protein molecular complexes, in which one or more anionic phospholipid molecules could bind to specific sites in SP-B. Such specific binding sites have recently been described at atomic detail for some membrane proteins [31]. Our results show that the strongest effects in promoting exposure of epitopes in SP-B are caused by PG, PI and PS. PG and PI are the prominent anionic phospholipids of natural surfactant, with PG being the major anionic phospholipid in most species and PI in some [32].

The effects described here also have relevance for the optimization of immunological methods to detect and quantitate SP-B in clinical samples. Determination of SP-B levels is an important analytical procedure to evaluate occurrence of several respiratory pathologies including congenital SP-B deficiencies [1], or secondary alterations such as those found in alveolar proteinosis [33], viral infections [34] or post-transplantation trauma [35]. Given that the anionic phospholipids might alter SP-B reactivity with antibodies, their proportion in surfactant samples needs to be considered when validating ELISA methods for SP-B measurements.

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